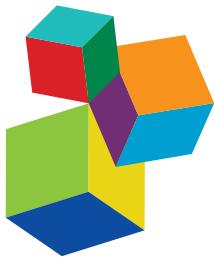


THE SEARCH FOR BIOLOGICAL ACTIVE AGENT(S) FROM ACTINOBACTERIA, 2nd Edition

EDITED BY: Learn-Han Lee, Kok-Gan Chan, Jem Stach,
Elizabeth M. H. Wellington and Bey-Hing Goh
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THE SEARCH FOR BIOLOGICAL ACTIVE AGENT(S) FROM ACTINOBACTERIA, 2nd Edition

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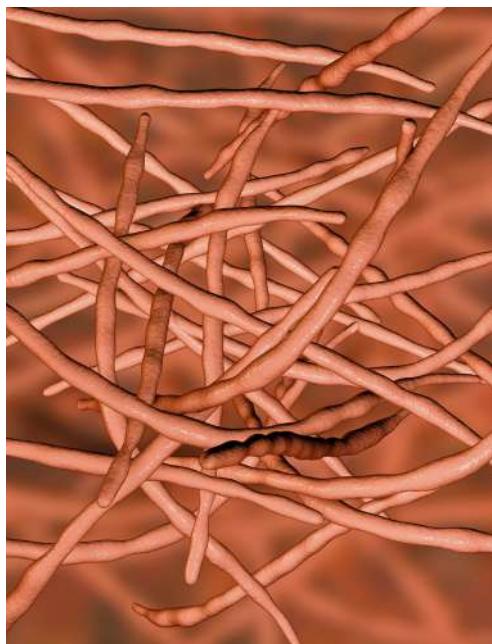
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There is a large market demand for new drugs. The existing chronic or common ailments without cures, development of new diseases with unknown causes, and the widespread existence of antibiotic-resistant pathogens, have driven this field of research further by looking at all potential sources of natural products. To date, microbes have made a significant contribution to the health and well-being of people globally. The discoveries of useful metabolites produced by microbes have resulted in a significant proportion of pharmaceutical products in today's market. Therefore, the investigation and identification of bioactive compound(s) producing microbes is always of great interest to researchers.

Actinobacteria are one of the most important and efficient groups of natural metabolite producers. Among the numerous genera, *Streptomyces* have been recognized as prolific producers of useful natural compounds, as they provide more than half of the naturally-occurring antibiotics isolated to-date and continue to emerge as the primary source of new bioactive compounds. Certainly, these potentials have attracted ample

research interest and a wide range of biological activities have been subsequently screened by researchers with the utilization of different *In vitro* and *In vivo* model of experiments. Literature evidence has shown that a significant number of interesting compounds produced by Actinobacteria were exhibiting either strong anticancer or neuroprotective activity. The further in depth studies have then established the modulation of apoptotic pathway was involved in those observed bioactivities. These findings indirectly prove the biopharmaceutical potential possessed by Actinobacteria and at the same time substantiate the importance of diverse pharmaceutical evaluations on Actinobacteria. In fact, many novel compounds discovered from Actinobacteria with strong potential in clinical applications have been developed into new drugs by pharmaceutical companies. Together with the advancement in science and technology, it is predicted that there would be an expedition in discoveries of new bioactive compounds producing Actinobacteria from various sources, including soil and marine sources. In light of these current needs, and great interest in the scope of this research, this book seeks to contribute on the investigation of different biological active compound(s) producing actinobacteria which are exhibiting antimicrobial, antioxidant, neuroprotective, anticancer activities and similar.

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Editorial: The Search for Biological Active Agent(s) From Actinobacteria

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Editorial on the Research Topic

The Search for Biological Active Agent(s) From Actinobacteria

INTRODUCTION

Nature has always been an interesting source for bioactive products, particularly those derived from microorganisms. Even though microorganisms can be found literally throughout Earth, more efforts are still needed to study the microbial biodiversity, given that there are still 99.999% of microbial taxa that awaits to be discovered (Locey and Lennon, 2016). As the largest phylum under the Bacteria kingdom, *Actinobacteria* has gained tremendous amount of attention from the scientific community, mainly due to their ability in producing a vast array of bioactive compounds with interesting chemical structures (Barka et al., 2016). To date, actinobacteria have contributed more than 65% of antibiotics used in medicine; out of which over 10,000 bioactive compounds were produced by the members of the genus *Streptomyces* (Bérdy, 2005; Bull and Stach, 2007; Subramani and Aalbersberg, 2012; Zotchev, 2012; Karuppiah et al., 2016). Even after decades of bioprospecting research, the genus *Streptomyces* remains in the spotlight of microbial product research, mainly due to its seemingly unsurpassed ability in synthesizing a vast array of compounds with various bioactivities (Forget et al.; Kamjam et al.; Law et al.; Lyu et al.; Maryam and Khan; Tan et al., 2016; Ser et al., 2017). The genus *Streptomyces* was firstly introduced by Waksman and Henrici in 1943, several years before Professor Waksman was bestowed with the Nobel Prize in Physiology or Medicine in 1952 for the discovery of streptomycin from *Streptomyces griseus* (Schatz et al., 1944; Nobelprize.org., 2018). Since then, continuous efforts have been put in to explore the potential of these Gram-positive bacteria and the genus now contains over 860 species and subspecies isolated from various habitats (www.bacterio.net). One of the reasons behind their ubiquitous nature is the unique developmental life cycle—these bacteria grow to form substrate mycelium and further develop spores when the environment becomes unfavorable for growth. Facing this environmental stress, some actinobacteria have also deployed defense system and/or survival mechanism like production of secondary metabolites that may have more functions than we initially thought.

Under the research topic “The search for biological active agent(s) from actinobacteria,” a total of 23 articles were published, covering a variety of topics revolving actinobacteria including their diversity in different habitats and the discovery of novel, bioactive strains along with some interesting bioactivities such as antibacterial, antifungal, antioxidant, and anticancer properties.

Due to the ease of sampling, numerous literature has previously indicated diversity of terrestrial-derived actinobacteria across the globe. For instance, Professor William C. Campbell and Professor Satoshi Omura have successfully discovered *Streptomyces avermitilis* (from golf course in Ito) which synthesized an antiparasitic compound, avermectin, and subsequently earn them (part of) the Nobel Prize of Physiology and Medicine in 2015 (Burg et al., 1979; Nobelprize.org., 2018). Even after extensive search of bioactive strains from terrestrial area, there are some actinobacteria hidden in parts of world/geographical region that have been previously overlooked (Sharma et al.; Khieu et al., 2015). For example, one of the study has discovered rich diversity of antimicrobial producing *Streptomyces* from moonmilk cave, which is locally used as traditional medicine against several ailments (Maciejewska et al.). The same study reported that 90% of the strains isolated from the cave induced strong growth suppression against the multi-drug resistant *Rasamonia argillacea*, thus possessed great potential to be developed for cystic fibrosis patients and those with chronic granulomatous diseases. On the other hand, researchers have also reported presence of bioactive *Streptomyces* sp. agricultural soil in Beni-Suef, Egypt and successfully discovered a diketopiperazine derivative (*m/z* 488.05) which may contribute to antimicrobial and anticancer activities (Ahmad et al.). On the other hand, *Streptomyces* sp. ASK2 was shown to produce an aromatic compound with aliphatic side chain (*m/z* 444.43) exhibiting antagonistic activity against multidrug resistant *K. pneumoniae* using adult zebrafish infection model (Cheepurupalli et al.). Furthermore, actinobacteria found in rhizosphere soil of plants and endophytic actinobacteria (e.g., reside on/within certain plant species) contribute as good source of bioactive compounds, as they could synthesize a wide diversity of secondary metabolites which may promote and/or ensure health of their host. Growing in the wetland area, 10 actinobacteria strains were recovered from the medicinal plant *Vochysia divergens* located in wetland area in Brazil, belonging to the *Aeromicrobium*, *Actinomadura*, *Microbacterium*, *Microbispora*, *Micrococcus*, *Sphaerisporangium*, *Streptomyces*, and *Williamsia* genera (Gos et al.). One of the extract produced by strain LGMB491 (a close relative of *Aeromicrobium ponti*) displayed the highest activity against methicillin-resistant *Staphylococcus aureus* (MRSA), with a minimum inhibitory concentration of 0.04 mg/mL. Strain LGMB491's extract contained 1-acetyl-β-carboline (1), indole-3-carbaldehyde (2), 3-(hydroxyacetyl)-indole (4), brevianamide F (5), and cyclo-(L-Pro-L-Phe) (6) as major compounds with antibacterial activity. Though, more than 50 years have passed since the discovery of streptomycin from soil streptomycete, these papers have once again demonstrated the pharmaceutical importance of terrestrial-derived actinobacteria.

Actinobacteria are abundance in nature, therefore some studies have expanded from the initial isolation source (i.e., terrestrial area) and began to search for actinobacteria from freshwater and marine environments. As over 70% of the Earth is covered by water (e.g., lake, sea, ocean), it is almost close to impossible not to encounter any actinobacteria with unique traits (Balasubramanian et al.; Dhakal et al.; Islam et al.; Quezada

et al.; Undabarrena et al.). As discussed by Kamjam et al., there have been more than 21 new species of 13 genera reported between year 2006 and 2016. Some of which could produce secondary metabolites, among which *Streptomyces* species is the richest producer. By the same token, marine soil/sediments have also demonstrated similar bioactive potential as those derived from terrestrial sources; several actinobacteria strains isolated in India displayed strong antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and *Candida albicans* (Dholakiya et al.; Kavitha and Savithri). Furthermore, bioprospecting of actinobacteria from dynamic environment such as the mangrove areas are gaining good results as well. Many studies were from Asia, an area that has the largest coverage of mangrove forests which equates to 42% of the total global mangrove area (Giri et al., 2011; ITTO, 2014). Malaysia is home to 3.7% of global total mangrove coverage and three studies in this research topic have emphasized the importance of this ecosystem, particularly for the search of bioactive compounds from actinobacteria (Arumugam et al., 2013; Lee et al., 2014; Ser et al., 2015, 2016; Azman et al., 2017). The hexane partition of *Streptomyces* sp. CCB-PSK207 was found to be able to protect *Caenorhabditis elegans* against with *Pseudomonas aeruginosa* strain PA14 infection via re-activation of lysozyme 7, without impairing feeding behavior of *C. elegans* (Fatin et al.). These findings have revealed a key component for *P. aeruginosa* PA14 infection—lysozyme 7 which functions as innate immunity defense molecule and this was the first report of marine actinobacteria producing metabolites which is capable of rescuing *C. elegans* from PA14 through modulation of lys-7 activity. Another two novel *Streptomyces* species have also been isolated from different parts of Malaysia: (a) *Streptomyces colonosanans* MUSC 93^T from Sarawak, East Malaysia, and (b) *Streptomyces antioxidant* MUSC 164^T from east coast of Peninsular Malaysia. MUSC 93^T was given the name as *S. colonosanans* given that the strain demonstrated anticancer activity against human colon cancer cell lines without significant cytotoxic effect against human normal colon cells (Law et al.). On the other hand, *S. antioxidant* MUSC 164^T was found to produce pyrazines and phenolic-related compounds which are capable of reducing free radicals and protect neurons against hydrogen peroxide damages (Ser et al.). Altogether, these studies suggested the importance of aquatic associated actinobacteria, especially against harmful pathogens and chronic human diseases like neurodegenerative diseases and cancer.

Exploring new taxa has always been successful strategy in the discovery of candidates for the development of new microbial drug(s). However, the subsequent steps to maximize yield and production from the species of interest are equally important. As part of the commonly used antibiotics, clavulanic acid is initially isolated from *Streptomyces clavuligerus*. Ser et al. discussed the importance of optimization in traditional fermentation technology, particularly fermentation conditions and media composition to increase clavulanic acid production in *S. clavuligerus*. In fact, the differences in major ingredient(s) of a medium is imperative as slight changes in composition (or concentration) could tip off the balance between the growth of the organism and the production of secondary metabolite(s).

Using clavulanic acid as example, the addition of its precursors like arginine and ornithine increase the supply of C5 precursors into the biosynthesis pathway, which ultimately lead to higher production of the antibiotics. Similarly, one could also increase the yield of compound of interest by reducing occurrence of competing pathway, either via addition of specific inhibitors or even reprogram metabolic pathways via genetic modifications (Pickens et al., 2011).

With the advancement in next-generation sequencing (NGS), actinobacteria are recognized as “hidden treasures” in the nature that awaits exploration (Jose and Jha). In point of fact, the availability of genome sequences has unlocked possibly new potential of actinobacteria (van Heel et al., 2013; Skinnider et al., 2015; Blin et al., 2017; Ser et al., 2018). Following stimulated much progress in computational resources, including bioinformatics tools like antibiotics and Secondary Metabolites Analysis SHell (antiSMASH) and Prediction Informatics for Secondary Metabolomes (PRISM) to assist in surveying of the genomic the announcement of the complete genome sequence for the model actinobacterium *Streptomyces coelicolor* A3(2), many researchers have discovered presence of “cryptic” or silent biosynthetic gene clusters among actinobacteria genomes, indicating that researchers may have underestimated the “true nature” of actinobacteria specifically in producing useful secondary metabolites (Bentley et al., 2002; Takagi and Shinya, 2011; Harrison and Studholme, 2014). As a result, several studies have employed the CRISPR/Cas9 system to increase production yield, by (a) either knock-in genes to activate silent biosynthetic gene clusters or (b) delete repressors genes (Pickens et al., 2011; Jia et al., 2017; Robertsen et al., 2017; Zhang et al., 2017). By introducing a heterologous promoter, kasO* promoter in the upstream region of the biosynthetic gene clusters, Zhang et al. (2017) have successfully “awaken” the silent biosynthetic gene cluster in *Streptomyces roseosporus*

NRRL15998 and stimulated production of two polycyclic tetramate macrolactam, which failed to be expressed in a heterologous system. Altogether, metabolic engineering offers an alternative to shorten time needed to optimize a strain, possibly simplifying the subsequent purification process to isolate compound of interest. Coupled with the cutting-edge molecular and analytical tools, the discovery of biosynthetic gene clusters could possibly increase the chemical diversity of actinobacteria from different natural sources, consequently amplifying the pharmaceutical potential of these beneficial microbes.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Antibacterial and Antioxidant Activities of Novel *Actinobacteria* Strain Isolated from Gulf of Kambhat, Gujarat

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Bacterial secondary metabolites possess a wide range of biologically active compounds including antibacterial and antioxidants. In this study, a Gram-positive novel marine *Actinobacteria* was isolated from sea sediment which showed 84% 16S rRNA gene sequence (KT588655) similarity with *Streptomyces variabilis* (EU841661) and designated as *Streptomyces variabilis* RD-5. The genus *Streptomyces* is considered as a promising source of bioactive secondary metabolites. The isolated novel bacterial strain was characterized by antibacterial characteristics and antioxidant activities. The BIOLOG based analysis suggested that *S. variabilis* RD-5 utilized a wide range of substrates compared to the reference strain. The result is further supported by statistical analysis such as AWCD (average well color development), heat-map and PCA (principal component analysis). The whole cell fatty acid profiling showed the dominance of iso/anteiso branched C15–C17 long chain fatty acids. The identified strain *S. variabilis* RD-5 exhibited a broad spectrum of antibacterial activities for the Gram-negative bacteria (*Escherichia coli* NCIM 2065, *Shigella boydii* NCIM, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas* sp. NCIM 2200 and *Salmonella enteritidis* NCIM), and Gram-positive bacteria (*Bacillus subtilis* NCIM 2920 and *Staphylococcus aureus* MTCC 96). Extract of *S. variabilis* strain RD-5 showed 82.86 and 89% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and metal chelating activity, respectively, at 5.0 mg/mL. While H₂O₂ scavenging activity was 74.5% at 0.05 mg/mL concentration. Furthermore, polyketide synthases (PKSs types I and II), an enzyme complex that produces polyketides, the encoding gene(s) detected in the strain RD-5 which may probably involve for the synthesis of antibacterial compound(s). In conclusion, a novel bacterial strain of *Actinobacteria*, isolated from the unexplored sea sediment of Alang, Gulf of Kambhat (Gujarat), India showed promising antibacterial activities. However, fractionation and further characterization of active compounds from *S. variabilis* RD-5 are needed for their optimum utilization toward antibacterial purposes.

Keywords: *Actinobacteria*, antibacterial, antioxidant, biolog, marine bacteria, novel strain, polyketide synthases, sea sediment

INTRODUCTION

More than 70% of the surface of the earth planet covers by the sea which contains exceptional diversity which is more than 95% of the whole biosphere (Qasim, 1999). It was observed that the living diversity is higher in some marine ecosystems, such as the deep sea and coral reefs, than the tropical rainforests (Edwards et al., 2006). The ocean is the habitat of several groups of life-forms which live in a complex environment with extreme variations in pressure, salinity, light, and temperature (Munn, 2004). Recently, it was proven that the ocean floor possesses many unique forms of *Actinobacteria* (Fenical and Jensen, 2006). *Actinobacteria* are widely distributed in intertidal zones, mangroves, seawaters, animals, plants, sponges, and in ocean sediments (Goodfellow and Williams, 1983; Castillo et al., 2005; Jensen and Mafnas, 2006; Ramesh and Mathivanan, 2009; Sun et al., 2010; Xiao et al., 2011; Rao and Rao, 2013). *Actinobacteria* from the marine environment are considered as a promising source of pharmaceutically important compounds because of a different kind of unique adaptation characteristics (Fenical and Jensen, 2006; Jose and Jha, 2017).

Actinobacteria are Gram-positive bacteria with filamentous structure. These are considered the most economical and biotechnological important prokaryotes which produce several secondary metabolites with significant biological activities. Out of these *Actinobacteria*, *Streptomyces* is an important industrial group of organisms that widely explored for the wide range of biologically active compounds (Berdy, 2005). *Actinobacteria* comprise of G + C rich microorganisms (Embley and Stackebrandt, 1994), live in varying habitats and well established for the synthesis of bioactive secondary metabolites (Sengupta et al., 2015). *Actinobacteria* inhabiting marine environment (such as sea sediments, etc.) gain much attention (Lane and Moore, 2011) because they are considered more challenging to culture compared to their terrestrial relatives. They have special growth requirements and media composition. Furthermore, several *Actinobacteria* genera produce novel secondary metabolites with several bioactivities (Jensen et al., 2005). The recent grasp of the fact that marine environment can be a potential source for the novel isolates with novel natural products encourages intensive search and efforts from several groups. Nearly seventy five percent of all the known industrial antibiotics (Kieser et al., 2000) and numerous economically important compounds (Okami and Hotta, 1988) were obtained from the streptomycetes. *Actinobacteria* have also ability to synthesize antiviral (Sacramento et al., 2004), antifungal (Zarandi et al., 2009), antitumor (Hong et al., 2009), insecticidal (Pimentel-Elardo et al., 2010), antioxidants (Janardhan et al., 2014), anti-inflammatory (Renner et al., 1999), anti-biofouling (Xu et al., 2010), immunosuppressive (Mann, 2001), anti-parasite (Pimentel-Elardo et al., 2010), plant growth promoting and herbicidal compounds (Sousa et al., 2008), enzyme inhibitors (Hong et al., 2009) and industrially important enzymes. Advance molecular tools such as metagenomics, metatranscriptomics, and metaproteomics can be employed directly for the extraction of DNA, RNA, and protein from environment samples (Mincer et al., 2005). Simultaneously, polymerase chain reaction

(PCR) amplified products were cloned and sequenced for identifying new *Actinobacteria* present in the environment samples (Monciardini et al., 2002; Riedlinger et al., 2004). Selective primer is now available to amplify the 16S rRNA gene from the specific *Actinobacteria* (Monciardini et al., 2002). Metabolic bioactive compounds obtained from marine or territorial *Actinobacteria* are commonly synthesized by enzymes polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS). The PKS is categorized into three different groups such as types I, II, and III. Both NRPS peptides and PKS-type I are encoded by a number of modules which are multifunctional in nature (Ayuso-Sacido and Genilloud, 2005; Smith and Sherman, 2008). They form a series of biosynthesis reaction including acyl (PKS-I) or peptidyl (NRPS) chain initiation, elongation, and termination (Walsh, 2008). PKS-II molecules which are non-modular, complex of several single module proteins and their group of enzymatic activity act in an iterative manner to produce a polyketide (Gallo et al., 2013). The core PKS module comprises of a ketoacyl-synthase (KS α), a chain elongation factor (KS β), and an acyl-carrier protein (Walsh, 2004; Das and Khosla, 2009). The PKS-III types are homodimer enzymes and act on the acyl-CoA without involving any acyl-carrier proteins (Shen, 2003). In continues searching potential bioactive, molecular methods will help for analyzing and comparing the genetic variations within these genes, in the normal laboratory condition strain's specialized metabolites is not routinely produced which are useful for screening for molecule production is remains mostly a trial-and-error approach (Metsa-Ketela et al., 1999; Ayuso-Sacido and Genilloud, 2005; Gontang et al., 2010).

Extensive study has been done on various coastal areas of India for isolation and cultivation of *Actinobacteria*. However, the coast of Gujarat and especially, Gulf of Khambhat is relatively unexplored so far. Therefore, the present study was aimed to investigate the novel marine *Actinobacteria* using molecular methods and phylogenetic comparisons of the isolates. Furthermore, the isolated bacterial strain was functionally characterized by antibacterial and antioxidant activities. The present study provides a useful insight of bacteria inhabiting sea sediment of Arabian Sea. The isolated bacterial strain can be utilized further for the developing novel antibacterial compounds.

MATERIALS AND METHODS

Isolation and Culture Characterization of Marine *Actinobacteria*

The sea sediment samples (25 g) were collected from coastal areas of Gulf of Khambhat, Gujarat, India near a ship scraping industries ($21^{\circ}24'35.85''N$, $72^{\circ}11'54.1''E$). Samples were transported to the laboratory under cool and control conditions, and immediately processed for the isolation of marine *Actinobacteria* (through serial dilution method) from sediment samples using modified Gause's Synthetic Agar medium (Ye et al., 2009). In brief, 0.5 g sea sediment was suspended in 9.5 ml of sterile saline solution (0.9% NaCl). The suspended solution was serially diluted up to 10^{-10} in saline solution. About

100 μL diluted solution (10^{-3} to 10^{-10}) was spread individually on modified Gause's Synthetic Agar medium containing 0.01% (w/v) potassium dichromate to prevent the early growth of other bacteria and fungus. Plates were incubated at 30°C for 4–7 days, and *Actinobacteria* were preliminarily screened based on traditional morphology.

BIOLOG Assay of Selected *Actinobacteria* Isolates

The isolated bacteria were categorized by GEN III MicroPlate test assay performed with a Biolog system. The test panel comprises of 71 carbon sources with 23 chemical sensitivity assays and thus provides a "Phenotypic Fingerprint" of the tested microorganism. The Biolog system dissects and analyses the ability of a cell to metabolize all major substrates. Furthermore, other important physiological properties such as salt, pH, reducing power, chemical sensitivity and lactic acid tolerance were also determined. Overnight grown bacterial suspensions were mixed with 0.85% saline solution (5 mL) and IF-a was adjusted for 90–98% transmittance (T90) with a Biolog turbidimeter. Into each well of Biolog microplate, about 100 μL bacterial suspension was dispensed and incubated at 30°C. The developed color is compared with the Biolog species library to identify the bacterial isolates.

Average Well Color Development Assay

BIOLOG plates are commonly used for the analysis of microbial community function and micro-organism may be identified by the specific phenotype color fingerprint. The average well color development (AWCD) quantification of individual plate or individual well is performed by continuous monitoring of OD absorbance at 590 nm. The measured data was expressed as AWCD in response to incubation time (Garland and Mills, 1991).

$$\text{AWCD} = \Sigma \text{OD}_i / 95$$

Chemotaxonomic Identification

Chemotaxonomic identification of isolates was done by fatty acid methyl ester (FAME) analysis using gas chromatography coupled with Sherlock microbial identification system (MIS). The MIS gives the data output includes fatty acids composition and sample chromatographic run. The software computes "Sim index" which congregates values of samples FAME with the library and gives a Euclidian distance (ED).

Molecular Identification

Isolate RD-5 was grown in 50 mL of Gause's Synthetic broth containing NaCl (4%, w/v) for 7 days. The mycelia were harvested by centrifugation at 10,000 rpm for 5 min and genomic DNA was extracted using phenol-chloroform extraction method (Hopwood et al., 1985). DNA quality and concentration were measured using a Nanodrop 1000 Spectrophotometer.

The 16S rRNA gene was amplified using genomic DNA and universal bacterial primers (Table 1). The 50 μL PCR mixture was contained; 1–2 μL DNA template, 0.5 μL 20 μM of each primer, 5 μL of 10X buffer, 5 μL of dNTPs (2.5 mM), 0.5 μL

Taq DNA (5 units/ μL), and 41.5 mL ddH₂O. PCR was done in MyCyclerT-100 (Bio-Rad, United States) using the optimized conditions (Yousuf et al., 2012, 2014a,b; Keshri et al., 2013; Keshri et al., 2015). The amplified products were analyzed on a 1.0% agarose gel, purified (QIAquick PCR Purification Kit, Qiagen, Germany) and sent to M/s Macrogen, S. Korea for the sequencing services. The 16S rRNA gene sequence was aligned using BioEdit software, compared with gene sequences available in the databases (NCBI + DDBJ + EMBL) and deposited in GenBank with an accession number KT588655. The putative phylogenetic affiliation was determined using the naïve Bayesian rRNA classifier and RDP-II database with the 95% confidence (Wang et al., 2007; Cole et al., 2009).

Bioactivity from Marine *Actinobacteria*

Primary Screening of Marine *Actinobacteria* for Antibacterial Activity

The isolated and purified *Actinobacteria* isolates were screened for antibacterial activity by cross streak method (Balagurunathan and Subramanian, 2001) using Mueller-Hinton agar (Himedia, India) against eight different pathogenic bacteria; Gram-negative (*Escherichia coli* NCIM 2065, *Shigella boydii* NCIM, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Pseudomonas* sp. NCIM 2200, *Salmonella enteritidis* NCIM, and two Gram-positive bacteria (*Bacillus subtilis* NCIM 2920 and *Staphylococcus aureus* MTCC 96). Plates containing well grown RD-5 strain was cross streaked with pathogenic bacteria at 90° angles and incubated at 37°C overnight. Antagonism was observed by noting the absence or presence of pathogenic bacterial growth.

Optimization of Growth Conditions for the Production of Bioactive Compounds

The promising strain RD 5 was cultured in six different media; starch casein agar, yeast malt extract agar (ISP2), glycerol asparagine agar (ISP5), inorganic salt agar (ISP-4), tyrosine agar (ISP-7) and gause's synthetic agar (GSA) and incubated at 30°C for 7–9 days. The cell mass was measured by the dry weight of cell biomass after 24 h interval and compound production was measured using well diffusion method at 24 h interval for 9 days. The experiments were repeated three times for each assay.

Extraction of Bioactive Compounds and Bioactivity Assay

The most promising isolate (RD-5) was grown in the optimized gause's synthetic broth (GSB) media for the isolation of the active compounds. The selected isolate was inoculated in GSB medium and incubated for 7 days in shaking condition at 180 rpm at 30°C. Culture media was harvested every 24 h, centrifuged for 15 min at 8,000 rpm and collected supernatant was mixed with an equal volume of ethyl acetate followed by extraction with separating funnel (Ismail et al., 2009). The crude extract was obtained by removing the solvent using rotary evaporator. The dried crude extract was dissolved in methanol, and stock concentration was prepared as 100 mg/mL. The crude extracts (3 to 7 mg) were used for the bioactivity against different pathogenic bacteria using well diffusion method with Mueller Hinton agar (Nandhini and Selvam, 2011). Methanol used as a control and the bioactivity of

extracts was noted based on the zone of inhibition. Furthermore, the bacterial extract was evaluated for the different antioxidant and radicals scavenging activity.

DPPH Radicals Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the bacterial extract was determined using method reported by (Bersuder et al., 1998) using different concentrations of melanin (0.05–5.0 mg/mL). In test tubes, different concentration of melanin was taken, and volume was made up to 2 mL with distilled water. About 2 mL of 0.002% DPPH solution was added to each tube, mixed and incubated for 30 min in the dark. Reduction of DPPH radical was quantified at 517 nm using UV-Vis spectrophotometer. The percentage of DPPH radical scavenging activity was calculated as:

$$\text{DPPH radical scavenging activity [\%]} = [(A_c - A_s)/A_c] \times 100$$

Where, A_c and A_s were the absorbance of the control and sample, respectively. The experiment was conducted in triplicates.

Hydrogen Peroxide Radical Scavenging Activity

A solution of hydrogen peroxide (40 mmol/L) was prepared in phosphate buffer (pH 7.4). To 4 mL of bacterial extract of different range of concentrations (0.05–5.0 mg/mL), 0.6 mL of H₂O₂ solution was added. The absorbance was measured at 230 nm by the UV-visible spectrometer and percentage inhibition of H₂O₂ scavenging activity was calculated (Keser et al., 2012; Mishra et al., 2015; Patel et al., 2016).

$$\text{H}_2\text{O}_2 \text{ scavenging activity [\%]} = [(A_c - A_s)/A_c] \times 100$$

Where A_c and A_s were the absorbance of control and test samples, respectively. The experiment was conducted in triplicates.

Metal Chelating Activity

The ferrous ions chelating activity of the bacterial extract was analyzed (Dinis et al., 1994). Different concentration of extract (0.05–5.0 mg/mL) was made up with final volume 0.5 mL and mixed with 0.05 mL of 2 mM FeCl₂. About 0.2 mL ferrozine solution (5 mM) was added to the reaction mix, shaken vigorously and kept for 10 min at room temperature. The absorbance of the reaction mix was estimated at 562 nm and percentage inhibition of ferrozine-Fe²⁺ complex formations was calculated:

$$\% \text{ of inhibition} = (A_s/A_c) \times 100$$

Where A_c and A_s were the absorbance of control and test samples, respectively.

Cloning of PKS-I and PKS-II Genes

Two set of degenerative primers were designed to amplify internal fragment of KSα and PKS-I biosynthetic genes fragments from RD-5 strain (Table 1). PCR was done in 25 μL volume that contained 1X Taq buffer, 2.5 μL of dNTPs (2.5 mM), 20 pM primers (forward and reverse), 0.05 U of Taq DNA polymerase enzyme (Sigma, United States) and 10–15 ng genomic DNA. PCR was carried out with denaturation of the template DNA at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, primer annealing at 58°C for 120 s, for the KS of PKS-II domain while 55°C for 2 min was used for the amplification of K1F/M6R PKS-I gene and finally extension at 72°C for 4 min. Amplified PCR products were analyzed on 1% agarose gel, purified, cloned into pGEM-T easy vector (Promega, United States) and transformed to *E. coli* DH5α. Recombinant plasmid DNA was extracted using alkaline lysis method and confirmed by PCR with vector-specific primers M13F and M13R. Both cloned genes fragments, PKS-I and PKS-II were sequenced from M/s Macrogen Inc, South Korea and deposited in GenBank with the accession numbers MG459176 and MG459177, respectively.

Phylogenetic Analysis

The 16S rRNA gene sequences (KT588655) were subjected to BLASTn for the comparison with the other 16S rRNA gene sequences exist in GenBank and closest relative 16S ribosomal RNA sequences were retrieved from NCBI database (Zhang et al., 2000). Sequence alignment was performed with cluster W (Altschul et al., 1997), phylogenetic trees were constructed (using Mega ver. 6) with the neighbor-joining method and a bootstrap value of 1000 replicates (Tamura et al., 2013). The resultant sequence of both PKS-I and PKS-II genes fragment was also analyzed with BLASTx search and protein sequence were retrieved from NCBI, aligned and the phylogenetic tree was constructed using the neighbor-joining tree-making algorithm.

Statistical Analysis

Average well color development (AWCD), diversity richness (R), and Shannon evenness (E) were calculated by analysis of variance (ANOVA) of each strain based on color development with every 24 h. The cluster analysis was used to evaluate the most utilized substrate for each strain. The AWCD data was

TABLE 1 | List of primers used for amplification of non-ribosomal peptide synthetases (NRPS) and PKS-1 gene fragments and 16S rRNA.

Primer Name	DNA sequences (5'-3')	Name of product	Target size	Reference
27F 1492R	5'- AGAGTTGATCMTGGCTCAG -3' 5'- ACCTTGTTACGACTT -3'	16S rRNA	1.5 Kb	Lane, 1991
K1F M6R	5'-TSAAGTCAACATCGBCA-3' 5'-CGCAGGTTCSGTCACCAGTA-3'	Type-I polyketide synthases (PKS-I)	1.4 Kb	Ayuso-Sacido and Genilloud, 2005
KSαF KSαR	5'-TSGCSTGCTTGGAYGCSATC-3' 5'-TGGAANCCGCCAABCCTCT-3'	Ketosynthase gene (PKS-II)	700 bp	Metsa-Ketela et al., 1999

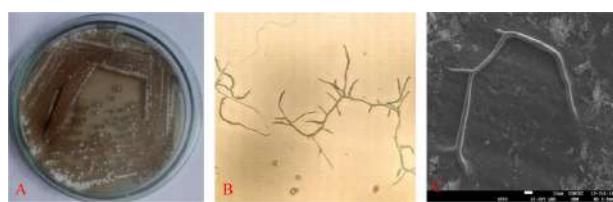


FIGURE 1 | Isolated colonies on **(A)** Gause's synthetic agar (GSA) medium, **(B)** microscopy and **(C)** SEM image of RD-5.

standardized to remove inoculum density effects. Ordination methods were used for principal component analysis (PCA) of the data taken at 96 h. The method categorised samples on scatter plots of two or more axes and the most closest micro-organism come together (Randerson, 1993; Podani, 2000). For the comparison of numerical responses in the 95 substrates, PCA plot reduced the multivariate data set (variables or individuals) and exhibited any changes in the variation of the data.

RESULTS

Isolation and Characterization of Actinobacterial Strains

A total of 11 different strains of *Actinobacteria* were isolated from Gulf of Khambat, Alang, Bhavnagar, Gujarat. The distinctly different isolates based on their morphological and pigmentation were purified by repeated streak method on Gause's Synthetic Agar medium and preserved at 4°C as on slant. All the isolates were screened with preliminary

cross streak assay. Out of them, Isolate RD-5 was found novel, additionally exhibits potent activity against pathogenic bacteria.

Selected strain was aerobic, Gram-positive and the colonies are dry, powdery, fuzzy with a concentric ring on agar surface which showed secondary metabolite production with diffusible brownish pigment were initially identified as *Actinobacteria* (Figure 1A). Microscopic examination of the strain was undertaken under a compound microscope. The short branched vegetative hyphae and aerial mycelia were sparse with a patchy distribution (Figures 1B,C).

Characterization of Microbial Strain(s) from the Selected Cultures Based on BIOLOG

All *Actinobacteria* were examined using Biolog System to obtain their metabolic profiles or biotyping. Biolog System analysis is based on carbon (C) utilization patterns of the *Actinobacteria* toward different carbon source. The ability to use a wide range of carbon source may indicate that the *Actinobacteria* were able to survive in the different environment in nature. Metabolic profiles resulted from Biolog GENE III System analysis indicated the 11 *Actinobacteria* were differentiated into different strains. As shown in (Supplementary Table S1) the eleven strains of *Actinobacteria* (RD-1 to RD-9 and RD-15 and RD-16) have the different capability to metabolize 95 carbon sources from GENE III microplates. The 95 carbon sources are categorized as polymers, sugar and sugar derivatives, carboxylic acids and methyl esters, carboxylic acids and methyl esters, alcohol, nucleosides and nucleotides and sugar phosphates. Of the 95 carbon sources, only 75 can be utilized by the all eleven strains of *Actinobacteria*. Strain RD-5 was one from the eleven colonies,

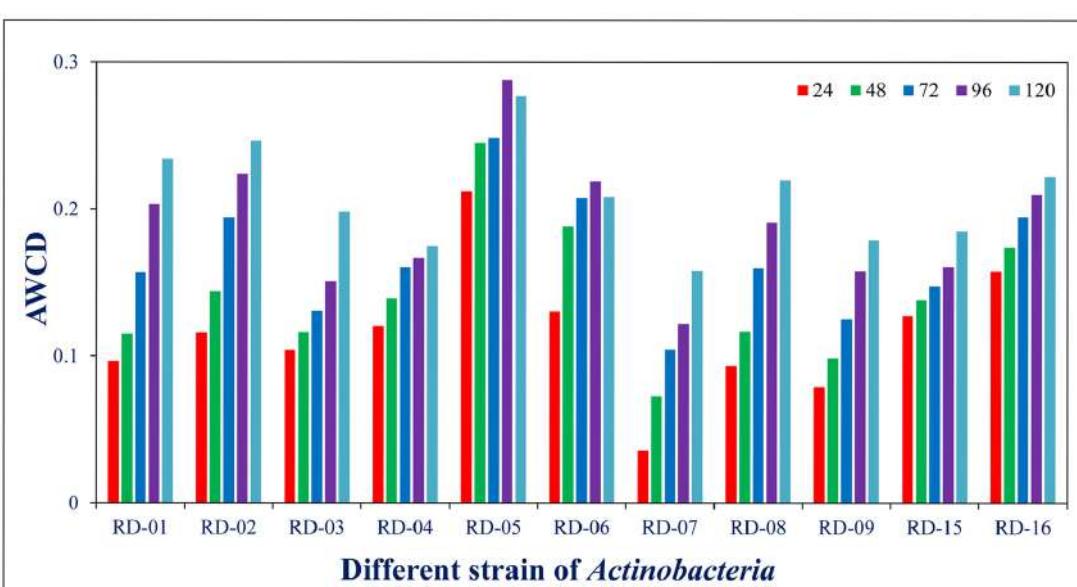
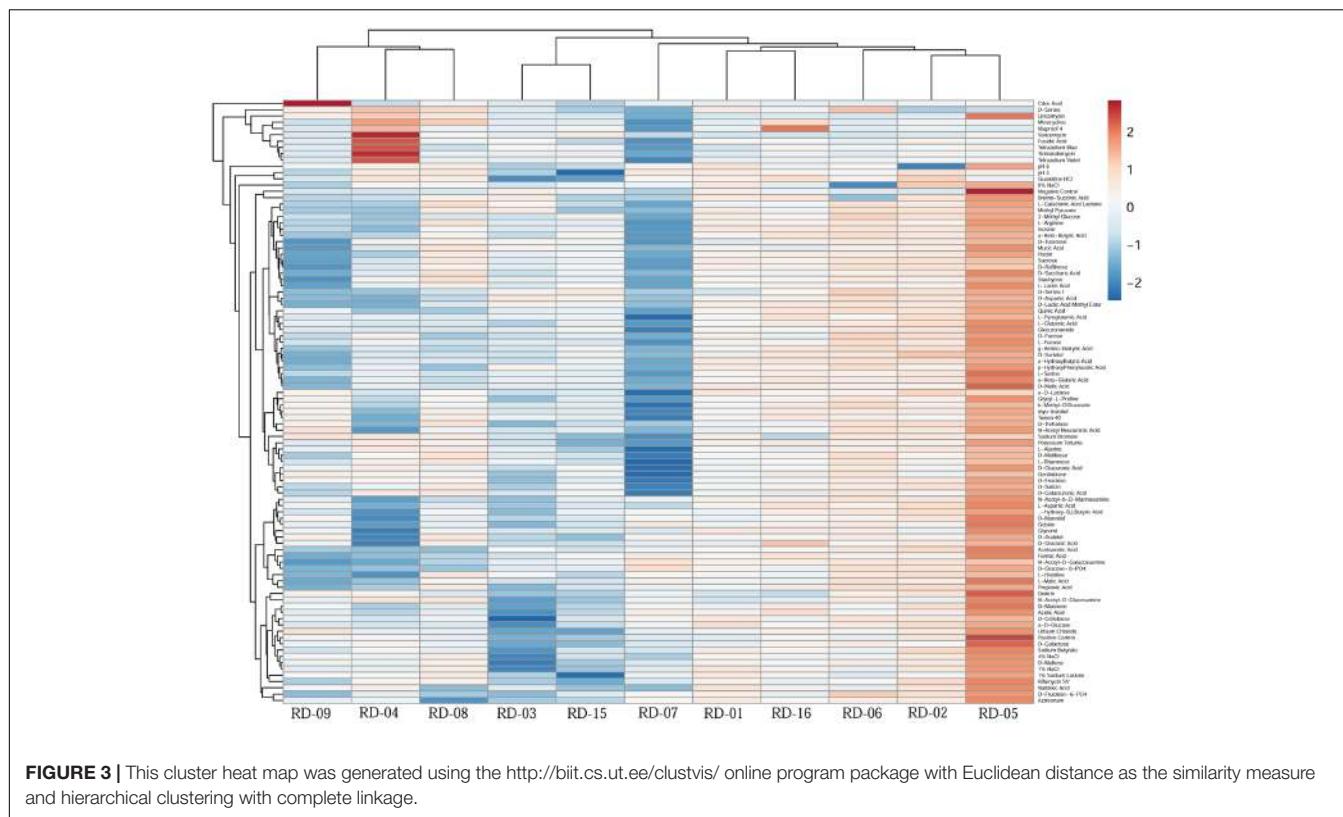


FIGURE 2 | Average well color development (AWCD) of metabolized substrates in BIOLOG GENE III in every 24 h.



showing significantly higher in carbon sources activity with 90 substrates followed by Strain RD-6 and RD-9 using 89 followed by RD-4, and RD-16 using 88 RD-15, RD-8 and RD-1, RD-2 and RD-7 and RD-3 with 87, 86, 82, and 81, respectively.

Monitoring Color Development in BIOLOG™ GENE III Plates with Other Reference Strain of *Actinobacteria*

Normalized value of AWCD further evidence that different strain cluster (**Figure 2**). In the hierarchical clustering with the complete linkage, RD-5 shows the most of the substrate is utilized in 96 h, but other strain is less used the substrate (**Figure 3**). PCA of ordinance methods scatter plot of each strain in BIOLOG allow the sample to be represented two or more axis PC1 (55.5%) second one PC2 (15%) RD-5 was scatter in PC2 axis (**Figure 4**).

FAME Analysis

The chemotaxonomic study of the potential isolate RD-5 revealed that it belongs to the *Actinobacteria*. Saturated iso/anteiso-branched fatty acids with C15–C17 long chain was detected as major cellular fatty acids. The cluster analysis of FAME profile showed correlation among organisms by Euclidian distance. Cluster containing isolates identified was delineated at 22.5 ED (**Figure 5**) were closely matched those of *Streptomyces*, but considerable differences were recorded among the eleven strains.

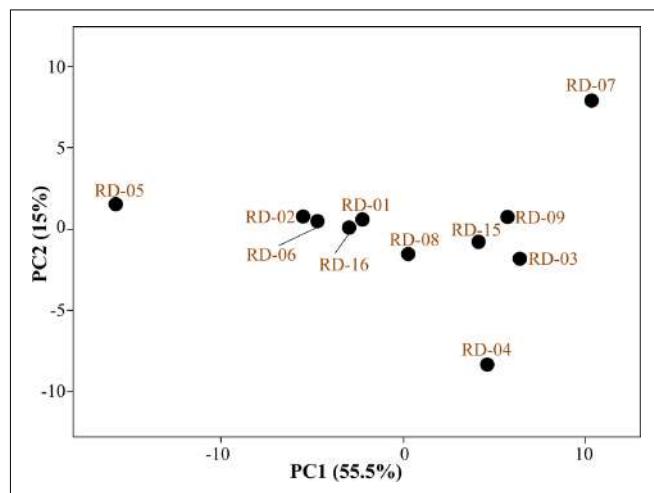


FIGURE 4 | Principal component analysis (PCA) of all 11 strain of *Actinobacteria*.

Phylogenetic Analysis of 16S rRNA

The 16S rRNA gene of RD-5 was amplified and sequenced (KT588655). The partial 16S rRNA gene sequence of RD-5 covered a stretch of 1382 bp having an average 54.8% G+C content. Nucleotides were subjected to BLASTn analysis (**Table 2**) which showed the 84% similarity with *Streptomyces variabilis*. The nucleotide sequences of the type strain were retrieved from the NCBI, and a phylogeny was studied

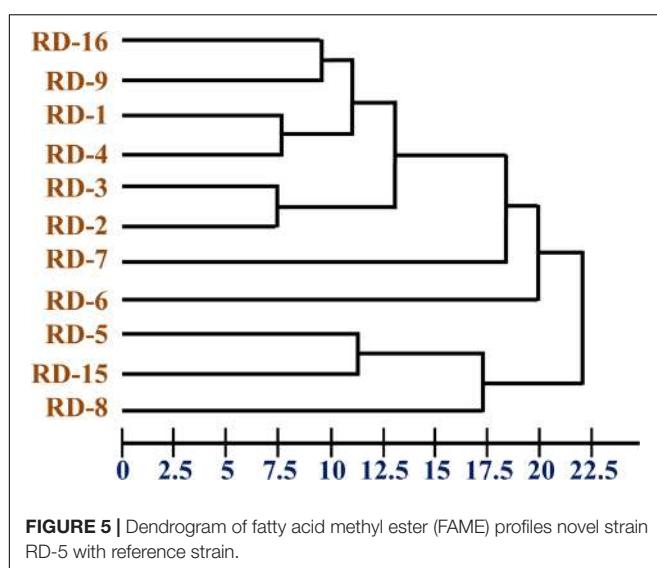


FIGURE 5 | Dendrogram of fatty acid methyl ester (FAME) profiles novel strain RD-5 with reference strain.

(Figure 6). The phylogenetic position of the strain was within a cluster that contains *Streptomyces fenghuangensis* (KJ575043), *Actinomycetales bacterium* (KT021825), and *Streptomyces* sp. RD-4 (KT588654). *Streptomyces* sp. RD-5 was posed with as single branch and shared with 99% Query cover and 82% sequence identity with a closed group. Another phylogenetic tree was constructed with the reference strain, and out-group were taken *E. coli*, and it does not show any similarity match with reference strain (Figure 7).

These 16S rRNA sequences were also classified in Rdp Naive Bayesian rRNA Classifier Version 2.11 database with >1200 Nucleotide and Confidence threshold is 95% it shows domain Bacteria unclassified_Actinomycetales at the genus level.

Bioactivity from Marine Actinobacteria

Primary Screening of Antibacterial Activity

Isolated different marine *Actinobacteria* were primarily screened with the cross streak method for bioactivity against pathogenic bacteria. On the basis of maximum inhibition of pathogenic strain, RD-5 was selected for the further screening.

Culture Media Study and Optimization of Cell Growth and Production of the Compound

To maximize the antibacterial production as well as cell mass, strain RD-5 was cultured in five different media, out of six different media, GSA medium supposed to maximize the cell mass as (Table 3 and Figure 8) well as the production of antibacterial activity. The growth curve for strain RD-5 and the antibacterial activity produced in the GSA medium was measured every 24 h of the interval (Figure 9). Strain RD-5 showed the first phase of growth 72 h post inoculation. The second phase occurred during 168 h, and thereafter stationary phase occurred. This strain produced compounds after around 72 h and production increased depending on cell growth. The compounds produced were maximized at the end of the second phase. *Shigella boydii* and *Klebsiella pneumoniae*

TABLE 2 | The BLASTn results, of 16S rRNA according to the NCBI database.

Description	Accession number	Maximum query cover	Maximum score	Total score	Maximum identity (%)
<i>Streptomyces variabilis</i> strain RD-5 16S ribosomal RNA gene, partial sequence	KT588655.1	100%	2553	2553	100%
<i>Streptomyces variabilis</i> strain HBUM173496 16S ribosomal RNA gene, partial sequence	EU841661.1	99%	1299	1299	84%
<i>Streptomyces variabilis</i> strain 173634 16S ribosomal RNA gene, partial sequence	EU570414.1	99%	1085	1085	81%
<i>Streptomyces variabilis</i> strain 173500 16S ribosomal RNA gene, partial sequence	EU570413.1	99%	1055	1055	81%
<i>Streptomyces</i> sp. RD4 16S ribosomal RNA gene, partial sequence	KT588654.1	99%	1168	1168	82%
<i>Streptomyces fenghuangensis</i> strain NIOT-Ch-34 16S ribosomal RNA gene, partial sequence	KJ575043.1	99%	1142	1142	82%
<i>Streptomyces radiopugnans</i> strain HBUM174024 16S ribosomal RNA gene, partial sequence	EU841699.1	99%	1127	1127	82%
<i>Streptomyces nanhaiensis</i> strain JA 24 16S ribosomal RNA gene, partial sequence	KJ947850.1	94%	1050	1050	81%
<i>Streptomyces atacamensis</i> strain C60 16S ribosomal RNA gene, partial sequence	NR_108859.1	99%	1092	1092	81%

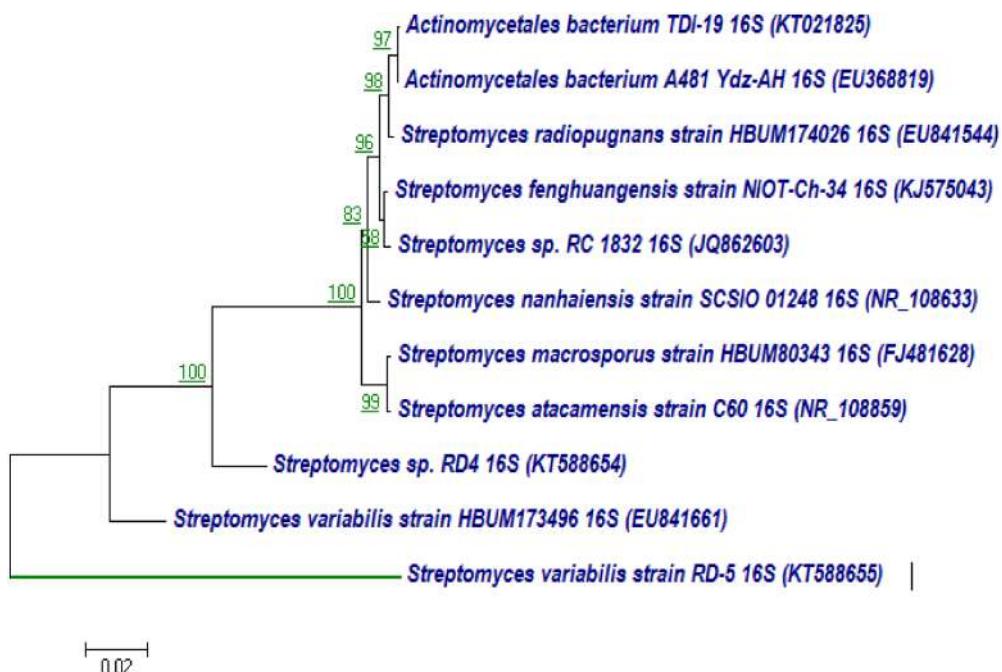


FIGURE 6 | Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain RD-5 and closely related members of the genus *Streptomyces*. Numbers at nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar, 0.02 substitutions per nucleotide position.

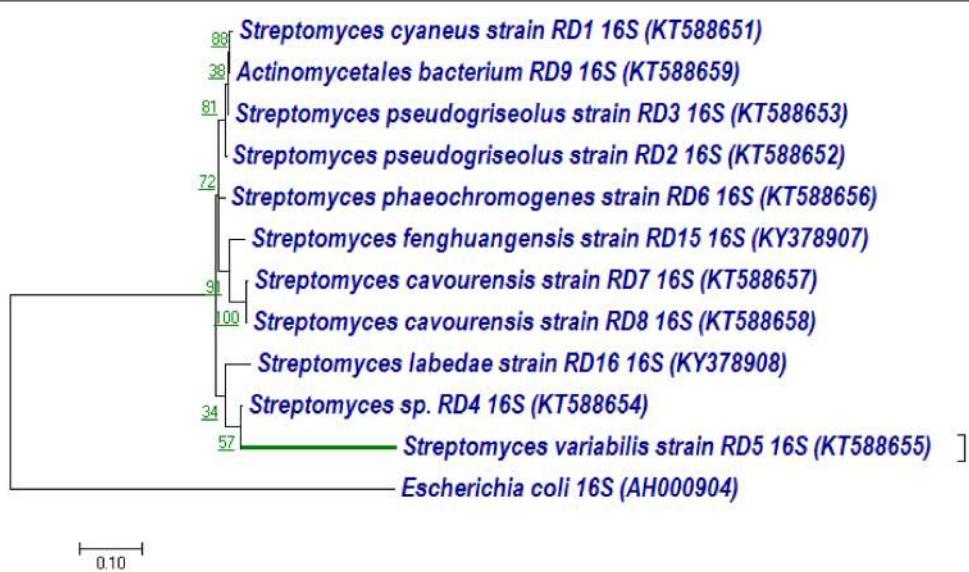


FIGURE 7 | Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain RD-5 and closely related members of the genus *Streptomyces* as reference strain with out-group *Escherichia coli*. Numbers at nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar, 0.10 substitutions per nucleotide position.

showed maximum antibacterial activity from an extract of RD-5 which was 27 mm in both. While the response of *Pseudomonas* sp. was less compared to other pathogenic strains (19 mm).

Secondary Screening of Antibacterial Compound

The bioactive compounds were extracted from the fermented broth using ethyl acetate solvent, and concentrated crude extract which was used as test compound was carried out by agar

TABLE 3 | Cultural characteristics of *Streptomyces variabilis* RD-5 on different media.

Medium	Growth	Aerial mycelium	Substrate mycelium	Pigment
Starch casein agar	Moderate	Brownish white	Brownish white	None
Yeast malt extract agar (ISP2)	Good	White	Brownish white	Yellow
Inorganic salt agar (ISP-4)	Good	Brownish white	Brownish white	Yellow
Glycerol asparagine agar (ISP5)	Good	Slight orange	Light brown	Light yellow
Tyrosine agar (ISP-7)	Moderate	Light brown	Brownish white	None

well diffusion method. The antibacterial activity of crude extract at concentration of 5 mg/well was assayed against pathogenic strain *Shigella boydii* (13 mm), *Klebsiella pneumonia* (24 mm), *Enterobacter cloacae* (16 mm), *Bacillus pumilus* (22 mm), *Salmonella enteritidis* (14 mm), *Staphylococcus* sp. (16 mm), *E. coli* (15 mm), *Pseudomonas* sp. (17 mm) (**Figure 10**).

Antioxidant and Scavenging Activity

DPPH is a stable free radical having absorption maxima at 517 nm. The results of DPPH radical scavenging activity of ethyl acetate extract of *S. variabilis* is depicted in (**Figure 11**). Bacterial extract showed 43.67–82.86% DPPH free radical scavenging activity at 0.05–5.0 mg/mL as compared to ascorbic acid which showed 86% activity at 0.05 mg/mL concentration. The activity of the extract was increased with an increase in concentration and reached to around 55% at 1.0 mg/mL concentration against 98% of ascorbic acid. Further, increase in concentration marginally influenced activity. It was observed that extract of *S. variabilis* showed maximum activity at 2 mg/mL concentration after that slight difference was observed. The activity of the extract increased up to 2.0 mg/mL concentration, a further increase in concentration did not influence activity. Metal chelating

activity of extracts of various *Actinobacteria* ranged from 16% as compared to Na-EDTA which showed 65% activity at 0.05 mg/mL concentration (**Figure 12**). With an increase in concentration of extract, the activity increased to 16–89% at 0.05–5 mg/mL while in case of Na-EDTA, 0.5 mg/mL, at concentration yielded 87.5% metal chelating activity. Here, *S. variabilis* exhibited maximum activity at 5 mg/mL concentration. H₂O₂ scavenging activity of extracts ranged from 64% as compared to ascorbic acid exhibiting 74.5% activity at 0.05 mg/mL concentration (**Figure 13**). As far as H₂O₂ scavenging activity is concerned, extract of *S. variabilis* exhibited activity almost at par with that of ascorbic acid.

Phylogenetic Analysis PKS-I and PKS-II Genes

BLASTx analysis of PKS-I and PKS-II amino acid biosynthetic genes of strain RD-5 showed the 99–92% of query cover and 54–52% sequences identity with their closest matches (**Tables 4, 5**). The phylogenetic tree was inferred by maximum likelihood method using the amino acid sequences of both PKS-I and PKS-II of RD-5 novel strain. PKS-I gene sequences showed the maximum identity with *S. hygroscopicus*, *Streptomyces* sp. NBRC 109436, *S. atratus*, *S. melanoporofaciens*, and *S. atratus* with 59–56% identity (**Figure 14**). The PKS-II

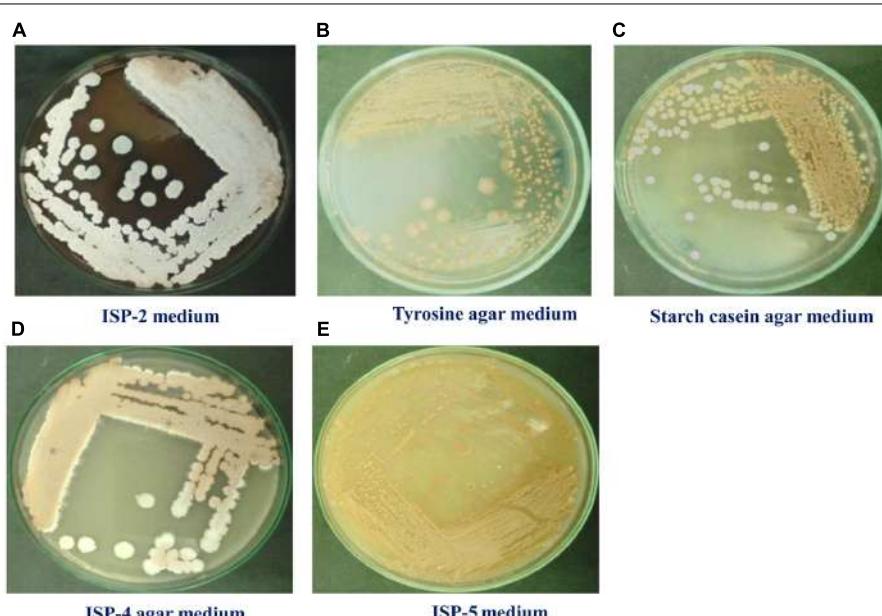
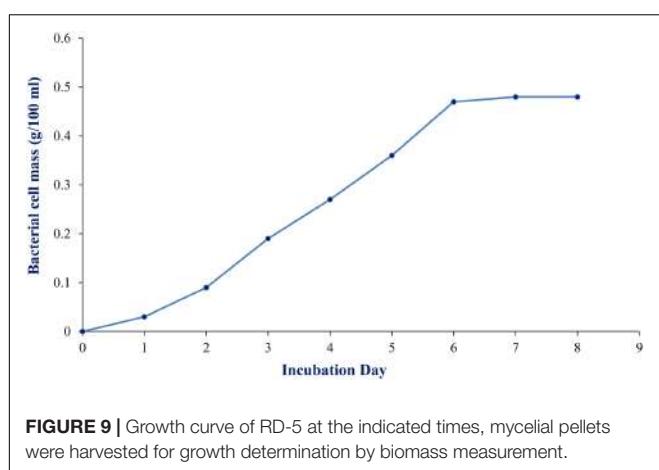


FIGURE 8 | Growth of RD-5 in different media such as **(A)** ISP-2 medium, **(B)** Tyrosine agar medium, **(C)** Starch casein agar medium, **(D)** ISP-4 agar medium and **(E)** ISP-5 medium.



gene sequences showed the maximum identity of 71–49% with previously reported sequences. Amino acid search analysis showed similarity with universal stress and hypothetical proteins from *Streptomyces* sp. NRRL F-5727 (WP_031002278.1), *S. globisporus* (WP_030690697.1), *S.*

exfoliates (WP_024756517.1), *S. laurentii* (BAU87338.1), and *Streptomyces* sp. CcalMP-8W (WP_018491225.1) (**Figure 15**).

DISCUSSION

Adaptations of marine bacteria have developed prodigious metabolic and physiological ability to survive in the extreme conditions that allows them to produce different kind of metabolites, which could not be produced by the terrestrial ones. *Actinobacteria* are well established for producing secondary metabolites with novel antibiotics which are of immense importance to prevent multi-drug resistant pathogens. The *Actinobacteria* produce spores which generally resist desiccation and show to some extent higher resistance toward environmental fluctuation to adopt the harsh condition comparative to others microbes (Hopwood and Wright, 1973).

In the present study, total 11 different isolates were screened, out of them, one promising marine *Actinobacteria* strain, identified as *S. variabilis* RD-5 showed the novelty with antagonistic properties. The phylogenetic position

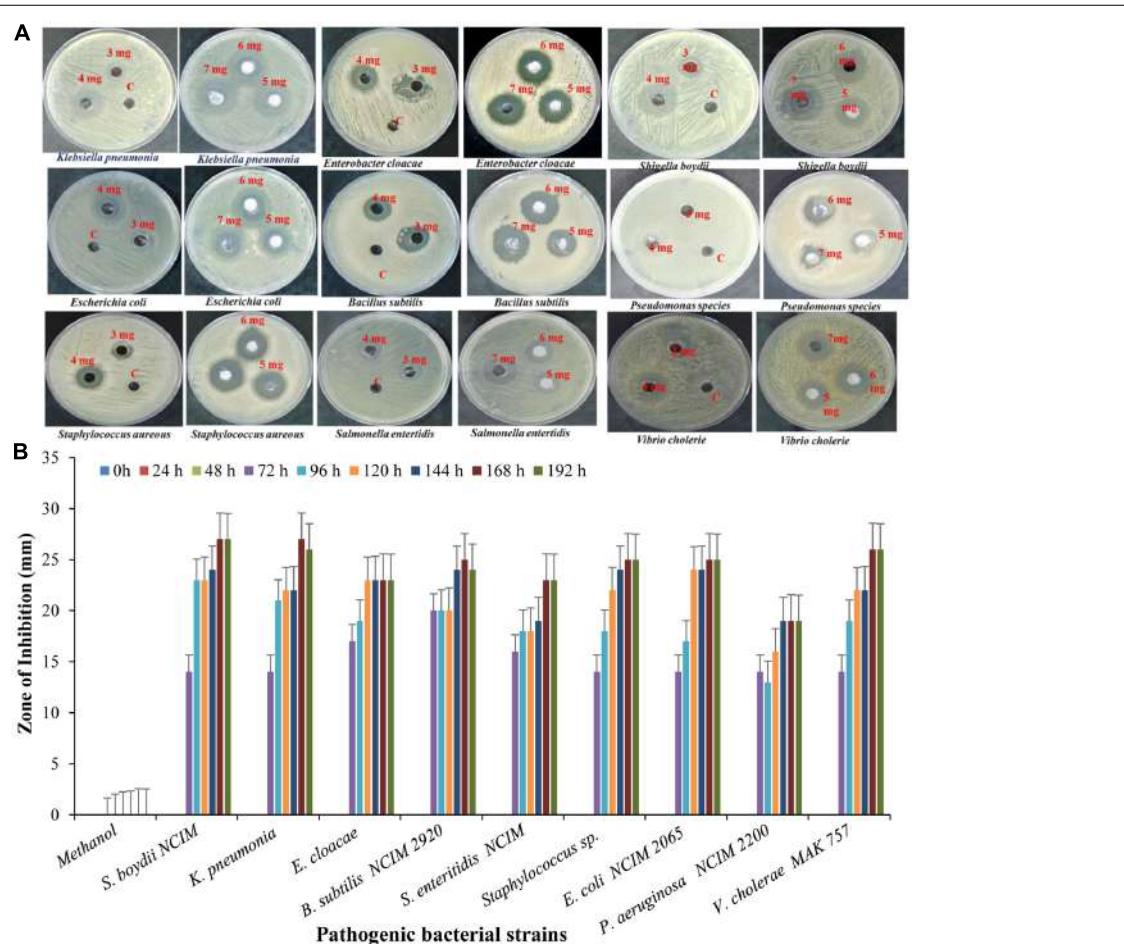


FIGURE 10 | Antibacterial activity of crude extract against different pathogenic strain with **(A)** different concentration and **(B)** different time interval.

of the *S. variabilis* RD-5 suggested that isolated strain from coastal areas of Gulf of Khambhat have a potential diverse arrangement with novelty which can be useful for many of the applications and can be explored broadly.

Culture medium, GCA, found to be the best for isolation of marine *Actinobacteria* *S. variabilis* RD-5. The strain showed optimum growth at 30°C on GSA and ISP-2 media. A retarded growth was also shown on Tyrosine agar medium and ISP-5 medium. *S. variabilis* RD-5 was characterized morphologically and microscopically which confirmed its identity as *Streptomyces* genus (Williams et al., 1989; Manfio et al., 1995).

BIOLOG analysis suggested higher AWCS in RD-5 compared to others reference strains of *Actinobacteria*. BIOLOG assay further showed that strain *S. variabilis* RD-5 utilized a wide range of substrates. The cluster and PC analyses showed substrate utilization pattern similar to other *Actinobacteria* community (Figures 5, 6). The cluster analysis and PCA showed the comparability of both experiments and further confirmed the overlap metabolic fingerprints among the different strains of *Actinobacteria*.

The 16S rRNA gene sequencing and phylogenetic analysis revealed that RD-5 is a novel strain, having identity below 85% as shown by RDP-II classifier. Phylogenetic analysis showed that RD-5 strain was closely related to novel *Actinobacteria* bacterium such as TDI19 (KT021825), *S. radiopugnans* strain HBUM174026 (EU841544), *Streptomyces* sp. RC 1832 (JQ862603), *S. nankaiensis* strain SCSIO 01248 (NR_108633), isolated from different geographical location including deep-sea sediment (Tian et al., 2012).

PCR amplification and identification of these biosynthetic genes was very important for assessing its potential for both culturable and unculturable microorganism (Minowa et al., 2007). Large numbers of biologically active compounds are identifying which is encoded by a set of genes, in which PKS-I and PKS-II are responsible for the biosynthesis of the active metabolite (Ayuso-Sacido and Genilloud, 2005). The presence of types I and II PKS gene in *S. variabilis* RD-5 showed a direct correlation with the identified bioactive compound, which is polyketide in nature.

The extracted compound of *S. variabilis* RD-5 was found the most active against pathogenic bacteria, and thus it can play an important role in clinical appliances. Extracellular enzymes play a key role in the recycling of organic carbon and nitrogen compounds in biotechnology. The strain RD-5 exhibited highest antibacterial activity against *Klebsiella pneumonia*. The result showed that secondary metabolite active compounds containing antibacterial activities were extracellular and it could be extracted, quantified and further explored for the discovery of new drugs (Passari et al., 2015). To best of our knowledge, this is the first report of *S. variabilis* RD-5 having strong antimicrobial activity against bacteria. From the results, we concluded that the results of morphological, biochemical characteristics and polyphasic approach; the isolate *S. variabilis* RD-5 was the member of *Actinobacteria*, which secretes bioactivity with novel characteristics.

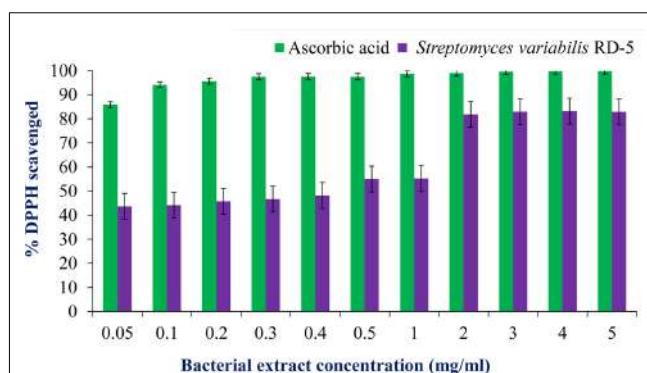


FIGURE 11 | Antioxidant activity of bacterial extract with different concentration.

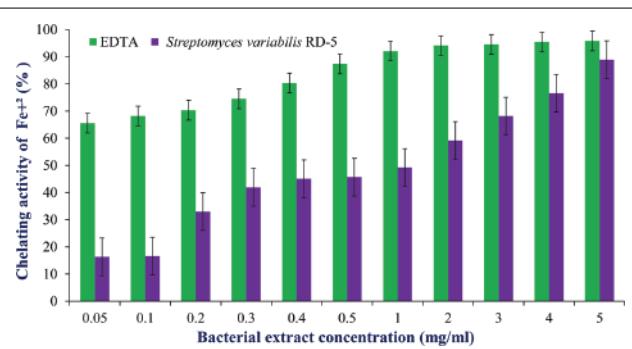


FIGURE 12 | Chelating activity of bacterial extract with different concentration.

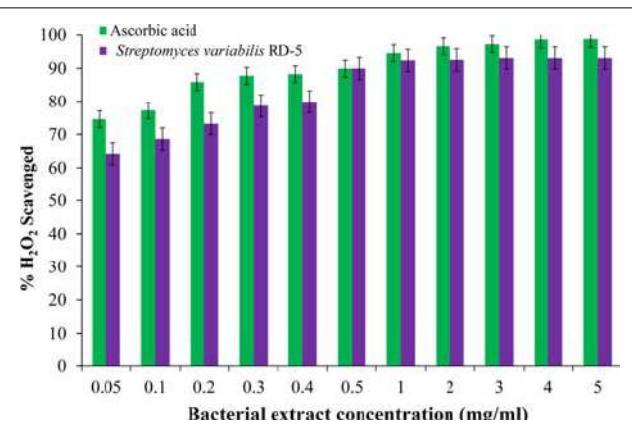


FIGURE 13 | H₂O₂ scavenged activity of bacterial extract with different concentration.

The crude extract was tested and found good antioxidant properties which can be useful for further research development to make it the industrially important. The radical scavenging activity of the extract was concentration dependent, and gradual increase of concentration increased the activity which was supported by the report of Kumaqai et al. (1993). The DPPH free radical scavenging assay was extensively used to measure antioxidant capacity. Antioxidants react with DPPH and reduce

TABLE 4 | The BLASTx results, of PKS-I according to the NCBI database.

Description	Accession no.	Maximum query cover	Maximum score	Total score	Maximum identity (%)
Polyketide synthase (<i>Streptomyces hygroscopicus</i>)	WP_060954384.1	99%	419	809	56%
Polyketide synthase (<i>Streptomyces</i> sp.) NBRC 109436	WP_064455733.1		418	813	56%
Polyketide synthase 12 (<i>Streptomyces atratus</i>)	SFY45126.1	99%	414	709	59%
Type I polyketide synthase (<i>Streptomyces caatingaensis</i>)	WP_053161268.1	99%	381	424	55%
type I polyketide synthase (<i>Streptomyces auratus</i>)	EJJ02441.1	99%	381	514	54%
AGR0001					
Type I polyketide synthase 3 (<i>Streptomyces</i> sp.)	APD71668.1	99%	379	1103	53%
Beta-ketoacyl synthase (<i>Streptomyces hygroscopicus</i>)	WP_078638584.1	99%	392	685	54%
Polyketide synthase 12 (<i>Streptomyces</i> <i>melanosporofaciens</i>)	SED16442.1	99%	408	813	55%
Polyketide synthase (<i>Streptomyces violaceusniger</i>)	WP_014057309.1	99%	408	817	56%
Polyketide synthase (<i>Streptomyces hygroscopicus</i>)	WP_078646099.1	99%	407	808	55%

TABLE 5 | The BLASTx results, of PKS-II according to the NCBI database.

Description	Accession number	Maximum query cover	Maximum score	Total score	Maximum identity (%)
Universal stress protein (<i>Streptomyces</i> sp.) WM6368	WP_053703232.1	96%	159	159	58%
Universal stress protein (<i>Streptomyces</i> sp.) 3211	WP_079403829.1	96%	157	157	58%
Universal stress protein (<i>Streptomyces</i> sp. H021)	WP_053631949.1	96%	155	155	57%
Universal stress protein (<i>Streptomyces virginiae</i>)	WP_030895366.1	96%	155	155	57%
Universal stress protein (<i>Streptomyces globisporus</i>)	WP_030690697.1	92%	188	188	66
Universal stress protein (<i>Microtetraspera glauca</i>)	WP_030493181.1	92%	184	184	70%
Universal stress protein (<i>Streptomyces</i> <i>flavochromogenes</i>)	WP_030326218.1	92%	173	173	62%
Universal stress protein (<i>Streptomyces venezuelae</i>)	WP_055640132.1	92%	171	171	92%
Universal stress protein (<i>Streptomyces griseus</i>)	WP_030748761.1	92%	169	169	68%
MULTISPECIES: universal stress protein (<i>Streptomyces</i>)	WP_030648525.1	92%	168	168	63%

the DPPH molecules equal to the number of freely available hydroxyl groups (Matthäus, 2002). The DPPH scavenging activity depends on the degree of due to its ability to donate hydrogen proton. With the same concentration, the isolate was capable of reducing Fe^{3+} ions which indicated the presence of active compounds in the solvent extracts (Kekuda et al., 2010). *S. variabilis* RD-5 is potential sources of antioxidants, which reflects by high hydrogen peroxide activity, is useful

in preventing the progress of various oxidative stress-related disorders (Poongodi et al., 2012). Hydrogen peroxide has ability to cross cell membrane easily and also reacts with metal ions (Fe^{2+} and/or Cu^{2+}) to produce ROS (reactive oxygen species) such as hydroxyl free radical which have toxic effects (Swant et al., 2009). Thus, the present study suggests that the Actinobacterial extract of RD-5 can act as better antioxidant agents for removing H_2O_2 .

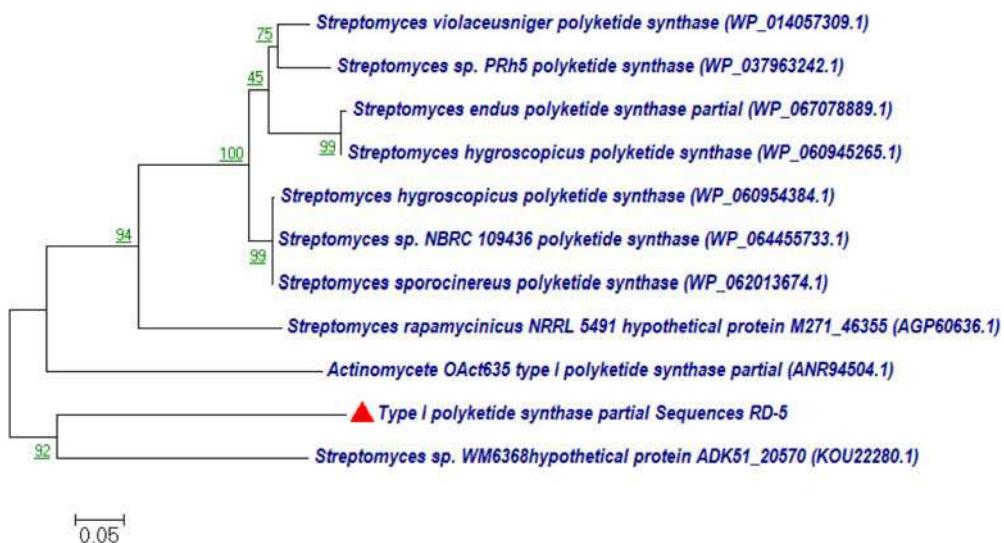


FIGURE 14 | Representative neighbor-joining tree of PKS-I amino acid sequences. The scale bar indicates the number of substitutions that occur per site.

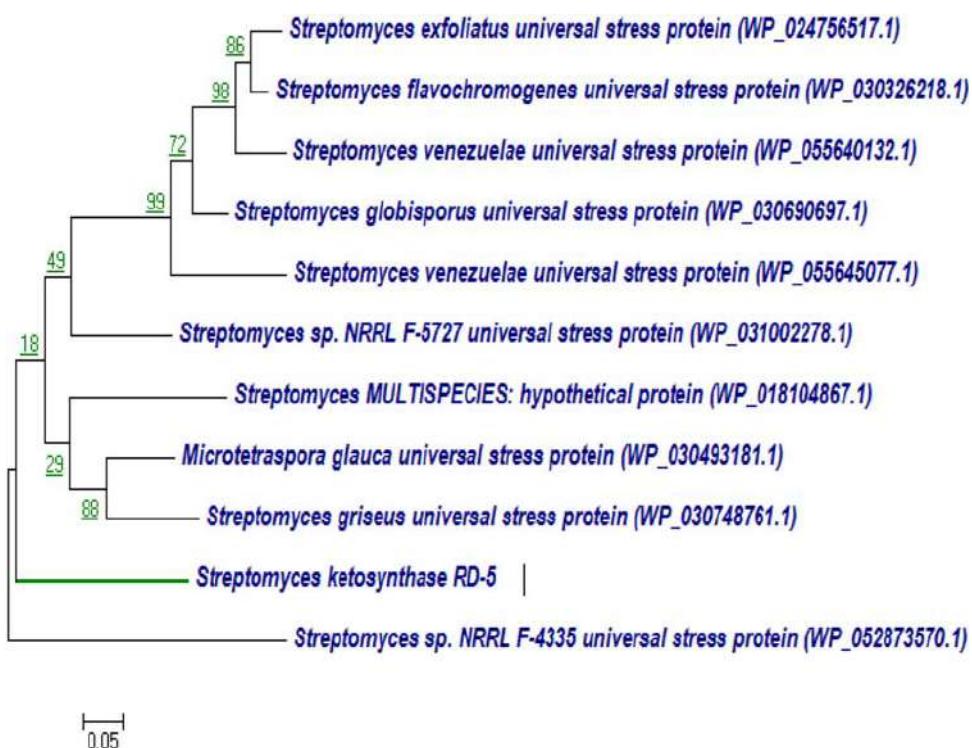


FIGURE 15 | Representative neighbor-joining tree of PKS-II amino acid sequences. The scale bar indicates the number of substitutions that occur per site.

According to a report of Thenmozhi and Kannabiran (2012), ethyl acetate extract of *Streptomyces* species VITSTK7, isolated from marine environment of the Bay of Bengal, exhibited 43.2% DPPH scavenging activity and 51% metal chelating activity at 10 mg/mL concentration. Similarly, Karthik et al. (2013) reported antioxidant activity of three marine *Actinobacteria* isolated from marine sediments of Nicobar Islands whereas phenolic

compounds extracted from *Streptomyces* sp. LK-3 exhibited 76% DPPH scavenging activity at 100 µg/mL. Two phenolic compounds from *Streptomyces* sp. JBIR-94 and JBIR-125 showed DPPH scavenging activity with an IC value of 11.4 and 35.1 µM, respectively. Sowndhararajan and Kang (2013) studied free radical scavenging potential of culture filtrate of *Streptomyces* sp. AM-S1 isolated from forest humus soil in Gyeongsan,

South Korea where ethyl acetate extract exhibited higher activity as compared to the lyophilised cell-free supernatant. According to Rao and Rao (2013), the extracts of *Actinobacteria* isolated from mangrove soil of Vishakhapatnam region showed 46–70% DPPH scavenging activity and 68–78% FRAP activity at 20 µg/mL concentration. Karthik et al. (2014) reported an extracellular protease produced by a marine *Streptomyces* sp. MAB 18 which exhibited antioxidant activity. *Nocardiopsis alba* isolated from mangrove soil collected from Andhra Pradesh, India, exhibited antioxidant activity. The potential fraction obtained by chromatography showed antioxidant activity at par with standard ascorbic acid (Janardhan et al., 2014). Nagaseshu et al. (2016) reported antioxidant activity of methanol extracts of *Actinobacteria* isolated from marine sediment collected from Kakinada coast. They also correlated the antioxidant activity of the extract which cytotoxic and antiproliferative activities.

The results of FAME of carbon chain length C15–C17 is consistent with the long carbon chain with saturated fatty acids which is used to produce phospholipids for *Streptomyces* cell membranes. Possibly *S. variabilis* RD-5 makes a triglyceride lyase which breaks bonds present between the carbon atoms, in resultant the FAMEs were generated (Lu et al., 2013). Further elucidation of genome sequences of strain RD-5 should be helpful for the investigation how the FAMEs were generated.

CONCLUSION

Adaptation of marine microorganism has developed prodigious physiological and metabolic capacities to survive in a harsh condition that triggered them to synthesize different metabolites, which could not be produced by the terrestrial ones. In the

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- present study, *S. variabilis* RD-5 was isolated from Gulf of Khambat, Alang, Bhavnagar, and screened for its ability to produce the bioactive compound. The extracted compounds show good antibacterial and antioxidant properties. Extracellular enzymes play a key role in recycling of organic carbon and nitrogen compounds in biotechnology.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KM and AM. Performed the experiments: RD and RK. Analyzed the data: RD, RK, and AM. Secured the funds to support this research: KM and BJ. Wrote the paper: RD and RK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmich.2017.02420/full#supplementary-material>

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Antibacterial Activity of Endophytic Actinomycetes Isolated from the Medicinal Plant *Vochysia divergens* (Pantanal, Brazil)

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Endophytic actinomycetes from medicinal plants produce a wide diversity of secondary metabolites (SM). However, to date, the knowledge about endophytes from Brazil remains scarce. Thus, we analyzed the antimicrobial potential of 10 actinomycetes isolated from the medicinal plant *Vochysia divergens* located in the Pantanal sul-mato-grossense, an unexplored wetland in Brazil. Strains were classified as belonging to the *Aeromicrombium*, *Actinomadura*, *Microbacterium*, *Microbispora*, *Micrococcus*, *Sphaerisporangium*, *Streptomyces*, and *Williamsia* genera, through morphological and 16S rRNA phylogenetic analyzes. A susceptibility analysis demonstrated that the strains were largely resistant to the antibiotics oxacillin and nalidixic acid. Additionally, different culture media (SG and R5A), and temperatures (28 and 36°C) were evaluated to select the best culture conditions to produce the active SM. All conditions were analyzed for active metabolites, and the best antibacterial activity was observed from metabolites produced with SG medium at 36°C. The LGMB491 (close related to *Aeromicrombium ponti*) extract showed the highest activity against methicillin-resistant *Staphylococcus aureus* (MRSA), with a MIC of 0.04 mg/mL, and it was selected for SM identification. Strain LGMB491 produced 1-acetyl-β-carboline (**1**), indole-3-carbaldehyde (**2**), 3-(hydroxyacetyl)-indole (**4**), brevianamide F (**5**), and cyclo-(L-Pro-L-Phe) (**6**) as major compounds with antibacterial activity. In this study, we add to the knowledge about the endophytic community from the medicinal plant *V. divergens* and report the isolation of rare actinomycetes that produce highly active metabolites.

Keywords: actinomycetes, endophytes, *Vochysia divergens*, pantanal, MRSA, secondary metabolites

INTRODUCTION

Endophytes are microorganisms that inhabit the internal tissues of plants without causing any negative effects, and actinomycetes isolated from plants have been widely studied due their ability to produce active metabolites (Kim et al., 2000; Zhao et al., 2011; Kadiri et al., 2014; Golinska et al., 2015; Savi et al., 2015a,b). Actinomycetes have been used for drug discovery for more than

five decades, producing more than 10,000 bioactive compounds. Of these ~75% are produced by *Streptomyces*, the by far mostly explored actinomycete genus. The remaining 25% bioactive compounds were isolated from “rare actinomycetes”, i.e., actinomycetes isolated in lower frequency than *Streptomyces* (Rong and Huang, 2012; Tiwari and Gupta, 2012). Since, the rare actinomycetes are an underexplored group, the use of these organisms, and their compounds have gained great importance in drug discovery programs (Rong and Huang, 2012; Tiwari and Gupta, 2012), mainly to combat infections caused by resistant microorganisms. The widespread use of broad-spectrum antibiotics has created a strong selective pressure, resulting in survival, and spread of resistant bacteria (Davies and Davies, 2010). The increase in bacterial resistance is a major concern for public health (Ventola, 2015). Unfortunately, many pharmaceutical companies have reduced or eliminated their search for new antibiotics, due to economic reasons, exasperating the problem further (Borrero et al., 2014). In order to find microorganisms with potential to produce active metabolites our group has been searching endophytic microorganisms from medicinal plants located in underexplored environments, such as the Brazilian wetland regions (Savi et al., 2015a,b; Hokama et al., 2016; Peña et al., 2016; Santos et al., 2016; Tonial et al., 2017). The Brazilian Pantanal is the largest wetland in the world, and it is characterized by two seasons: flooding and the dry. Hence, the Pantanal has developed a peculiar biological diversity regarding its fauna and flora (Alho, 2008). According to Arieira and Cunha (2006), only 5% of the species of plants of the Pantanal can survive the stress caused by drought and flood periods. Among them is the medicinal plant *Vochysia divergens*, which is commonly used in form of syrups and teas for the treatment of colds, coughs, fever, pneumonia, and other diseases (Pott et al., 2004). In a study carried out with endophytes from *V. divergens*, Savi et al. (2015a) identified actinomycetes able to produce highly active metabolites. However, the study was performed with a small number of isolates, and the diversity of *V. divergens* remained little explored. Thus, the focus of this study is to identify endophytic actinomycetes from the medicinal plant *V. divergens* and to assay their secondary metabolites, dependent on different culture conditions, against clinical pathogens associated with antibiotic resistance.

MATERIALS AND METHODS

Sample Collection

V. divergens leaves with no marks or injuries were collected from 21 plants located in the Pantanal sul-mato-grossense/Brazil, specifically in two regions of the Pantanal of Miranda, Abobral ($19^{\circ}30'09.5''S$, $57^{\circ}02'32.2''W$) and São Bento ($19^{\circ}28'53.9''S$, $57^{\circ}02'36.9''W$).

Isolation of Actinomycetes

The leaves from *V. divergens* were subjected to surface sterilization according to a protocol described by Petri (1986). The leaves were fragmented (8×8 mm) and deposited on petri dishes containing starch casein agar (SCA) (Mohseni et al., 2013), with nalidixic acid ($50 \mu\text{g/mL}$) and cycloheximide ($50 \mu\text{g/mL}$).

The plates were incubated at 28°C for 30 days, and were examined daily for the presence of colonies. The actinomycetes isolates were deposited in the Laboratório de Genética de Microrganismos (LabGeM) culture collection, Federal University of Paraná, Brazil (<http://www.labgem.ufpr.br/>).

Identification

Morphological Analysis

Four different culture media were used to access the macro-morphological characteristics, ISP2—Agar yeast-malt extract; ISP3—Oat Agar; ISP4—Agar Starch and inorganic salts; ISP5—Glycerol Asparagine Agar (Shirling and Gottlieb, 1966). The isolates were streaked on the plates and incubated at 28°C for 21 days. The characteristics evaluated were growth rate, the formation and color of aerial spore mass and substrate mycelia.

Molecular Taxonomy

Total genomic DNA was extracted from 3 day old cultures using the method described by Raeder and Broda (1985). Partial sequence of the 16S rRNA gene was amplified using primers 9F ($5'\text{GAGTTTGATCCTGGCTCAG}3'$) and 1541R ($5'\text{AAGGAGGTGATCCAGCC}3'$), as described by Savi et al. (2016). The PCR product was purified using Exo1 and FastAP enzymes (GE Healthcare, USA), and sequenced using the BigDye[®] Terminator v3.1 Kit. The products were purified with SephadexG50 and submitted to an ABI3500[®] automated sequencer (Applied Biosystems, Foster City, CA, USA). Consensus sequences were analyzed and aligned using Mega 6.0 (Tamura et al., 2013) and BioEdit, and compared to sequences available in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Type strain sequences were found through search in the List of Prokaryotic Names with Standing Nomenclature database (<http://www.bacterio.net/>). All sequences obtained were deposited in the GenBank, the accession numbers are listed in Table 1. For Bayesian inference analysis, a Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with posterior probabilities using MrBayesv3.2.6 x86 (Ronquist et al., 2011). GRT evolutionary model was determined using the Akaike Information Criterion (AIC) in R software (R Core Team, 2017) and the phangorn package (Schliep, 2011). Comparisons of sequences with respect to their percentile similarity were estimated using the R software (R Core Team, 2017) and the pegas package (Paradis, 2010).

Antibiotic Sensitivity

The susceptibility of the endophytes to 11 antibiotics, oxacilin (a penicillin), vancomycin (a glycopeptide), chloramphenicol (an amphionicol), meropenem (a carbapenem), streptomycin (an aminoglycoside), tetracycline (a tetracycline), gentamicin (another aminoglycoside), rifampicin (a macrolactam), ampicillin (another penicillins), ceftazidime (a third generation cephalosporin), and nalidixic acid (a quinolone) were evaluated as described by Passari et al. (2015). The analysis was performed considering the isolate sensitive (S) with an inhibition zone > 20 mm, intermediate (I) with an inhibition zone of 10–19.9 mm and resistant (R), if the inhibition zone was between 0.0–9.9 mm (Williams et al., 1989).

TABLE 1 | Identification, place, and source of isolation and morphological characteristic of endophytic actinomycetes isolates, morphological characteristics 21 days after inoculation in four different culture media at 28°C.

Strain genera	NCBI genbank accession n°	Place/Source of isolation	ISP2—Agar yeast-malt extract			ISP3—Oat agar			ISP4—Agar starch and inorganic salts			ISP5—Glycerol asparagine agar		
			Aerial spore mass	Substrate mycelium	Grown	Aerial spore mass	Substrate mycelium	Grown	Aerial spore mass	Substrate mycelium	Grown	Aerial spore mass	Substrate mycelium	Grown
<i>Actinomadura</i> sp. LGMB466	KY458125	Abobral Leaf	Moderated: White	Brown	++ +	Abundant: White	Yellow	++ +	Low: White	Yellow	+	Low: White	Pink	++
<i>Actinomadura</i> sp. LGMB487	KY421547	Abobral Leaf	Moderated: White	Ivory-white	++ +	Abundant: White	Yellow	++ +	Low: White	Yellow	+	Low: White	Ivory- white	++
<i>Aeromicrobium ponti</i> LGMB491	KY411896	Abobral Leaf	None	Yellow	++	None	Yellow	++	None	Yellow	++ +	None	Yellow	++ +
<i>Microbacterium</i> sp. LGMB471	KY423334	São Bento Leaf	None	Yellow	++ +	None	Yellow	++ +	None	Ivory-white	++	None	Ivory- white	++ +
<i>Microbispora</i> sp. LGMB461	KY411900	São Bento Stem	Abundant: White	Ivory-white	++ +	Abundant: White	Ivory-white	++ +	Abundant: White	Ivory-white	+	Abundant: White	White	++ +
<i>Microbispora</i> sp. LGMB465	KY411898	São Bento Stem	Moderated: White	Ivory-white	++ +	Abundant: White	Ivory-white	++ +	Abundant: White	Ivory-white	+	Abundant: White	Ivory- white	++
<i>Micrococcus</i> sp. LGMB485	KY423496	Abobral Leaf	None	White	++ +	None	White	++ +	None	White	++ +	None	White	++ +
<i>Sphaerisporangium</i> sp. LGMB482	KY458126	Abobral Stem	Abundant: White	Brown	++ +	Abundant: White	Pink	++ +	Abundant: Pink	Red/ Ivory-white	++ +	Abundant: White	Ivory- white	++
<i>S. thermocarboxydus</i> LGMB483	KY423333	Abobral Stem	Abundant: Gray	Gray	++ +	Moderated: White	Ivory-white	++ +	Abundant: White	Gray	++ +	Abundant: White	Brown	++ +
<i>Williamsia</i> <i>serinedens</i> LGMB479	KY421546	Abobral Stem	None	Orange	++ +	None	Light orange	++ +	None	Orange	++ +	None	Orange	++ +

+++, Abundant; ++, Moderated; +, low.

Biological Activity

Screening of Culture Conditions

Isolates were inoculated in 50 mL of SG medium (Shaaban et al., 2011), incubated for 3 days at 36°C and 180 rpm. Subsequently, 1 mL from the pre-culture was inoculated in SG and R5A media (100 mL) (Fernandez et al., 1998), and incubated for 10 days at two different temperatures, 28 and 36°C, and 180 rpm. The culture was filtered-off on Whatmann 4 filters, the water fraction was extracted with EtOAc (3 × 100 mL). The combined organics were evaporated *in vacuo* at 40°C and diluted in methanol at 10 mg/mL.

Antibacterial Activity–Disk Diffusion Assays

The antibacterial activity of crude extracts and the isolated compounds **1–9** was evaluated against methicillin-sensitive *Staphylococcus aureus* (MSSA) (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (BACHC-MRSA), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231), *Acinetobacter baumannii* (BACHC-ABA), *Klebsiella pneumoniae*, the producer of the enzyme KPC (*K. pneumoniae* carbapenemase) (BACHC-KPC), *Stenotrophomonas maltophilia* (BACHC-SMA), and *Enterobacter cloacae* a producer of the enzyme VIM (Verona integron-encoded metallo-β-lactamase) (BACHC-VIM). The bacteria were cultivated for 12 h at 37°C, and diluted according to the McFarland standard 0.5 scale. Each test organism was streaked on a sterile Mueller-Hinton agar plate with a cotton swab. Extracts were aliquoted in 100 µg amounts per 6 mm sterile filter disc. The discs were placed on plates and incubated for 24 h at 37°C. The diameter halos were measured in millimeters. As a positive control, a disc with a standard antibiotic with activity against each of the bacteria was used, and pure methanol was used as negative control (CLSI, 2015; Savi et al., 2015b).

MIC–Minimum Inhibitory Concentration and

MBC–Minimum Bactericidal Concentration

Extracts from strain LGMB491 that showed high antibacterial activity were selected to determine the minimum inhibitory concentration. The MIC of extracts against the clinical pathogens was performed as described by Ostrosky et al. (2008) and CLSI. The minimum bactericidal concentration was determined as described by Soltani and Moghaddam (2014).

Statistical Analyses

The statistical analysis was performed using analysis of variance (ANOVA) to compare extract effects to their respective controls. We also performed Post-hoc tests using Tukey's honest significant difference. All tests premises were fulfilled; the significance level used was 0.05 (α).

Large-Scale Fermentation, Extraction and Isolation

A large-scale fermentation (10 L) of strain LGMB491 was performed using SG culture medium at 36°C for 10 days. The culture was subjected to extraction with EtOAc (3 × v/v), and the combined organic layers were evaporated *in vacuo* at 40°C to yield 653 mg of crude extract. The crude extract was subjected to reverse phase C₁₈ column chromatography (20 × 8 cm, 250 g),

eluted with a gradient of H₂O-MeOH (100:0–0:100) to produce fractions FI–FV. The single fractions were subjected to HPLC and Sephadex LH-20 (MeOH; 1 × 20 cm) purifications to yield compounds **1–9** in pure form (Figure 9, Figure S9). NMR spectra were measured using a Varian (Palo Alto, CA) Vnmr 400 (¹H, 400 MHz; ¹³C, 100 MHz) spectrometer, δ-values were referenced to the respective solvent signals (CD₃OD, δ_H 3.31 ppm, δ_C 49.15 ppm; DMSO-*d*₆, δ_H 2.50 ppm, δ_C 39.51 ppm). HPLC-MS analyses were accomplished using a Waters (Milford, MA) 2695 LC module (Waters Symmetry Anal C₁₈, 4.6 × 250 mm, 5 µm; solvent A: H₂O/0.1% formic acid, solvent B: CH₃CN/0.1% formic acid; flow rate: 0.5 mL min⁻¹; 0–4 min, 10% B; 4–22 min, 10–100% B; 22–27 min, 100% B; 27–29 min, 100–10% B; 29–30 min, 10% B). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array detector (PDA) and a Phenomenex C₁₈ column (4.6 × 250 mm, 5 µm; Phenomenex, Torrance, CA). Semi-preparative HPLC was accomplished using Phenomenex (Torrance, CA) C₁₈ column (10 × 250 mm, 5 µm) on a Varian (Palo Alto, CA) ProStar Model 210 equipped with a photodiode array detector and a gradient elution profile (solvent A: H₂O, solvent B: CH₃CN; flow rate: 5.0 mL min⁻¹; 0–2 min, 25% B; 2–15 min, 25–100% B; 15–17 min, 100% B; 17–18 min, 100–25% B; 18–19 min, 25% B). All solvents used were of ACS grade and purchased from the Pharmco-AAPER (Brookfield, CT). Size exclusion chromatography was performed on Sephadex LH-20 (25–100 µm; GE Healthcare, Piscataway, NJ).

RESULTS

Isolation of Endophytic Actinomycetes

From 2,988 fragments analyzed, 10 endophytic actinomycetes were isolated (Table 1), thus the isolation frequency was 0.34%. From the 10 isolates, 70% ($n = 7$) were isolated from the Abobral, and 30% ($n = 3$) from the São Bento region. Five isolates were obtained from stems, and five from leaf tissues of the plant (Table 1).

Morphological Identification

A great macro-morphological diversity was observed, with white, ivory-white, pink, brown, gray, orange, and yellow colony colors. Most of isolates showed abundant to moderate growth after 21 days of incubation, and six isolates showed abundant to moderate spore formation on ISP2 and ISP3 media. Isolates LGMB461 and LGMB465 showed high morphological similarity, and probably represent the same species (Table 1).

Molecular Analysis

Using a BLAST analysis in the GenBank database, the isolates were classified as eight genera: *Aeromicrobium*, *Williamsia*, *Microbacterium*, *Sphaerisporangium*, *Micrococcus*, *Microbispora*, *Actinomadura*, and *Streptomyces*. Each genus was analyzed in a separate phylogenetic tree based on Bayesian inference.

Actinomadura (LGMB466 and LGMB487)

The alignment consisted of strains LGMB466 and LGMB487, 55 type strains representative of *Actinomadura* genus, and *Streptomyces glauciniger* (AB249964) as out group taxa. The

analysis comprises of 1,402 characters, 1,011 of these were conserved, 124 were parsimony informative and 131 were uninformative. Strains LGMB466 and LGMB487 showed high similarity among themselves (98.86%), and in the phylogenetic analysis these isolates did not cluster with any species from the *Actinomadura* genus (**Figure 1**, Table S1), and probably represent a new species.

Aeromicrombium (LGMB491)

Strain LGMB491 was aligned with all type strains from the *Aeromicrobium* genus (12 species), and *Nocardioides albus* (X53211) was used as out group taxa. The alignment consisted of 1,336 characters, 1,164 of these were conserved, 89 were parsimony informative and 68 were uninformative. Based on

this phylogenetic analysis, strain LGMB491 is close related to *Aeromicrobium ponti* (**Figure 2**), sharing high sequence similarity, 99.25 % (Table S2).

Microbacterium (LGMB471)

Strain LGMB471 was aligned with type strains from the *Microbacterium* genus, and *Agrococcus jenensis* (X92492) as outgroup taxa. The alignment comprised of 1,314 characters, of those 721 conserved sites, 122 were parsimony informative, and 57 uninformative. In the phylogenetic tree, isolate LGMB471 ended up in a single branch related to species *Microbacterium liquefaciens*, *Microbacterium maritipicum*, *Microbacterium oxydans*, *Microbacterium luteolum*, *Microbacterium saperdae*, and *Microbacterium paraoxydans* (Figure 3, Table S3).

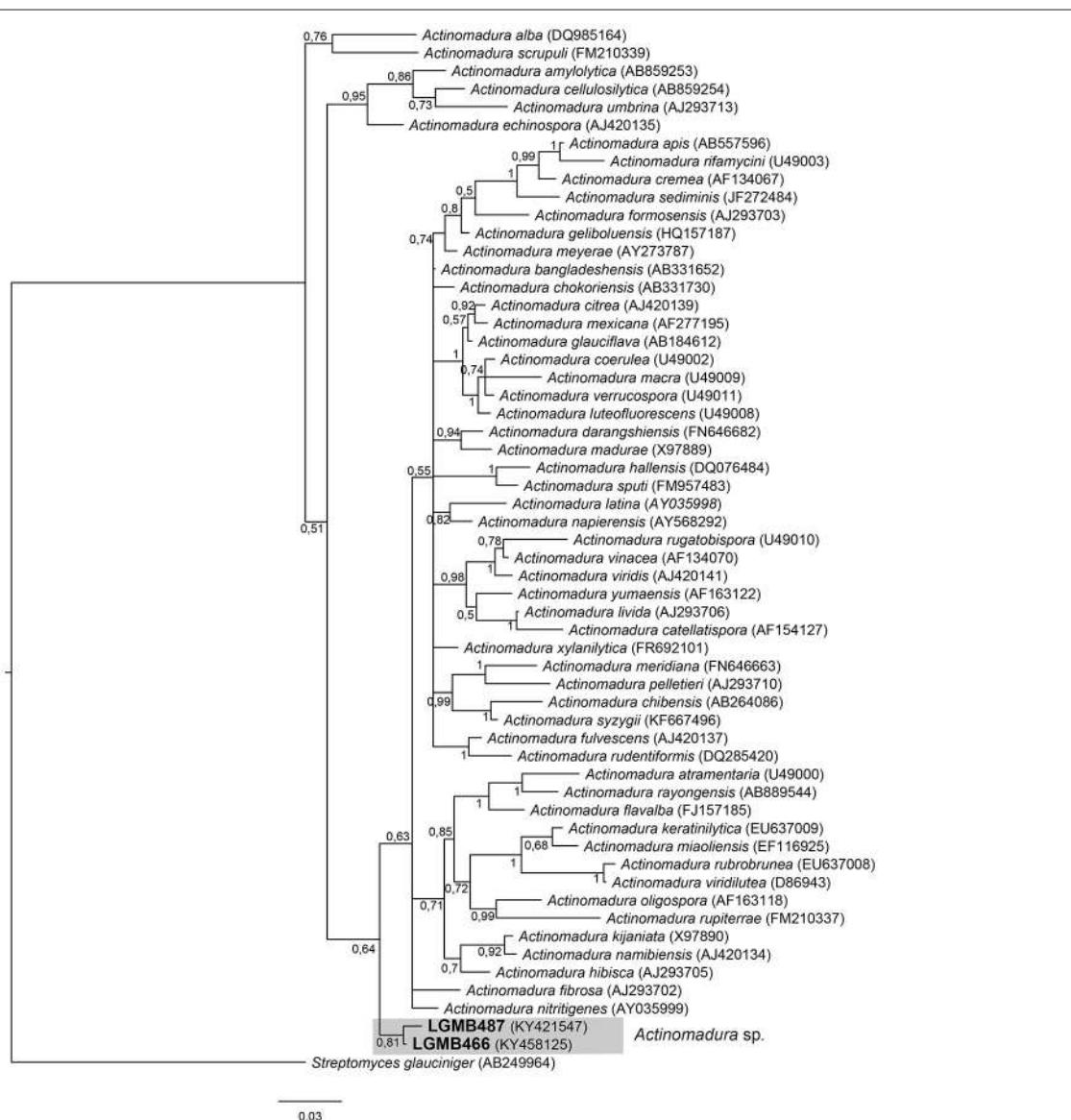


FIGURE 1 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB466, LGMB487, and the 53 type strain of *Actionomadura* genus. Values on the node indicate Bayesian posterior probabilities. The species *Streptomyces glaucescens* was used as out group. Scale bar indicates the number of substitutions per site.

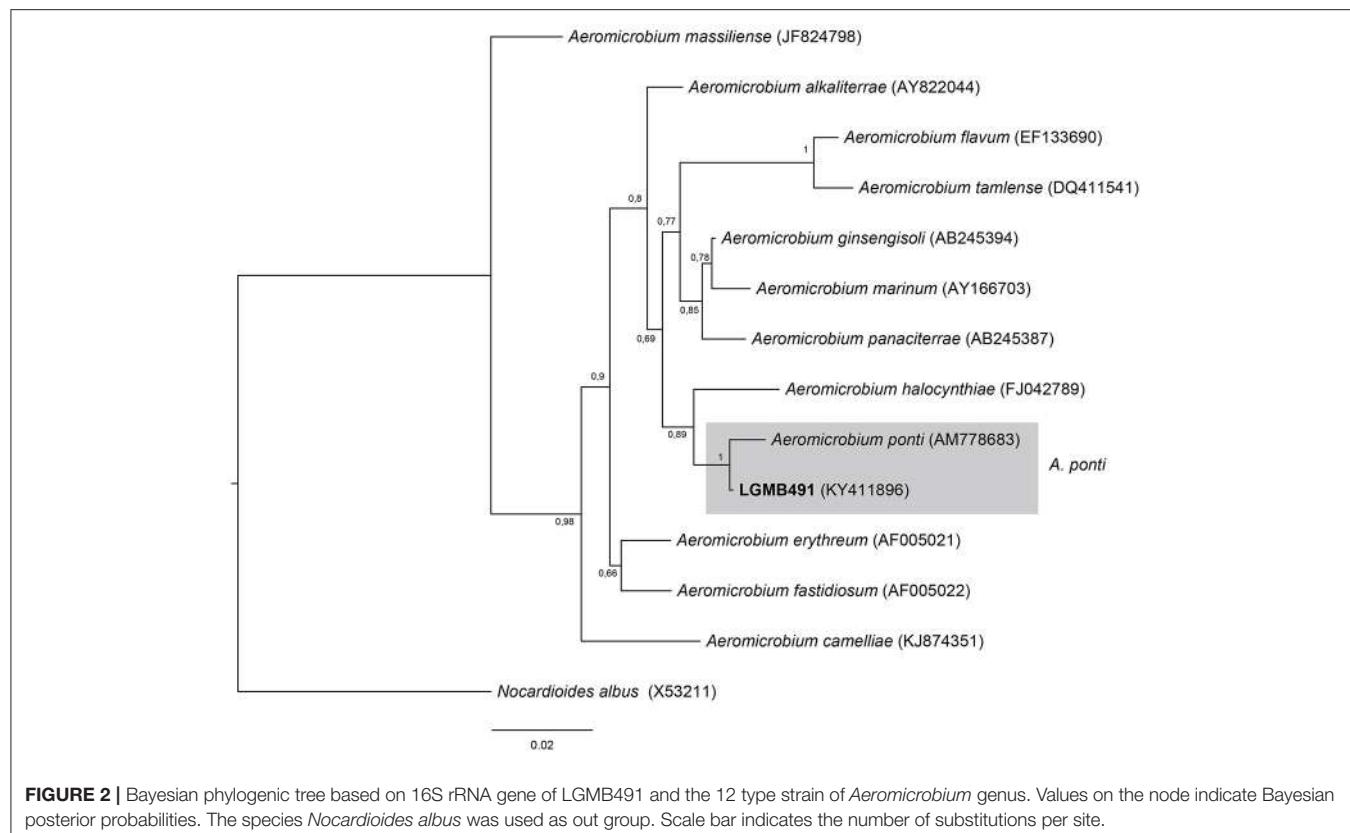


FIGURE 2 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB491 and the 12 type strain of *Aeromicrobium* genus. Values on the node indicate Bayesian posterior probabilities. The species *Nocardioides albus* was used as out group. Scale bar indicates the number of substitutions per site.

TABLE 2 | Antibiotic sensitivity pattern of endophytic actinomycetes.

Strain/Genera	Antibiotic sensitivity										
	Oxa 1 µg	Van 30 µg	Clo 30 µg	Mer 10 µg	Est 10 µg	Tet 30 µg	Gen 10 µg	Rif 5 µg	Amp 10 µg	Caz 30 µg	Nal 30 µg
<i>Actinomadura</i> sp. LGMB466	R	S	I	S	S	S	S	I	R	R	R
<i>Actinomadura</i> sp. LGMB487	S	I	R	S	S	S	S	S	I	S	S
<i>Aeromicrobium ponti</i> LGMB491	R	S	R	S	S	S	S	S	R	S	S
<i>Microbacterium</i> sp. LGMB471	R	S	I	S	S	S	S	I	R	R	R
<i>Microbispora</i> sp. LGMB461	R	S	R	R	S	S	S	I	R	R	R
<i>Microbispora</i> sp. LGMB465	R	S	R	R	S	S	I	I	R	R	R
<i>Micrococcus</i> sp. LGMB485	R	I	R	S	I	I	I	I	R	S	R
<i>Sphaerisporangium</i> sp LGMB482	R	S	I	S	S	S	S	I	R	R	R
<i>Streptomyces thermocarboxydus</i> . LGMB483	R	S	R	S	S	I	S	R	R	R	R
<i>Williamsia serinedens</i> . LGMB479	R	S	R	S	S	I	S	I	I	S	R

Degree of susceptibility: >20 mm—Sensitive; 10–19.9 mm—intermediate; 0.0–9.9 mm resistant. Oxa, Oxacillin (1 µg/disc); Van, Vancomycin (30 µg/disc); Clo, Chloramphenicol (30 µg/disc); Mer, Meropenem (10 µg/disc); Est, Streptomycin (30 µg/disc); Tet, Tetracycline (30 µg/disc); Gen, Gentamicin (10 µg/disc); Rif, Rifampicin (5 µg/disc); Amp, Ampicillin (10 µg/disc); Caz, Ceftazidime (30 µg/disc); Nal, Nalidixic acid (30 µg/disc).

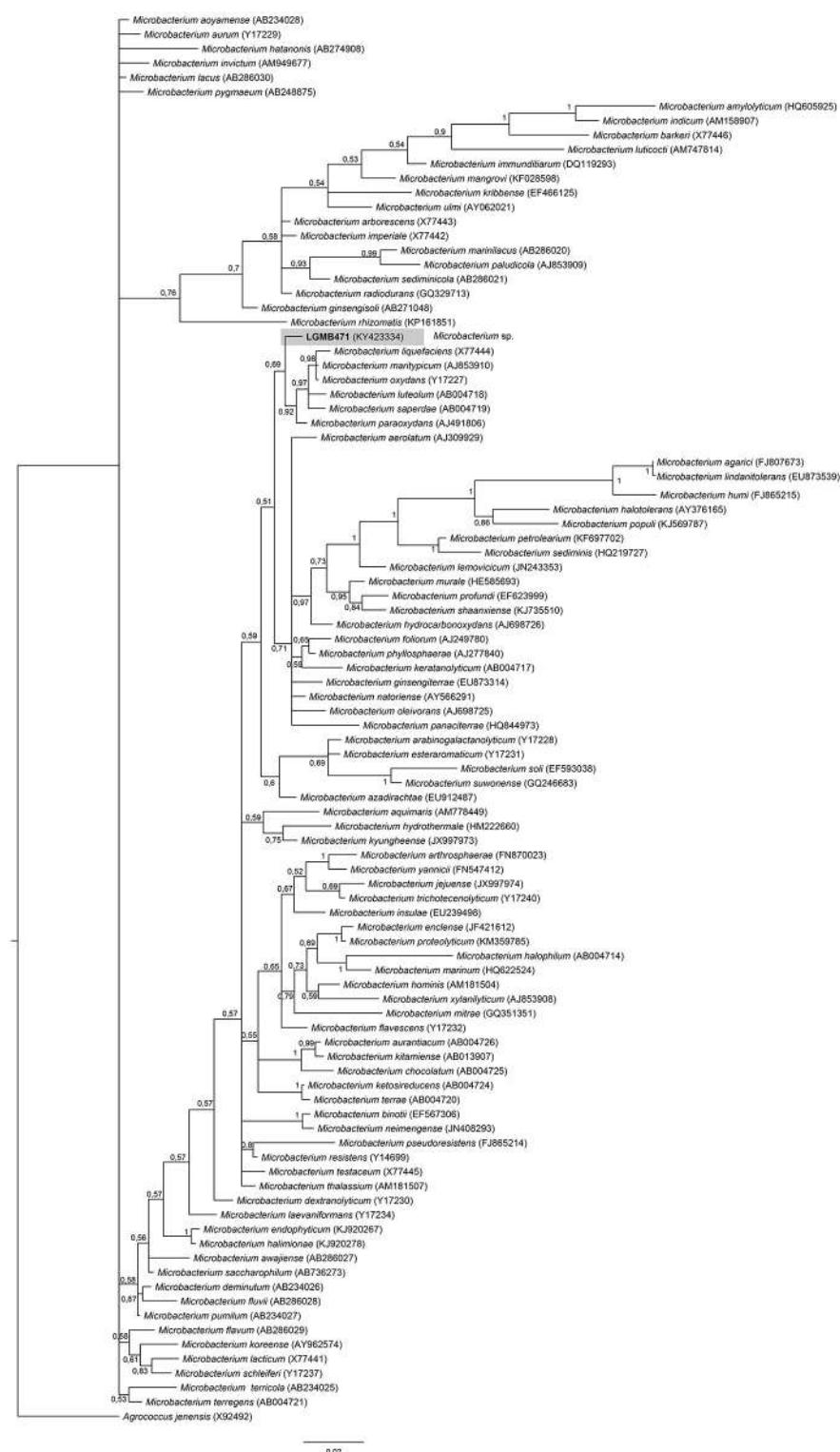


FIGURE 3 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB471 and the 94 type strain of *Microbacterium* genus. Values on the node indicate Bayesian posterior probabilities. The species *Agrococcus jenensis* was used as out group. Scale bar indicates the number of substitutions per site.

***Microbispora* (LGMB461 and LGMB465)**

The analysis comprises of strains LGMB461 and LGMB465, 10 species accepted in *Microbispora* genus, and the isolates previously reported as *Microbispora* sp.1, *Microbispora* sp.2, and *Microbispora* sp.3 (Savi et al., 2016). *Actinomadura echinospora* (AJ420135) was used as out group taxa. The alignment consists of 1,371 characters, 1,309 of these were conserved, 33 were parsimony informative, and 29 uninformative. In the phylogenetic analysis strains LGMB461 and LGMB465 presented similarity with *Microbispora* sp.1 (LGMB259) with 99.84 and 100% f similarity, respectively (Figure 4, Table S4).

***Micrococcus* (LGMB485)**

The Bayesian analysis comprised of all *Micrococcus* type strains, strain LGMB485 and *Citricoccus parietis* (FM9923367) as out group taxa (Figure 5). The alignment consisted of 1,340 characters with 452 conserved sites, nine were parsimony informative and 19 uninformative. Since the sequences were very similar (Table S5) and the alignment had only nine parsimony informative sites, a species designation cannot be assigned, and isolate LGMB485 was identified as *Micrococcus* sp.

***Sphaerisporangium* (LGMB482)**

For the Bayesian analysis, the sequence from LGMB482 was aligned with strains of the *Sphaerisporangium* genus, and *Actinomadura madurae* (X97889) was used as out group taxa. The alignment consisted of 1,320 characters, 886 of these were conserved, 51 were parsimony informative and 47 were uninformative. Strain LGMB482 is closely related to *S. melleum* AB208714 (99.4% similarity) and *S. viridalbum* X89953 (97.89% similarity), however, it is in an isolated branch and may represent a new species of the *Sphaerisporangium* genus (Figure 6, Table S6).

***Streptomyces* (LGMB483)**

The phylogenetic analysis was performed using 23 type strains closely related with LGMB483; including *Streptomyces albus* subsp. *albus* (X53163) as out group taxa. The alignment consisted of 1,391 characters, with 1,291 conserved sites, 45 were parsimony informative, and 39 uninformative. In the phylogenetic tree, isolate LGMB483 grouped with *Streptomyces thermocarboxydus*, sharing 99.86% of similarity (Figure 7, Table S7), and thus we suggest this isolate may belongs to this species.

***Williamsia* (LGMB479)**

The analysis consists of 11 sequences, including all type strains of the *Williamsia* genus, the strain LGMB479, and *Mycobacterium tuberculosis* (X58890) was used as out group taxa. The alignment comprises of 1,346 characters, of these 1,185 were conserved, 81 were parsimony informative and 56 were uninformative. Strain LGMB479 was in the same clade with *Williamsia serinedens* (AM283464) (Figure 8) and share 99.85% sequence similarity (Table S8), and may belongs to this species.

Antibiotic Sensitivity Test

In order to characterize the susceptibility profiles of the endophytes, 11 antibiotics with different mechanisms-of-action were utilized. Isolates were susceptible to vancomycin (80% sensitive and 20% intermediate), streptomycin (90% sensitive and 10% intermediate), tetracycline (70% sensitive and 30% intermediate), and gentamicin (80% sensitive and 20% intermediate). The two isolates of *Microbispora* sp. (LGMB461 and LGMB465) showed resistance to meropenem, and 90% of the isolates showed resistance to oxacillin, and nalidixic acid (Table 2).

Antibacterial Activity of Crude Extracts

All strains and culture conditions analyzed produced active extracts (Table 3, Table S9), however, the extract from LGMB491 (close related to *A. ponti*) cultured in SG medium at 36°C showed great antibacterial activity against *S. aureus* (22 mm) and MRSA (19.8 mm), and moderate activity against others clinical pathogens (Table 3, Figures S1–S8). The MIC and MBC of extract from LGMB491 against *S. aureus* and methicillin-resistant *S. aureus* were 0.02, and 0.04 mg/mL, respectively, and the MBC was 5 mg/mL for both bacteria (Table 4). In addition, the crude extract from LGMB491 had an MIC of 0.63 mg/mL against gram-negative bacteria associated with antibiotic resistance, *K. pneumoniae* KPC, *S. maltophilia*, and *E. cloacae* VIM, and a MIC of 0.31 mg/mL against *A. baumannii* and *P. aeruginosa*, respectively (Table 4).

Structure Determination of Secondary Metabolites from Strain LGMB491

Scale-up fermentation of strain LGMB491 (10 L) using SG medium, followed by extraction afforded 653 mg of crude extract. Fractionation, isolation and purification of the obtained extract using various chromatographic techniques resulted in compounds 1–9 in pure forms (Figure S9). Thorough analyses of the HPLC/UV, ESIMS and NMR spectroscopy data (Figure S10–S43), and by comparison with literature data (Laatsch, 2012), the compounds were identified as 1-acetyl-β-carboline (1) (Shaaban et al., 2007; Savi et al., 2015b), indole-3-carbaldehyde (2) (Zendah et al., 2012; Savi et al., 2015b), tryptophol (3) (Rayle and Purves, 1967), 3-(hydroxyacetyl)-indole (4) (Zendah et al., 2012), brevianamide F (5) (Shaaban, 2009), cyclo-(L-Pro-L-Phe) (6) (Barrow and Sun, 1994), cyclo-(L-Pro-L-Tyr) (7) (Barrow and Sun, 1994), cyclo-(L-Pro-L-Leu) (8) (Yan et al., 2004), and cyclo-(L-Val-L-Phe) (9) (Pickenhagen et al., 1975) (Figure 9). In order to determine the compounds responsible for the biological activity observed for the crude extract of strain LGMB491, we evaluated the antibacterial activity of compounds 1–9 against *S. aureus* and methicillin-resistant *S. aureus*. 1-Acetyl-β-carboline (1) showed an equivalent activity as the antibiotic methicillin against *S. aureus*, however, different from this antibiotic, compound 1 also showed activity against MRSA (Table 5). In addition, compounds 2, 4–6 also showed moderate activity against both MSSA and MRSA.

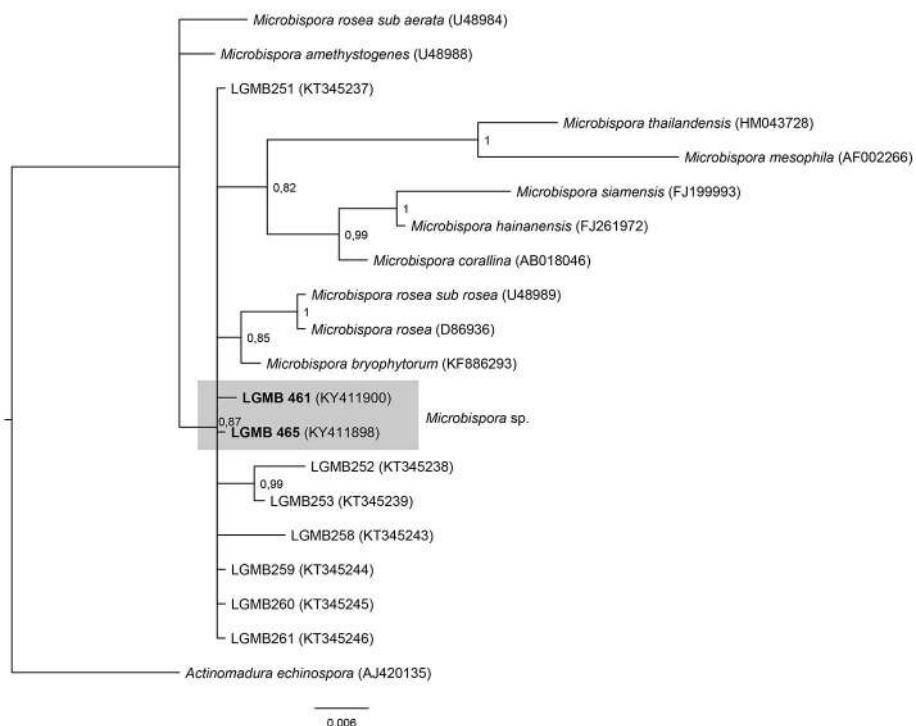


FIGURE 4 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB461, LGMB465, the 10 type strain of *Microbispora* genus, and 7 strains previously reported by Savi et al. (2016). Values on the node indicate Bayesian posterior probabilities. The species *Citricoccus parietis* was used as out group. Scale bar indicates the number of substitutions per site.

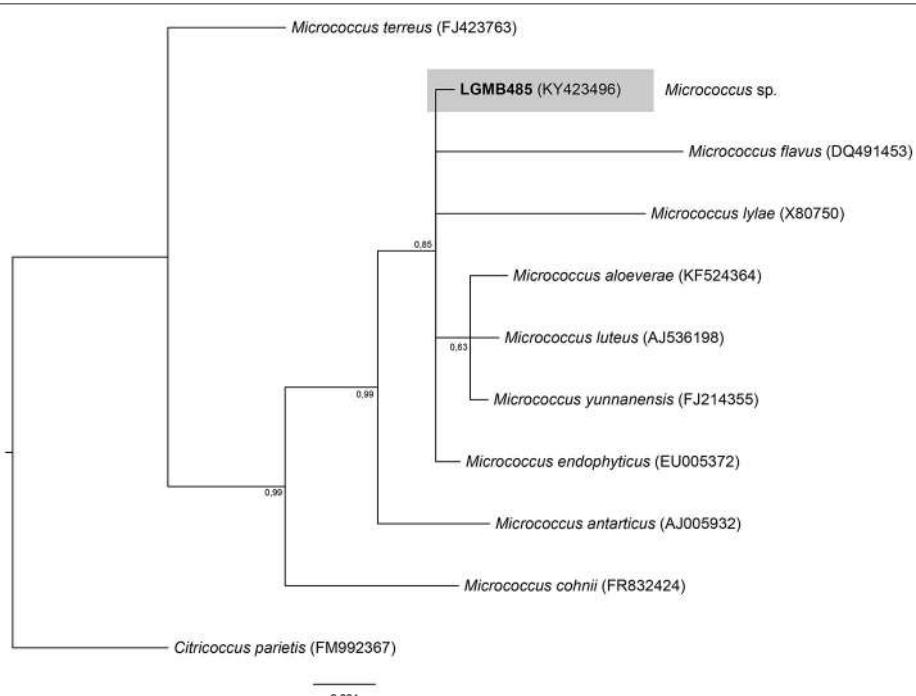


FIGURE 5 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB485 and the 9 type strain of *Micrococcus* genus. Values on the node indicate Bayesian posterior probabilities. The species *Citricoccus parietis* was used as out group. Scale bar indicates the number of substitutions per site.

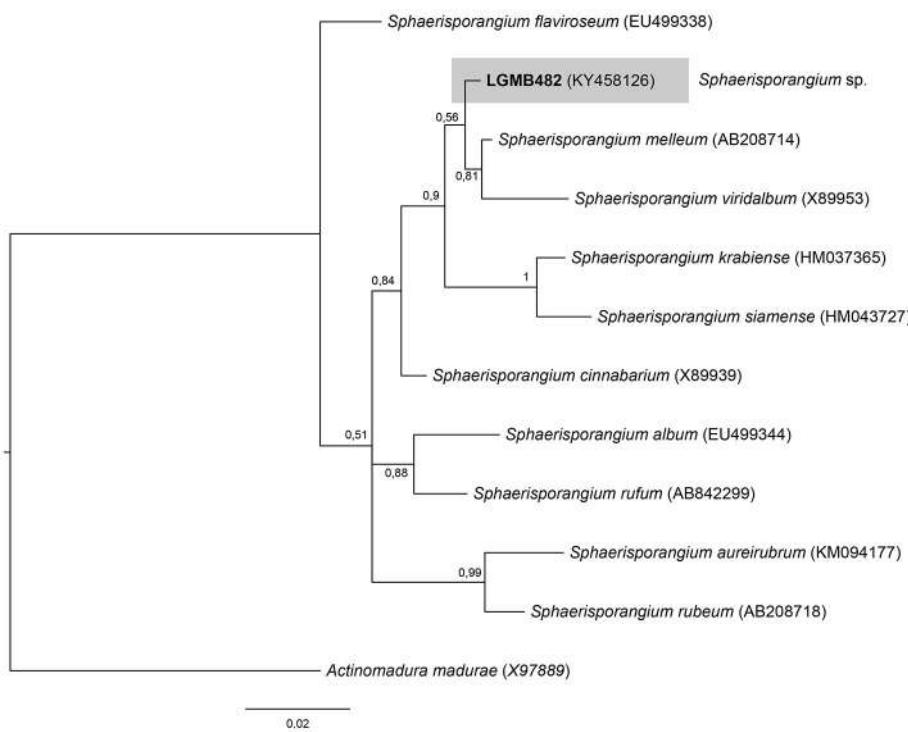


FIGURE 6 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB482 and the 10 type strain of *Sphaerisporangium* genus. Values on the node indicate Bayesian posterior probabilities. The species *Actinomadura madurae* was used as out group. Scale bar indicates the number of substitutions per site.

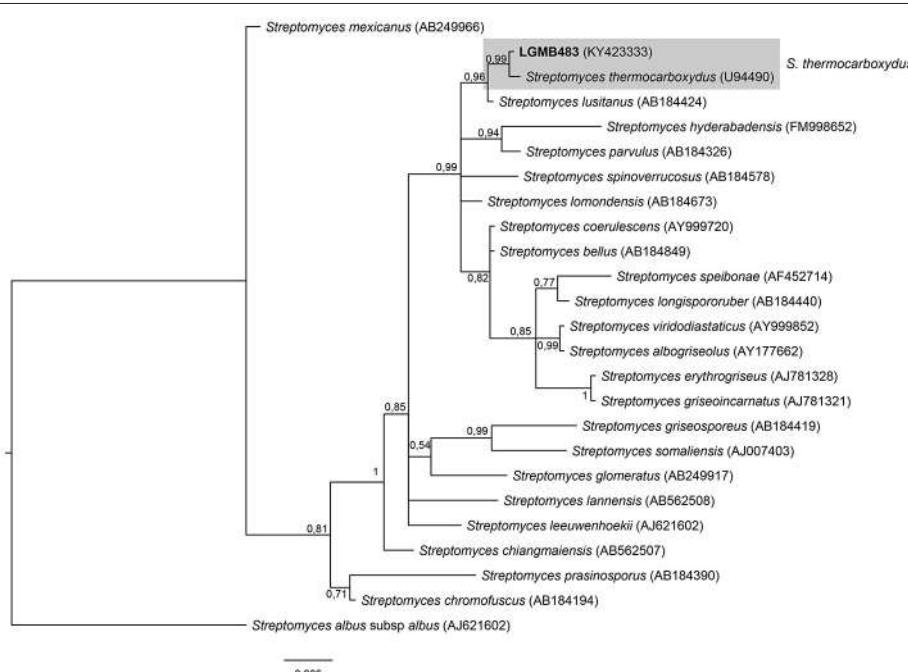


FIGURE 7 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB483 and the 33 type strain of *Streptomyces* genus. Values on the node indicate Bayesian posterior probabilities. The species *Streptomyces albus* subsp. *albus* was used as out group. Scale bar indicates the number of substitutions per site.

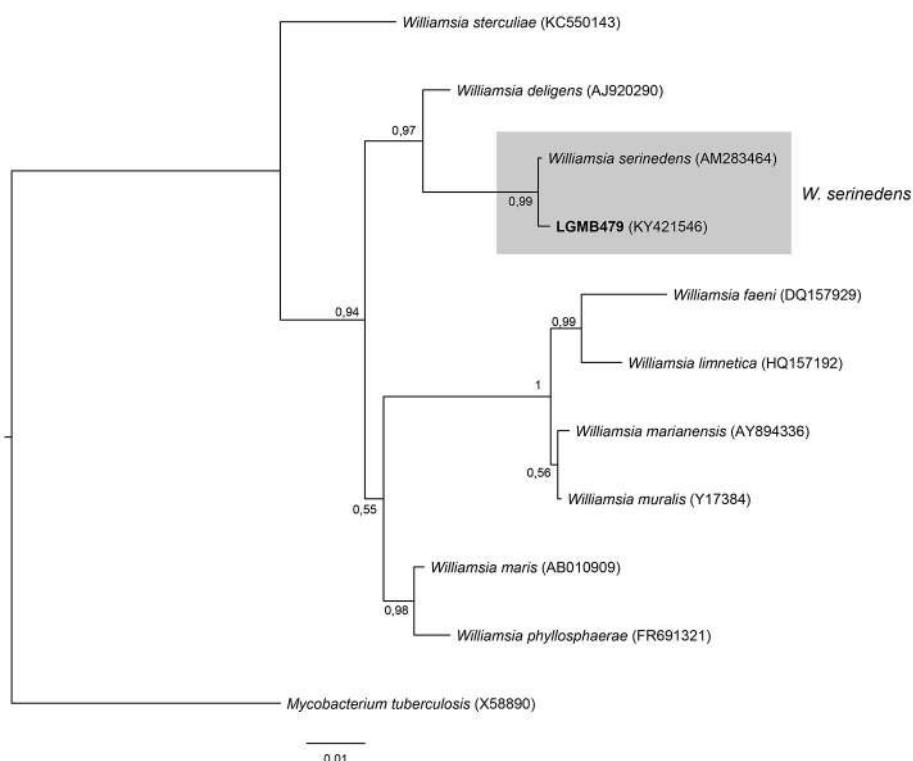


FIGURE 8 | Bayesian phylogenetic tree based on 16S rRNA gene of LGMB479 and the 9 type strain of *Williamsia* genus. Values on the node indicate Bayesian posterior probabilities. The species *Mycobacterium tuberculosis* was used as out group. Scale bar indicates the number of substitutions per site.

DISCUSSION

Endophytes Isolation and Identification

Actinomycetes from medicinal plants are the source of several secondary metabolites with biological activity (Qin et al., 2015; Savi et al., 2015b), and their metabolites may be associated with the medicinal properties of the plant host (Kusari et al., 2013; Santos et al., 2015). We explored the endophytes from the medicinal plant *V. divergens*, in order to catalog the species richness and biological properties. A low frequency of isolation (0.34%), compared with the isolation of terrestrial actinomycetes, was observed, in agreement with literature data (Passari et al., 2015). However, despite the lower isolation frequency a higher richness of genera was observed (Passari et al., 2015; Saini et al., 2016). We reported for the first time the isolation of strains close related to the species *A. ponti* (LGMB491) and *Williamsia serinedens* (LGMB479) as endophytes. *A. ponti* was originally isolated from seawater (Lee and Lee, 2008), and has been found in this environment (Jiang et al., 2010; Claverias et al., 2015). *W. serinedens* was first isolated from an oil-contaminated soil sample and it is common isolated from different types of soil (Yassin et al., 2007). In addition, species *S. thermocarboxydus* was isolated from soil (Kim et al., 2000), and was recently described as endophyte from a medicinal plant in India (Passari et al., 2015). Based on the 16S rRNA

phylogenetic analysis we suggest that strains LGMB471 and LGMB482 may represent new species within the *Microbacterium* and *Sphaerisporangium* genera, respectively (Figures 3, 6), and isolates LGMB466 and LGMB487 seem to be a new species within the *Actinomadura* genus (Figure 1). Isolates LGMB461 and LGMB465 belong to genus *Microbispora*, and showed high sequence similarity with strains belonging to *Microbispora* sp.1 group, previously isolated from *V. divergens* (Savi et al., 2016). However, sequencing others genes than 16S rRNA, and DNA-DNA hybridization would be required for species description (Meyers, 2014). *Microbacterium*, *Sphaerisporangium*, and *Micrococcus* species are common associated with medicinal plants in different regions, and climate conditions (Kim et al., 2000; Kamil et al., 2014; Xing et al., 2015). However, none of these has been isolated from wetland regions. Savi et al. (2015a) performed the first report about actinomycetes from the medicinal plant *V. divergens*. However, despite the higher number of isolates, the authors then just identified three genera as endophytes from this plant, *Microbispora*, *Micromonospora*, and *Streptomyces*. In addition to those genera previously mentioned (*Microbispora* and *Streptomyces*) we isolated species belonging to *Actinomadura*, *Aeromicrobium*, *Microbacterium*, *Sphaerisporangium*, *Micrococcus*, and *Williamsia* (Figures 1–8), thereby significantly increasing the knowledge regarding endophytes from *V. divergens*.

TABLE 3 | Antibacterial activity of the extracts from endophytic actinomycetes in two culture media (SG and R5A) and two temperatures (28°C and 36°C) against Clinical pathogens.

Strain/Genera	Antimicrobial activity (inhibition zone in mm)											
	Staphylococcus aureus (Figure S1)						Meticillin-resistant <i>S. aureus</i> (Figure S1)					
	Medium SG			Medium R5A			Medium SG			Medium R5A		
	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C
<i>Actinomadura</i> sp. LGMB466	9.75 ± 0.5	11.25 ± 0.5	11.25 ± 0.5	9 ± 0.8	9.5 ± 0.58	9.25 ± 0.5	9.5 ± 0.6	9.5 ± 0.6	12 ± 0	12.75 ± 0.5	12.75 ± 0.5	10.75 ± 0.5
<i>Actinomadura</i> sp. LGMB487	9.75 ± 0.5	11.75 ± 1	11.75 ± 1	9.5 ± 0.6	10.5 ± 1	9.5 ± 0.58	9.5 ± 0.6	9 ± 0.8	11.0	11.5 ± 0.6	12.25 ± 1	11.0
<i>Aeromicrobium ponti</i> LGMB491	20.5 ± 0.6	22 ± 1.3	19.25 ± 1.3	15.75 ± 1.7	24.2 ± 2.06	19.8 ± 0.5	11.5 ± 1.3	17.25 ± 1.7	11.5 ± 1	12 ± 0.8	10.5 ± 0.6	10.25 ± 0.5
<i>Microbacterium</i> sp. LGMB471	11.25 ± 1	9.5 ± 1	9.5 ± 1	11 ± 1.2	11.25 ± 0.96	8.25 ± 1.3	11.75 ± 1.5	9.25 ± 0.5	11.25 ± 0.5	12.25 ± 0.5	13 ± 0.8	11.0
<i>Microbispora</i> sp. LGMB461	8.75 ± 1.5	9.5 ± 1.0	9.5 ± 1.0	9.25 ± 1.5	9.75 ± 1.7	8.5 ± 1	10.75 ± 0.5	8.75 ± 0.5	12.75 ± 0.5	9.5 ± 0.6	12.25 ± 1	12.5 ± 0.6
<i>Microbispora</i> sp. LGMB465	10 ± 0.8	9.25 ± 0.5	9.25 ± 0.5	9.5 ± 0.6	9.5 ± 1	9.75 ± 0.96	8.0	11.5 ± 0.6	13.25 ± 1	11.5 ± 0.6	8.0	10.5 ± 0.6
<i>Micrococcus</i> sp. LGMB485	10.25 ± 0.5	12.5 ± 1.7	12.5 ± 1.7	10.5 ± 1.3	10 ± 0.82	11.2 ± 1.26	10 ± 1.4	8.75 ± 0.5	10.5 ± 0.6	10.75 ± 0.5	12.75 ± 0.5	10 ± 0.8
<i>Sphaerotilus</i> sp. LGMB482	10.5 ± 1.3	10.25 ± 1.7	10.25 ± 1.7	11 ± 0	9.5 ± 0.58	10.5 ± 1.3	9.5 ± 0.6	9.5 ± 0.6	11 ± 0.8	9.75 ± 1	12 ± 0.8	11 ± 0.8
<i>S. thermocarboxydus</i> LGMB483	10.75 ± 1.5	10.0	10.0	11.25 ± 1.0	11.2 ± 0.96	11.2 ± 0.96	8.75 ± 1.5	8.5 ± 1	11.5 ± 1.3	11 ± 0.8	11 ± 0	8.25 ± 0.5
<i>Williamsia serinegensis</i> LGMB479	12 ± 1.8	9.75 ± 2.2	9.75 ± 2.2	10.25 ± 0.5	12 ± 1.83	10.2 ± 0.96	10.0	10.25 ± 0.96	11.5 ± 0.6	9.5 ± 0.6	13.5 ± 1.3	12.25 ± 1

Strain/Genera	Antimicrobial activity (inhibition zone in mm)											
	P.aeruginosa (Figure S3)						A.baumannii (Figure S4)					
	Medium SG			Medium R5A			Medium SG			Medium R5A		
	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C	28°C	36°C
<i>Actinomadura</i> sp. LGMB466	9.25 ± 1	10 ± 0.8	9 ± 0.8	8.5 ± 1.3	10.5 ± 0.6	10 ± 0.8	10.5 ± 0.6	9.75 ± 1.3	9.75 ± 1	10.25 ± 0.5	9.75 ± 1.3	10 ± 0.8
<i>Actinomadura</i> sp. LGMB487	10.25 ± 1	7.75 ± 1	9.25 ± 0.5	7.75 ± 1	9.25 ± 0.9	10 ± 0.8	9.25 ± 1.9	9.5 ± 0.6	8 ± 0.8	7.5 ± 0.6	10.75 ± 1.9	7.75 ± 0.5
<i>Aeromicrobium ponti</i> LGMB491	10.5 ± 1	10.5 ± 2.9	9 ± 1.2	13 ± 1.8	12.5 ± 1	10.5 ± 2.1	9 ± 0.8	11.75 ± 1	10.25 ± 1.3	8.5 ± 1	8 ± 1.4	10.00
<i>Microbacterium</i> sp. LGMB471	9.0	10 ± 0.8	10.25 ± 1	10 ± 1.4	9.5 ± 0.6	10.5 ± 1.7	9.75 ± 0.5	9.25 ± 1	13.5 ± 0.6	10.75 ± 1.3	11.00	9.75 ± 0.5
<i>Microbispora</i> sp. LGMB461	8.25 ± 0.5	9.0	11.25 ± 1.5	9.0	8.5 ± 1	9.75 ± 0.5	9.00	10.75 ± 1	8.75 ± 1.5	9.25 ± 1	8.5 ± 0.6	8.75 ± 0.5
<i>Microbispora</i> sp. LGMB465	11 ± 1.4	10 ± 0.8	9.5 ± 0.6	10.5 ± 1	11.00	9 ± 0.8	10.25 ± 1	11.00	10.75 ± 0.5	10.25 ± 1	9.75 ± 0.5	11 ± 2
<i>Micrococcus</i> sp. LGMB485	9.0	10 ± 1.2	8.75 ± 1	9.0	10 ± 0.8	9.5 ± 1	9.25 ± 1.5	11.25 ± 0.5	7.5 ± 1	10.25 ± 0.5	7.5 ± 0.6	9.5 ± 1
<i>Sphaerotilus</i> sp. LGMB482	10.5 ± 1.7	8.75 ± 0.5	10.25 ± 1	9.75 ± 1	11.25 ± 0.9	9.75 ± 1.7	10 ± 0.8	11.75 ± 1.5	10.75 ± 1	13 ± 0.8	11.75 ± 1.5	
<i>S. thermocarboxydus</i> LGMB483	11 ± 1.4	11.25 ± 1.5	7.5 ± 0.6	8.5 ± 0.6	9.75 ± 0.5	11 ± 1.4	7.75 ± 1	8.75 ± 0.9	10 ± 1.4	8.75 ± 0.5	7.75 ± 0.5	7.5 ± 0.6
<i>Williamsia serinegensis</i> LGMB479	10.75 ± 2.1	10 ± 1.2	10 ± 1.2	9.5 ± 0.6	12.5 ± 1.9	9.5 ± 0.6	10.75 ± 1.3	11 ± 2	11 ± 2	12.25 ± 1.7	10.25 ± 1.5	

(Continued)

TABLE 3 | Continued

Strain/Genus	Antimicrobial activity (inhibition zone in mm)											
	<i>E. Cloacae</i> producer of VIM (Figure S6)						<i>S. maltophilia</i> (Figure S7)					
	Medium SG			Medium R5A			Medium SG			Medium R5A		
	28°C	36°C	28°C	28°C	36°C	36°C	28°C	36°C	36°C	28°C	36°C	36°C
<i>Actinomadura</i> sp. LGMB466	9.75 ± 0.5	10 ± 0.8	10.25 ± 0.5	10.5 ± 0.6	10.5 ± 1.3	11.75 ± 1	9.5 ± 0.6	9.25 ± 0.5	10.75 ± 0.5	10.5 ± 0.6	11.25 ± 1	10.25 ± 0.5
<i>Actinomadura</i> sp. LGMB487	11.25 ± 0.5	10.5 ± 0.6	10.75 ± 0.5	9.5 ± 0.6	11.25 ± 1	9 ± 0.8	11 ± 0.8	9.00	9.75 ± 1	8.25 ± 0.5	10.25 ± 0.5	8.5 ± 0.6
<i>Aeromicrobiumponi</i> LGMB491	10.75 ± 1	10.5 ± 1.3	12.25 ± 1.7	12.25 ± 1	13.5 ± 0.6	13.25 ± 1.5	9.00	12.25 ± 0.5	10 ± 0.8	10.75 ± 1	8.75 ± 0.5	10.5 ± 1
<i>Mycobacterium</i> sp. LGMB471	11.25 ± 0.5	10.00	10.00	10.25 ± 0.5	12.5 ± 0.6	9.75 ± 0.5	10.75 ± 0.5	10.5 ± 0.6	11.75 ± 0.5	9.75 ± 1	11.25 ± 0.5	10 ± 0.8
<i>Microbispora</i> sp. LGMB461	8.75 ± 1	10.25 ± 1	10.5 ± 1.3	10.75 ± 1	10.25 ± 1.3	10.5 ± 1.3	11 ± 0.8	10.25 ± 0.5	11 ± 1.8	10.25 ± 0.5	9.25 ± 0.5	11.00
<i>Microbispora</i> sp. LGMB465	10.25 ± 1	9.5 ± 0.6	10.75 ± 0.5	10.25 ± 1.3	9.25 ± 1	9.75 ± 0.5	9.5 ± 1	9.5 ± 0.6	9.75 ± 0.9	11.5 ± 0.6	10 ± 0.8	9.25 ± 0.5
<i>Micrococcus</i> sp. LGMB485	12.25 ± 1.3	11.5 ± 0.6	10.75 ± 1	10 ± 1.4	11.75 ± 0.5	10.00	10.5 ± 0.6	10.5 ± 0.6	9.5 ± 0.6	11.25 ± 0.5	8.5 ± 0.6	10.00
<i>Sphaerotilus</i> sp. LGMB482	11.5 ± 0.6	10.5 ± 0.6	10.75 ± 0.5	9.00	11.5 ± 1	10.75 ± 0.5	11.00	10.25 ± 1	12.75 ± 1.2	10.75 ± 0.5	12.00	9.75 ± 0.5
<i>Sthermocarboxydus</i> LGMB483	11.5 ± 0.6	12.00	10.25 ± 0.5	8.5 ± 0.6	11.5 ± 0.6	11.75 ± 1	9.75 ± 1	10.75 ± 1	11.00	12 ± 0.8	9.75 ± 0.9	8.5 ± 0.6
<i>Williamsia</i> serinegensis LGMB479	10.75 ± 0.5	10.5 ± 0.6	11.25 ± 0.5	9.25 ± 0.5	12.00	12.25 ± 1	12 ± 1.2	11.25 ± 1	12.75 ± 1.2	12.25 ± 1	12 ± 0.8	12.25 ± 1

Mean (±SD).

TABLE 4 | Minimum Inhibitory and Minimum Bactericidal Concentrations of the extract from strain *Aeromicrobium ponti* LGMB491.

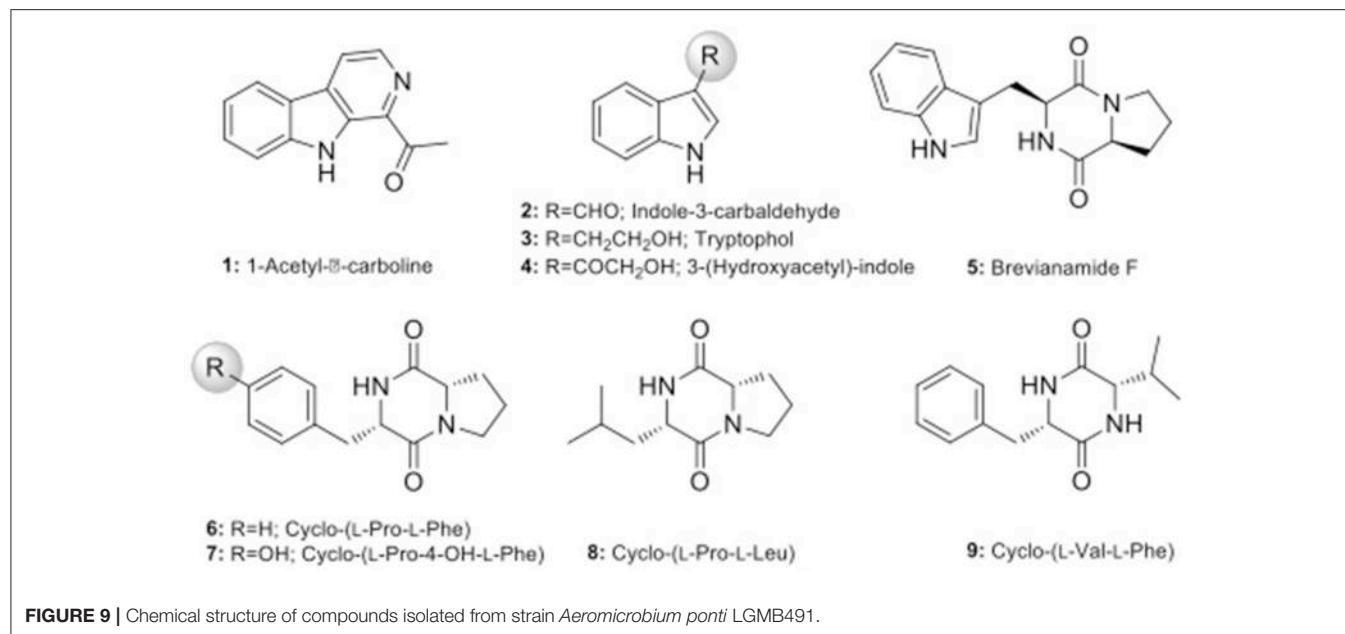
Microrganism	MIC (mg/mL)	MBC
Methicillin-sensitive <i>S. aureus</i> (MSSA)	0.02	5.0
Methicillin-resistant <i>S. aureus</i> (MRSA)	0.04	5.0
<i>Acinetobacter baumanii</i>	0.31	0.63
<i>Pseudomonas aeruginosa</i>	0.31	0.63
<i>Enterobacter cloacae</i> producer of VIM	0.63	1.25
<i>Klebsiella pneumoniae</i> producer of KPC	0.63	1.25
<i>Stenotrophomonas maltophilia</i>	0.63	1.25

Antibiotic Sensitivity Assay

In order to characterize the susceptibility profile as well as to suggest antibiotics to be used in actinomycete isolation, we evaluated the susceptibility profile of endophytes. We detected significant resistance to antibiotics oxacillin and nalidixic acid, only strain *Actinomadura* LGMB487 was sensitive to both compounds (Table 2). Nalidixic acid is the antibiotic used to inhibit bacterial growth during actinomycete isolation, however, even with the use of this compound, the presence of contaminating bacteria was common (Baskaran et al., 2011; Kadiri et al., 2014). Therefore, based on the high resistance to oxacillin observed in this study, we suggest the use of this antibiotic to inhibit bacterial growth during the isolation of actinomycetes. Strains LGMB466 and LGMB487, both characterized as *Actinomadura* sp., showed a complete different sensitivity pattern: strain LGMB487 was resistant only to chloramphenicol, and LGMB466 showed resistance to four antibiotics, and intermediate resistant to chloramphenicol, and rifampicin, suggesting that the resistance profile of isolates is not associated with the intrinsic factors of *Actinomadura* genus. The resistance observed in these strains can result from the presence of plasmids, which contributes to the well-known problem of antibiotic resistance (Wintersdorff et al., 2016). In addition, vancomycin, streptomycin, tetracycline, and gentamicin were previously reported from actinomycetes (Gonzalez and Spencer, 1998; Chopra and Roberts, 2001; Levine, 2006; Zumla et al., 2013), however, all strains evaluated here showed some sensitivity level to these antibiotics, which suggest that these compounds are not present as secondary metabolites from our isolates.

Biological Activity and Secondary Metabolites Identification

All isolates and conditions analyzed produced active secondary metabolites, ratios superior than observed in previous studies (Higginbotham and Murphy, 2010; Passari et al., 2015; Tonial et al., 2016), suggesting the high biotechnological potential of the evaluated strains. This may be related to the culture conditions used to obtain the secondary metabolites. Extracts from LGMB491 (close related to *A. ponti*) showed great activity against MRSA, with inhibition zones higher than caused by vancomycin, the clinical antibiotic used for the treatment of this resistant bacterium (Table 3). In addition, extracts from strain LGMB491 also had considerable MIC, and MBC values

**FIGURE 9 |** Chemical structure of compounds isolated from strain *Aeromicrobium ponti* LGMB491.**TABLE 5 |** Inhibition zone (mm) growth of methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) of compounds **1–9** (100 μ g/disk).

Microorganism \ Compounds	1	2	3	4	5	6	7	8	9	Methicillin
MSSA	18	10	10	10	11	10	–	–	–	20
MRSA	15	9	–	8	9	9	–	–	–	–

against *S. aureus*, MRSA, *K. pneumoniae* KPC, *S. maltophilia*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* VIM. These data suggest the presence of metabolites with broad spectrum activity (Smith et al., 2011). Compounds with broad spectrum activity are required to treat multidrug resistant pathogens, such as MRSA, *S. maltophilia*, *P. aeruginosa*, and *A. baumannii* (Bonomo and Szabo, 2006; Çikman et al., 2016), bacteria that are considered one of the most urgent issues in modern healthcare (Paulus et al., 2017). Therefore, due to the good activity observed, and the absence of studies about metabolites with biological activity from *A. ponti* species, we decided to characterize the major compounds produced by strain LGMB491. From the nine secondary metabolites isolated, 1-acetyl- β -carboline (**1**) turned out to be the compound responsible for the antibacterial activity of the LGMB491 extract. The compound displayed high activity against the MRSA (Table 5). β -carbolines are normally isolated from plants with a large spectrum of biological activity (Lee et al., 2013). Savi et al. (2015b) reported the production of four β -carbolines by the *Microbispora* sp. **1** also isolated from *V. divergens*. The authors isolated as the major metabolite the compound 1-vinyl- β -carboline-3-carboxylic acid, and attributed the vinyl chain as the likely responsible structural feature causing the antibacterial activity of this natural product. However, 1-acetyl- β -carboline (**1**), found during this study, showed also high biological activity, which is unlikely associated with the acetyl chain in position 1. Several studies demonstrated great

activity of compound **1** against MRSA, and suggest the use of this compound for an effective treatment of this resistant bacterium (Shin et al., 2010; Lee et al., 2013). In addition to 1-acetyl- β -carboline (**1**), compounds **2–6** displayed moderate antibacterial activity, and may act synergistically with compound (**1**), contributing for the activity observed. Brevianamide F (**5**), an alkaloid, was isolated for the first time from *Penicillium brevicompactum* (Birsh and Wright, 1969), and has nematocidal (Shiomi and Omura, 2004), anti-inflammatory (Rand et al., 2005), and antibacterial activity against methicillin-sensitive and resistant *S. aureus* (Kumar et al., 2014; Alshaibani et al., 2016). Cyclo-(L-Pro-L-Phe) (**6**) is a diketopiperazine, i.e., a member of these cyclic dipeptides commonly isolated from microorganisms that have been associated with antimicrobial activity, and plant growth regulation (Zhang et al., 2013; Kalinovskaya et al., 2017). Interesting, several diketopiperazines, including cyclo-(L-Pro-L-Phe), were previously isolated from *Aspergillus fumigatus* from a soil sample of the Pantanal, and showed high antibacterial activity against *S. aureus* (Furtado et al., 2005), which supports the idea of synergy of the compounds produced by strain LGMB491. The indoles isolated from strain LGMB491 are commonly produced by plants and endophytic microorganism (Braga et al., 2016). 3-(Hydroxyacetyl)-indole (**4**) showed a broad-spectrum antibacterial activity against methicillin-resistant *S. aureus*, and against vancomycin-sensitive or resistant *Enterococci*, attributed to disruption of cell membrane (Sung and Lee, 2007). In plants,

indole-3-carbaldehyde (**3**) is associated with the innate immunity to microbial pathogen infections (Stahl et al., 2016). This compound was also produced by *Microbispora* sp. 1 previously isolated from the medicinal plant *V. divergens* (Savi et al., 2015b). Some studies suggested that indole compounds play an important role in plant-microorganism interaction and plant defense (Gamir et al., 2012; Lin and Xu, 2013; Jeandet et al., 2014).

CONCLUSION

In this study, we increased the knowledge regarding the endophytic community of the medicinal plant *V. divergens*, through the isolation of rare actinomycetes, some of which were never described as endophytes. We identified for the first time some secondary metabolites produced by one strain close related to the species *A. ponti*, and demonstrated that this species is able to produce indoles, β -carbolines, brevianamide, and diketopiperazines. Future studies to evaluate the potential of these compounds in animal models are required to better understand the potential of compound 1-acetyl- β -carboline as an alternative to treat MRSA infections. Our results indicate that actinomycetes from *V. divergens* have biotechnological potential as producer of bioactive compounds.

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AUTHOR CONTRIBUTIONS

All the authors contributed to the experimental design of the work; as well as to the acquisition, analysis, and interpretation of the obtained results; moreover, all the authors contributed to the writing and the critical revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.01642/full#supplementary-material>

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Bioactive Molecule from *Streptomyces* sp. Mitigates MDR *Klebsiella pneumoniae* in Zebrafish Infection Model

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The emergence and spread of multi-drug resistant (MDR) especially carbapenem-resistant *Klebsiella pneumoniae* is a major emerging threat to public health, leading to excess in mortality rate as high as 50–86%. MDR *K. pneumoniae* manifests all broad mechanisms of drug resistance, hence development of new drugs to treat MDR *K. pneumoniae* infection has become a more relevant question in the scientific community. In the present study a potential *Streptomyces* sp. ASK2 was isolated from rhizosphere soil of medicinal plant. The multistep HPLC purification identified the active principle exhibiting antagonistic activity against MDR *K. pneumoniae*. The purified compound was found to be an aromatic compound with aliphatic side chain molecule having a molecular weight of 444.43 Da. FT-IR showed the presence of OH and C=O as functional groups. The bioactive compound was further evaluated for drug induced toxicity and efficacy in adult zebrafish infection model. As this is the first study on *K. pneumoniae* – zebrafish model, the infectious doses to manifest sub-clinical and clinical infection were optimized. Furthermore, the virulence of *K. pneumoniae* in planktonic and biofilm state was studied in zebrafish. The MTT assay of ex vivo culture of zebrafish liver reveals non-toxic nature of the proposed ASK2 compound at an effective dose. Moreover, significant increase in survival rate of infected zebrafish suggests that ASK2 compound from a new strain of *Streptomyces* sp. was potent in mitigating MDR *K. pneumoniae* infection.

Keywords: carbapenem resistant, MDR *Klebsiella pneumoniae*, zebrafish, *Streptomyces* sp. ASK2, bioactive compound

INTRODUCTION

The emergence and spread of pathogens harboring extended spectrum β -lactamase-like carbapenem-resistant *Klebsiella pneumoniae* (CR-KP), *Escherichia coli*, and other gram negative bacteria are major emerging threat to public health (Schwaber and Carmeli, 2008; Vatopoulos, 2008). Of particular concern is the spread of multi-drug resistant (MDR) strains of *K. pneumoniae*, an encapsulated opportunistic pathogen, colonizes the human gastrointestinal tract, skin, nasopharynx and urinary tract. The pathogen is characterized by the presence of major virulence

factors such as capsule (Siu et al., 2012), type 1 and type 3 pili (Di Martino et al., 2003), LPS (Vuotto et al., 2014) and siderophores (Schembri et al., 2005). Among which, the thick polysaccharide capsule is a promising virulence factor in *K. pneumoniae* that facilitate its evasion of host defenses (Yu et al., 2008). During infectious state, *K. pneumoniae* grow as a biofilm on medical equipments like catheters and this is not only crucial for the establishment of infection but also makes treatment difficult (Zowawi et al., 2015). *K. pneumoniae* is the major cause of infections in catheterized patients, and hence categorized as one of the top eight significant nosocomial pathogens (Ullmann, 1998; Khan et al., 2015). Added to all, *K. pneumoniae* is emerged as a new MDR varieties of human pathogens that can have drastic consequences on health care worldwide (Patel et al., 2008; Struelens et al., 2010; Ulu et al., 2015). One such best example is the evolution and spread of CR-KP. Extended spectrum β -lactamase (ESBL) and carbapenamase producing *K. pneumoniae* have been shown to manifest all broad mechanisms of drug resistance (Nordmann et al., 2009) and also confer resistance to other class of antibiotics such as aminoglycosides, sulphonamides, trimethoprim, tetracyclines, and chloramphenicol, as the plasmids coding for ESBLs may also carry additional genes conferring resistance to many other antimicrobial classes (Rawat and Nair, 2010). Various drug resistance mechanisms including gene mutation, acquisition of novel antibiotic catalytic genes, modification of target site and membrane proteins, differential expression of specific genes for efflux pumps which mediate drug effects and biofilm formation (Tenover, 2006; Nordmann et al., 2009; Cooksey, 2011). Hence infections caused by CR-KP are hard to treat (Kumar et al., 2011; Thakur et al., 2013). Currently, clinicians follow one of the three strategies for the treatment of CR-KP, the first line antibiotic such as meropenem, fluoroquinolone, and aminoglycoside. However, at higher concentrations, these drugs are highly toxic. The second choices of drugs are colistin, tigecycline, and fosfomycin, are more toxic than the first-line drugs (Melano et al., 2003; Petrosillo et al., 2013).

Unfortunately, the emergence of colistin resistance limits further treatment options leading to increase in mortality rates to as high as 50–86% (Hirsch and Tam, 2010; Tzouvelekis et al., 2012; Capone et al., 2013). The third approach, combination therapy has been shown to be a promising choice to overcome the emergence of drug resistance and minimizing drug toxic effect, but in terms of outcomes there are very few reports illustrating combination therapy in humans (Kontopidou et al., 2014). Hence to combat the occurrence of resistant bacteria, development of new antimicrobials has become a more relevant question in the scientific community.

Rhizosphere soil of medicinal plants constitutes one such source for exploring antimicrobials owing primarily to their rich biodiversity (Khamna et al., 2009). *Streptomyces* spp. are one such group of microbes with huge and untapped potential for antimicrobial production. Thus many scientific communities are exploring actinomycetes spp. from various habitats for the development of novel anti-infectives. Rhizosphere soil are rich source of microbes, as rhizodeposition provides an important substrate for soil microbial community (Haichar et al., 2008),

especially medicinal plants due to their unique and enormously divergent bioactive molecules. Hence, we have selected few medicinal plants to isolate actinomycetes spp. antagonistic against MDR *K. pneumoniae*.

During early stage of drug development, drug validation is an important step. Zebrafish (*Danio rerio*) are widely accepted model in the current decade for *in vivo* assessment of drug efficacy (Kari et al., 2007). The major advantages of zebrafish as a predictive model for assessing drug induced toxicity includes the similarity of toxicity profiles to that of humans, requirement of small amount of drug, simple route of administration and also the efficacy of drug can be studied for different infectious dose (Li and Hu, 2012).

In the present study, we have selected five different medicinal plants to isolate potential *Streptomyces* sp. antagonistic against MDR *K. pneumoniae*. The bioactive molecule was isolated by multistep HPLC purification. The toxicity and efficacy of the bioactive molecule were evaluated using adult zebrafish. Also we optimized infectious dose to manifest sub-clinical and clinical infections using planktonic and biofilm of *K. pneumoniae*. Even though many zebrafish infectious models have been developed, this is the first study to report *K. pneumoniae* – zebrafish model and *ex vivo* organ culture of zebrafish liver to screen drug induced toxicity.

MATERIALS AND METHODS

Screening of Multi-drug Resistance in *K. pneumoniae*

Multi-drug resistant *K. pneumoniae* was obtained from Department of Microbiology, Aravind Eye Hospital, Madurai, India. The presumptive identification of the isolate was done using HiCrome UTI agar (HiMedia, India). Further identification were made by performing biochemical tests such as Indole production, MR-VP test, urease test, and lactose fermentation at 44.5°C for 5 days (Ullmann, 1998; Podschun et al., 2001).

Further, the strain was screened for drug resistance by performing Kirby-Bauer disk diffusion test against various selected antibiotics according to Clinical Laboratory Standards Institute (CLSI) guidelines. The selected antibiotics cover a range of β -lactams, fluoroquinolones, aminoglycosides, tetracyclines and polypeptides. Commercially available antibiotics disks (HiMedia, India) were used. The details of antibiotics used in the present study are given in **Table 1**. Triplates were maintained and the results were recorded as sensitive or resistant according to CLSI standard chart.

Screening of ESBL Production

Double disk synergy method was performed to detect ESBL production as recommended by CLSI (2011b). The method was executed using cefotaxime (30 μ g) alone and in combination with clavulanic acid (10 μ g). The cefotaxime disks were placed 30 mm apart from clavulanic-ceftazidime disk. The increase in zone toward the disk of cefotaxime-clavulanate was considered as positive for ESBL production (CLSI, 2011a; Kumar et al., 2014).

TABLE 1 | Antimicrobial susceptibility pattern of clinical strain of *K. pneumoniae*.

Antibiotic name	Quantity of antibiotic	Resistance/ sensitive/intermediate
β-lactums		
I. Penicillins		
Oxacillin	1 µg	Resistance
Methicillin	5 µg	Resistance
II. Cephalosporins		
a. First generation antibiotics		
Cefazolin	30 µg	Resistance
Cefadroxil	30 µg	Resistance
Cefalexin	30 µg	Resistance
b. Second generation antibiotics		
Cefuroxime	30 µg	Resistance
c. Third generation antibiotics		
Cefotaxime	30 µg	Resistance
Ceftazidime	30 µg	Resistance
Ceftazoxime	30 µg	Resistance
d. Fourth generation antibiotics		
Cefepime	30 µg	Resistance
Cefazolin	30 µg	Resistance
III. Carbapenems		
Imipenem	10 µg	Resistance
Meropenem	10 µg	Resistance
Ertapenem	10 µg	Resistance
Doripenem	10 µg	Resistance
IV. Aminopenicillins		
Amoxicillin	10 µg	Resistance
Ampicillin	10 µg	Resistance
Fluoroquinolones		
Levofloxacin	5 µg	Resistance
Gatifloxacin	5 µg	Resistance
Moxifloxacin	5 µg	Resistance
Ofloxacin	5 µg	Resistance
Aminoglycosides		
Gentamicin	30 µg	Resistance
Tobramycin	10 µg	Resistance
Amikacin	10 µg	Sensitive
Tetracyclines		
Tetracycline	30 µg	Resistance
Polypeptides		
Polymyxin B	300 units	Sensitive

Rhizosphere Soil Collection and Pretreatment

Rhizosphere soil of five different medicinal plants (*Solanum trilobatum*, *Ocimum tenuiflorum*, *Cardiospermum halicacabum*, *Justicia adhatoda*, and *Wedelia chinensis*) were collected from local medicinal plant garden. The plants were removed with intact roots and root zone soil were collected in a sterile container and kept at 4°C for further processing (Turnbull et al., 2012). Ten gram of each soil samples was incubated at 60°C for 40 min and re-suspended in 50 ml of saline. The re-suspended mixture was diluted with 50 ml of saline containing 1.5% (v/v)

phenol and shaken for 30 min at 28°C (Ramakrishnan et al., 2009).

Isolation of Actinomycetes spp.

The actinomycetes spp. were isolated by serially diluting the pretreated rhizosphere soil sample and inoculating by spread plate method onto various agar medium recommended for Actinomycetes spp. such as glycerol asparagine agar, actinomycetes isolation agar (Nanjwade et al., 2010), tryptone yeast extract medium (ISP1), yeast extract malt extract agar (ISP2) (Busarakam et al., 2014) and Czapek dox agar (Mangamuri et al., 2016). The media were supplemented with gentamicin (1 µg/ml) and fluconazole (50 µg/ml) after sterilization to inhibit the growth of bacteria and fungi, respectively (Eccleston et al., 2008). The plates were then incubated for 21 days at 30°C. All the morphologically different actinomycetes sp. grown on different agar medium were sub-cultured. Further, 20% of glycerol stocks at -80°C were maintained.

Antagonistic Activity against MDR

K. pneumoniae

Anti-bacterial activity of 51 actinomycetes isolates were assayed to select a potential strain according to Ramakrishnan et al. (2009). Spore suspensions of individual isolates were spot inoculated (10 µl per spot) on Muller Hinton agar plates, and incubated at 30°C for 3 days. The cells were then killed with chloroform vapors and were subsequently overlaid with 15 ml of medium containing 1% (w/v) agar, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract and inoculated with 100 µl of test isolate MDR *K. pneumoniae* on the agar surface. The resulting clear zone of inhibition (ZOI) was measured after 24 h of incubation. The experiment was repeated thrice. Mean diameter of ZOI and standard deviations were calculated. The strain ASK2 which exhibited the antagonistic activity against MDR *K. pneumoniae* was selected for taxonomical investigation.

Taxonomical Investigation of ASK2

Genomic DNA from *Streptomyces* sp. ASK2 was isolated using the procedure described by Kimura (1980). The gene fragments were amplified by using PCR Kit (GENEI Pvt. Ltd, India) and the 16S rRNA gene was amplified using Mastercycler pro thermal cycler (Eppendorf) with the following profile: initial denaturation at 95°C for 4 min, 30 amplification cycles of (95°C for 1 min, annealing temperature at 50°C for 60 s, 72°C for 1 min) and a final extension step at 72°C for 4 min. The PCR product was electrophoresed and purified from 1.5% agarose gel using QIAquick PCR purification kit (QIAGEN) and sequenced using the primers 8F and U1492R (Abdel Azeiz et al., 2016). Sequencing was done at Chromous Biotech, Bengaluru, India using ABI 3100 sequencer (Applied Biosystems). The sequence was edited using FinchTV (Geospiza, Inc.) and BioEdit (Ibis Biosciences, Abbott Labs). Sequence similarity search was made using 16S rRNA gene and taxid specific BLAST tool. Representative 16S rRNA sequences of related type strains of *Streptomyces* sp. were retrieved from National Center for Biotechnology Information (NCBI) database, and were aligned with *Streptomyces* sp.

ASK2 using CLUSTAL-X software (Ser et al., 2015a). MEGA version 5.2.2 neighbor-joining algorithm used to construct phylogenetic tree (Tan et al., 2015). Kimura's two-parameter model was used to compute evolutionary distances for the neighbor-joining algorithm (Ser et al., 2015b).

Fermentation and Recovery of Biomolecules

The seed culture of ASK2 was grown on seed medium (1% starch, 0.5% glucose, 0.5% yeast extract, 0.5% K₂HPO₄, 0.05% MgSO₄·7H₂O) and incubated at 30°C for 3 days on a rotary shaker. 15% (v/v) of seed culture was used to seed 10 liters of production medium (1% starch, 0.5% glucose, 0.5% yeast extract, 0.5% K₂HPO₄, 0.05% MgSO₄ and 0.5% peptone) at 30°C for 11 days. The fermented broth was centrifuged at 4300 × g for 10 min at 4°C. The culture filtrate was extracted with equal volume of ethyl acetate (EtOAc) and concentrated using rotary vacuum evaporator. The EtOAc extract was further concentrated *in vacuo* and dissolved in 10X phosphate buffer saline pH 7.0.

Purification

The EtOAc extract dissolved in phosphate buffer was purified using Semi-preparatory HPLC (Agilent Technologies, India). Reverse-phase analytical C18 column (Agilent Technologies, Netherlands: 4.6 mm × 250 mm) was used for standardizing the experimental conditions. The chromatographic separation was carried out using C18 preparatory column (Agilent Technologies, Netherlands: 10 mm × 250 mm) with water and methanol, 60:40, flow rate of 4 ml/min and injection volume of 0.5 ml. The elution pattern was monitored at 250 nm, peaks were then eluted out separately and each of them was screened for bioactivity by means of broth dilution method. The broth system consisted of 30 µl of 0.5 McFarland of *K. pneumoniae* culture in 2 ml nutrient broth with 200 µl of purified compound. The similar protocol was followed to find the activity against the standard isolate obtained from Microbial culture collection, Pune, MCC 2570-*Klebsiella pneumoniae*, NDM type drug resistant strain.

The fraction which displayed antimicrobial activity was further processed for second and third step purification by using water and methanol (40:60) as a mobile phase.

Characterization

Fourier Transform-infra-red Spectroscopy

The HPLC purified ASK2 was studied by Fourier transform-infra-red spectroscopy and the characteristics bands between 4000 and 400 cm⁻¹ were recorded using Perkin Elmer, Spectrum RX I.

Mass Spectroscopy Analysis

The purified compound was subjected to Electron Spray Ionization and the molecular weight was determined from m/z values.

¹H-NMR

The purified ASK2 was dissolved in DMSO and spectral analysis was performed using BRUCKER 300MHz AVANCE-II.

MIC Determination

The MIC of the compound ASK2 against *K. pneumoniae* was determined for the active fraction using microbroth dilution assay as described in the CLSI protocols. The overnight *K. pneumoniae* culture was diluted to obtain the final concentration of 10⁵ cfu/ml. The compound was dissolved in PBS and was tested at concentrations from 0.5 to 64 µg in twofold step intervals. Each well contains 100 µl of nutrient broth, 10 µl of culture and 100 µl of compound with varying concentrations. Triplicates were maintained for each concentration. The OD values were recorded at 600 nm, as well, the colony counts were recorded on Nutrient agar plates. The OD values and colony forming units were used to determine the MIC.

Animals

Zebrafish (*D. rerio*) irrespective of sex measuring approximately 4 to 5 cm in length, and weighing 300 mg, were purchased from a local aquarium. Proper care and maintenance such as feed, aeration and 14:10-h light/dark cycle at 25°C were ensured to allow acclimatization for a week before the study. All experiments were performed in compliance with applicable national and/or institutional guidelines for the care and use of animals (Animal Biosafety Level 2) were followed.

Preparation of Different Concentration of *K. pneumoniae*

Klebsiella pneumoniae were grown overnight in nutrient broth at 37°C. Different concentrations of cells were prepared by observing optical density and by plating on solid media ranging from 10⁴ to 10¹⁴ cfu/ml.

Biofilm Formation

A loopful of *K. pneumoniae* cells were inoculated in 50 ml nutrient broth and incubated at 37°C for 18 h. The cells were collected by centrifugation and adjust to 0.5 McFarland (10⁸ cfu/ml) with nutrient broth. 500 µl of inoculum were added to wells of 6 well cell culture plates containing glass coverslip of 1 cm² and incubated at 37°C for 72 h. The media were discarded and non-adherent cells were removed by washing with PBS. The biofilms that remained over the coverslips were scrapped and suspended in PBS and optical density of biofilm was measured at OD₆₀₀.

Induction of *K. pneumoniae* Planktonic and Biofilm Infection

To establish *K. pneumoniae* infection in zebrafish, planktonic cells of different density (10⁴–10¹⁴ cfu/ml) and biofilm (10⁹–10¹⁴ cfu/ml) were used. Each test group (*n* = 5 for each group) were infected by injecting 10 µl of different density of planktonic cells of intramuscularly 45° angle to the spine into a position immediately lateral to the Dorsal fin at a depth of 2 mm using sterile 1 ml insulin syringe (U-40, 0.30 mm × 8 mm). One uninjected group was maintained as control. The fish were observed daily to monitor pathological changes such as infection and lethality. The fish which were alive were sacrificed after 24 h and the dead fish were dissected immediately (~6 hpi).

The selected infected and uninfected whole fish were placed on iced wax plate, the organs were dissected out and the muscle tissue was used to estimate *K. pneumoniae* loads in. 100 mg of dissected muscle tissue were placed in a 2 ml microcentrifuge tube and homogenized using micropesle in 0.5 ml phosphate buffer saline and made upto 1 ml. This was followed by serial dilutions of 10^{-1} to 10^{-5} homogenates using sterile PBS and counts of blue mucoid colonies were estimated on UTI agar medium using 10^{-5} dilution. The colonies were counted following 24 h incubation at 37°C.

Toxicity Assessment

The compound was dissolved in phosphate buffer (10X) and fixed dose procedure was followed to assess the non-lethal toxicity. The following concentrations of compound were evaluated (8, 16, 32, 64, and 128 µg/ml). Zebrafish were challenged with intramuscular injection of 10 µl of different doses of compound ($n = 5$), and control groups received phosphate buffer as vehicle control ($n = 5$). Zebrafish were monitored for behavioral changes and mortality for upto 120 h. The experiments were repeated thrice on three independent days.

Ex Vivo Culture of Liver and Cell Viability Assay

Liver toxicity assay were performed for low dose (24 µg/ml), effective dose (48 µg/ml), and high dose (128 µg/ml) of ASK2. Effective dose is the one that was sufficient to achieve the desired clinical improvement (survival) after being challenged with infectious dose of *K. pneumoniae* cells. The toxicity was evaluated by MTT cell viability assay in *ex vivo* organ culture of zebrafish liver. The protocol developed for hepatotoxicity assessment in *ex vivo* organ culture of zebrafish is provided in Figure 1.

Each test group ($n = 5$) was administered with 24, 48, and 128 µg/ml of drug concentrations, and the control group received PBS. After 12 h, the fish were anesthetized by 150 mM of tricaine-S (MS-222) and euthanized by decapitation, the liver was isolated and washed thrice with PBS (experiment was carried at 4°C). The cleaned and chilled liver was trypsinized for 5 min to obtain single cells. To allow cell adherence, trypsin was then neutralized with culture media (DMEM + 10% FCS + 1X antibiotics). The dissociated cells were centrifuged ($43 \times g$ for 5 min) at 4°C and the cell pellets were re-suspended in growth media. The cells were seeded in a 96 well flat bottom microtitre plate at a density of 10^4 cells/ml with 100 µl of culture medium. A control well (culture medium alone) was maintained. The plate was incubated at 37°C for 12 h to allow the liver cells to grow and adhere. After incubation, the medium from the wells were removed carefully and MTT assay was performed. Briefly, 10 µl of MTT solution was added and the plates were incubated for 4 h at 28°C. At the end of the incubation, the culture medium was removed and 100 µl of DMSO was added and allowed to react for 45 s. The readings were obtained at 570 nm using ELISA plate reader. Meanwhile, the viability of cells were also assessed using fluorescent microscopy using FITC and propidium iodide stain to discriminate live and dead cells.

After the removal of cell culture medium, the staining solution was added and allowed to stain for 15 min in dark. The staining solution was removed by washing with PBS and the samples were analyzed using Nikon Trinocular microscope (Nikon Eclipse Ni-U, Japan).

Histopathology

Selected whole zebrafish were fixed following wash with 0.9% ice-cold saline and fixed immediately by immersion in Dietrich's fixative (10 ml per fish), with overnight incubation at room temperature. Fixed samples were routinely processed and then embedded in paraffin; 5 mM thick longitudinal sections were prepared which were de-waxed and rehydrated by standard methods and stained by haematoxylin and eosin. The histological observation of muscle tissue were made using Nikon Trinocular microscope (Nikon Eclipse Ni-U, Japan).

Efficacy of Compound to Treat MDR *K. pneumoniae* Infection

Two groups of fish were infected with 10 µl of 10^{12} cells intramuscularly. After 3 h of infection, the treatment group ($n = 20$) were intramuscularly administered with effective dose (48 µg/10 µl) of ASK2. The control group received sterile PBS as vehicle control. All the groups were monitored for the behavioral changes and mortality for 120 h. The survival rate and *K. pneumoniae* burden were recorded.

Statistical Analysis

All experiments were performed in triplicates. Mean, Standard deviation and Student's *t*-test was performed to test the statistical significance in MTT assay studies and graphs were prepared by using Graphpad prism 6.

RESULTS

Screening of Multi-drug Resistance in *K. pneumoniae*

Presumptive identification of *K. pneumoniae* using chromogenic agar results in the formation of blue mucoid colonies (Rajaratnam et al., 2014). Further, the use of conventional phenotypic methods allowed the characterization of *K. pneumoniae* as mucoid, non-motile, encapsulated isolate exhibiting positive reactions for VP test, urease test, lactose fermentation at 45°C and Indole negative, which basically distinguish it from *K. oxytoca* (Brisse et al., 2006; Patel et al., 2008). The antimicrobial susceptibility of *K. pneumoniae* against various antibiotics are given in Table 1. It was observed that the clinical *K. pneumoniae* exhibits resistance to all antibiotics of different classes including β-lactams, fluoroquinolones, aminoglycosides, tetracyclines and polypeptides and shows sensitivity to polymyxins alone. Further, the test strain shows positive for ESBL screening using cefotaxime-clavulanate combined disk test as recommended by CLSI. The test strain showed 25 mm of ZOI for cefotaxime-clavulanate combined disk (30/10 µg) whereas absence of ZOI was observed for cefotaxime disk. This result confirms that the

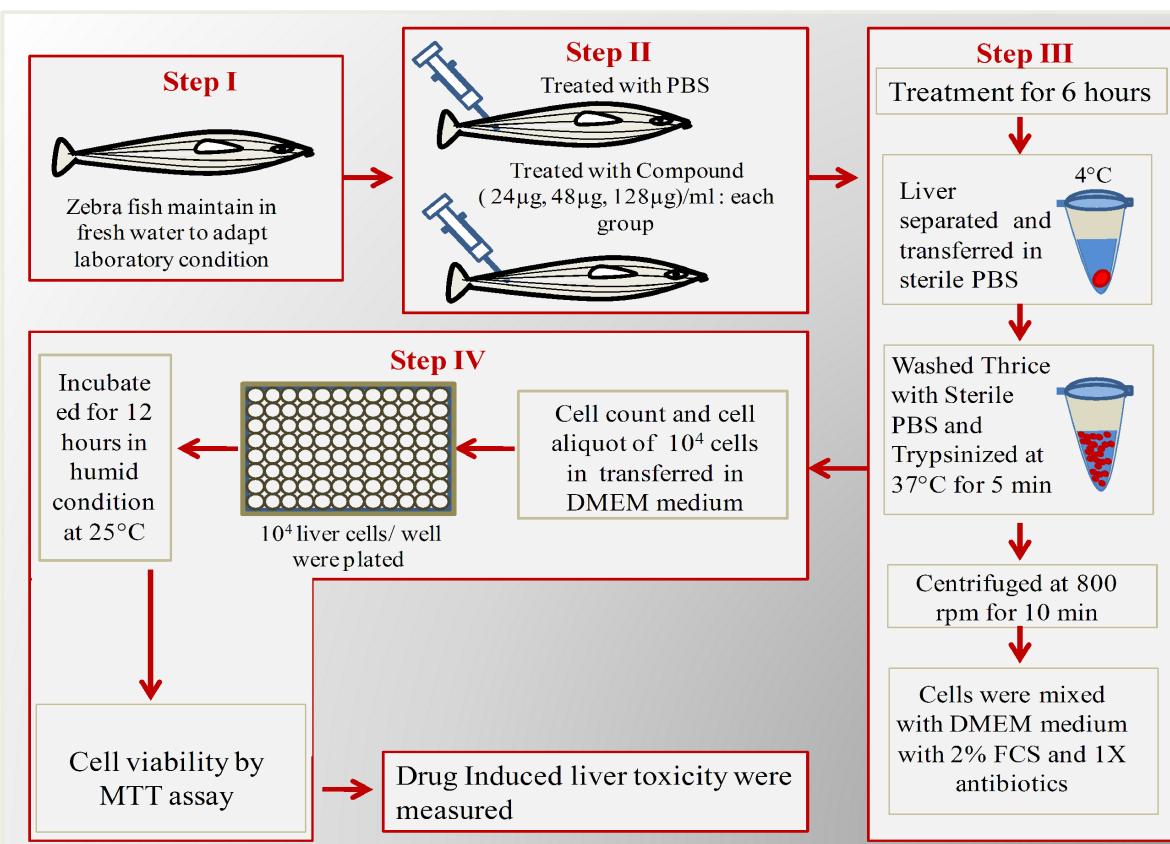


FIGURE 1 | Schematic overview of ex vivo organ culture of zebrafish liver cells. Step I: Acclimatization of zebrafish for 1 week; Step II: Injected with different concentrations of ASK2 and observe for 6 h; Step III: Separation and trypsinization of liver; Step IV: Plating of liver cells in 96 well plate for over night and checking cell viability by MTT assay.

clinical strain *K. pneumoniae* used in the present study produces ESBL. Hence the clinical strain was designated as MDR bacteria as per ECDC and CDC standard definition (Magiorakos et al., 2011).

Anti CR-KP Activity and Taxonomical Characterization of Potential Isolate

During the study, 51 morphologically different actinomycetes spp. were isolated from rhizosphere soil of five different medicinal plants. However, only one isolate was shown to have a potential activity against MDR *K. pneumoniae*. The isolate *Streptomyces* sp. ASK2 obtained from *Solanum trilobatum* soil sample displayed promising antagonistic activity with ZOI of 33 ± 0.5 mm by colony over lay assay (Figure 2B). Similarly the culture supernatant exhibited strongest activity against drug resistant *K. pneumoniae* and thus the strain ASK2 was subjected for taxonomical studies. The strain ASK2 was characterized by wrinkled, rough, irregular, dry and white aerial mycelia on ISP2 agar medium (Figure 2A). The scanning electron micrograph of ASK2 was found to have branched ribbon like spores with smooth surface (Figure 2C). The sequence (1053 base sequences) similarity search using BLAST tool reveals that ASK2 (Gen-

Bank Accession Number: KR187109) belongs to a distinct phyletic line in *Streptomyces* sp. The isolate was closely related to the type strain of *S. rimosus* subsp. *paromomycinus* strain NBRC 15454 sharing a homology of 97%. On the basis of cultural characteristics, spore structural properties, and 16S rRNA sequence, ASK2 was identified as a new strain belonging to *Streptomyces* sp. (Figure 3). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987; Ser et al., 2016). The optimal tree with sum of branch length = 0.04392832 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and were in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 10). The analysis involved nucleotide sequences of 20 *Streptomyces* sp. All ambiguous positions were removed for each sequence pair. There were a total of 1520 positions in the final dataset. Evolutionary analyses were conducted using MEGA5 (Tamura et al., 2011).

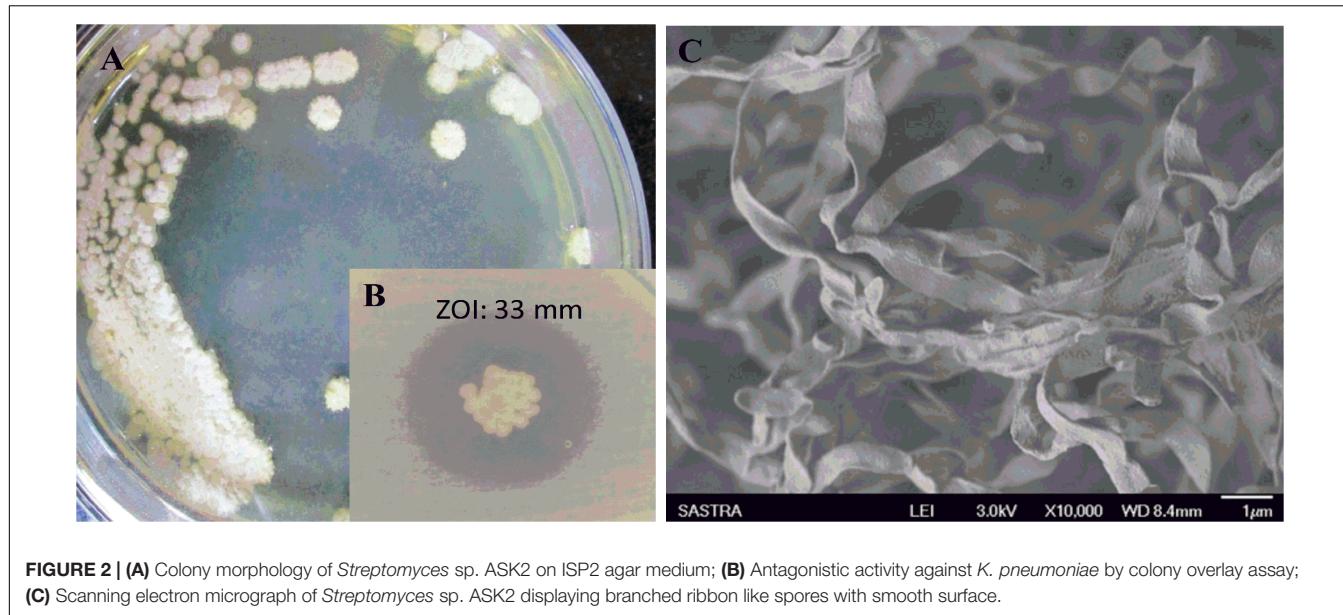


FIGURE 2 | (A) Colony morphology of *Streptomyces* sp. ASK2 on ISP2 agar medium; **(B)** Antagonistic activity against *K. pneumoniae* by colony overlay assay; **(C)** Scanning electron micrograph of *Streptomyces* sp. ASK2 displaying branched ribbon like spores with smooth surface.

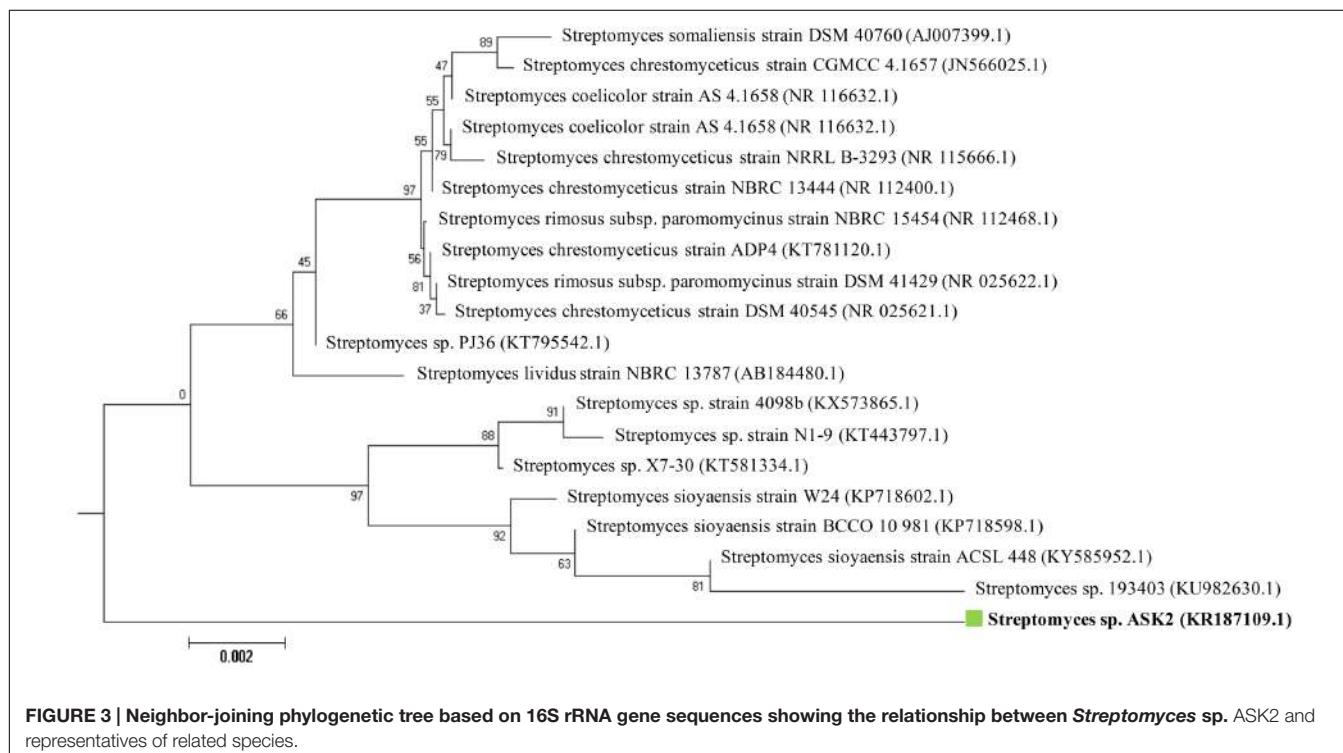


FIGURE 3 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *Streptomyces* sp. ASK2 and representatives of related species.

Multistep Purification and Identification of Active Peak

To isolate the active component, the crude extract (3 g) was subjected to reverse phase HPLC using optimized experimental parameters. Among the 12 fractions collected, the 3rd fraction with a retention time of 4.57 min showed potential antibacterial activity using broth dilution method. The re-passage of the active peak over the same column under the same experimental conditions yielded three peaks, among which one prominent

peak at a retention time of 4.4 min was found to have bioactivity against *K. pneumoniae*. The compound purity obtained at the second re-passage was 86%. Hence the active peak was subjected to third round of re-passage with 40% water and 60% methanol solvent system. The compound purity was increased to 93% (**Supplementary Image S1**). The compound showed the killing effect on standard NDM type *K. pneumoniae*. The pure compound obtained was white solids and are soluble in H₂O, DMSO, methanol and insoluble in CDCl₃ and petroleum ether.

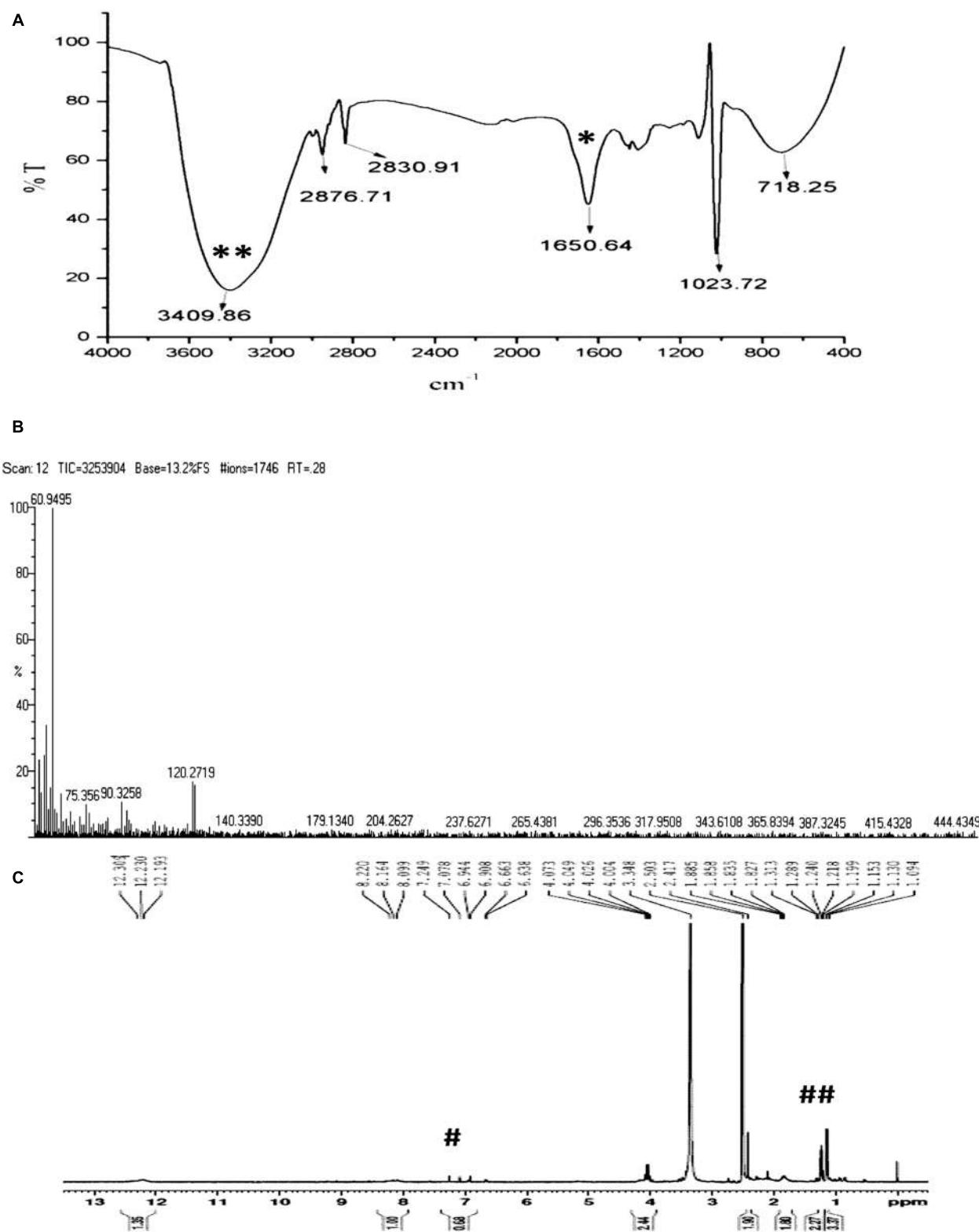


FIGURE 4 | Characterization of purified ASK2 bioactive molecule from *Streptomyces* sp. ASK2: (A) FT-IR Spectra: Presence of strong absorption troughs at 3400 cm^{-1} () and 1651 cm^{-1} (*) representing the presence of OH and C=O groups; (B) ESI-MS spectra; (C) ^1H NMR spectra represents the presence of aromatic compounds (#) and presence of hydrogen in aliphatic carbon chain (##).**

In order to verify whether the compound is similar to that produced by *S. rimosus*, the HPLC comparison of ASK2 and oxytetracycline were made. HPLC comparison of oxytetracycline and ASK2 does not appear to have similar separation profile. Moreover, the FT-IR spectrum of oxytetracycline and ASK2 were different. IR spectrum of ASK2 showed strong absorption troughs at 3400 and 1651 cm⁻¹ that represents the presence of OH and C=O groups, respectively (Figure 4A). Whereas, primary amine is the main functional groups in oxytetracycline, the ASK2 did not show any absorption peaks for primary amine (two peaks at \approx 3400 cm⁻¹ and \approx 3500 cm⁻¹), (Ramasamy et al., 2014). ESI MS data reveals that the molecular weight of ASK2 is found to be 444.43 Da (Figure 4B), which was found to be differing from oxytetracycline (460.43 Da) (Singh et al., 2011). In addition the ¹H NMR showed the triplet peaks at 6.944 to 7.249 ppm. It indicates the presence of hydrogen moiety in aromatic environment. Peaks in the range of 1.199 to 1.313 ppm indicate the presence of aliphatic nature of carbon chain (Figure 4C). Hence the bioactive compound isolated from *Streptomyces* sp. ASK2 is warrant to be a new compound, however, other NMR spectrometric methods are required to elucidate the complete structural details of the compound.

Screening of Toxicity and Efficacy of ASK2 in Zebrafish Model

Prior to testing of compound, the MIC of ASK2 was estimated for the purified compound. 16 µg/mL was found to be the MIC. The agar plates were recorded for the absence of colonies at this concentration. And the OD₆₀₀ was comparable with the control (Nutrient broth + compound) for final concentrations of 16 µg/mL and above.

Optimization of Subclinical and Clinical Infection for Planktonic Cells

We examined the susceptibility of zebrafish to *K. pneumoniae*. The adult fish were injected intramuscularly with various infectious dose (10^4 – 10^{14} cfu/ml) to induce subclinical and clinical infection in zebrafish. The survival rate of each infectious dose is given in Table 2. Upon infection with 10^{14} and 10^{12} cfu/ml, zebrafish readily became infected displaying 100% mortality within 24 and 120 h, respectively. Whereas, upon infection with less than 10^{12} cfu/ml, zebrafish were active and no mortality was observed (Table 2). Even though the aquarium water was highly loaded with *K. pneumoniae*, cells less than 10^{12} cfu/ml does not lead to clinical symptoms, suggesting that intramuscular injection with optimum dosage of *K. pneumoniae* are crucial to enhance the intracellular invasion and spread. Further, the estimation of colony forming units from tissue homogenates of the infected fish of each group on UTI agar reveals the presence of large numbers of *K. pneumoniae* organisms. These results demonstrated that *K. pneumoniae* successfully infects zebrafish and cause clinical illness. *K. pneumoniae* is considered to be the most important histamine producing bacteria in fish (Kanki et al., 2002). It is also known to cause fin and tail disease in Rainbow trout and more reports on *Klebsiella* infection

in different fishes are documented (Diana and Manjulatha, 2012).

Virulence of *K. pneumoniae* Planktonic and Biofilm

The role of different phenotypic expression (planktonic and biofilm) of *K. pneumoniae* were examined in zebrafish free living and biofilm model. Adult zebrafish were challenged with different concentrations of *K. pneumoniae* in planktonic and biofilm state. Biofilm at the dose of 10^{12} cells, caused a significant clinical symptoms and 100% mortality within 120 h. The lower dose ($<10^{12}$ cfu/ml) leads to sub-clinical infection alone. These observations were similar to that of planktonic cells. Furthermore, the colony counts of tissue homogenate were similar to that of planktonic cells. The results show no correlation between severity of infection and different phenotypes of *K. pneumoniae* in zebrafish planktonic and biofilm infection model (Table 3).

Toxicity Assessment by Fixed Dose Procedure

To assess the non-lethal toxicity, various concentrations of ASK2 were evaluated. Zebrafish challenged with different concentrations were observed for behavioral changes and mortality for upto 120 h. The compound was non-lethal upto 64 µg/ml concentration, whereas doses greater than 64 µg/ml was toxic, and 100% mortality were observed for 128 µg/ml (Table 4). However, to screen for liver toxicity, the low dose (24 µg/ml), effective dose (48 µg/ml) and high dose (128 µg/ml) of ASK2 were evaluated by performing MTT reduction assay, fluorescent microscopic technique, and histopathology.

Hepatotoxicity Assessment in Ex Vivo Culture of Zebrafish Liver

To evaluate cytotoxic activity of ASK2, the zebrafish were challenged with either low dose, effective dose or high dose of the compound. The treated and control groups were sacrificed after 7 h exposure and liver was obtained. The liver were carefully washed, trypsinized and grown in cell culture medium and incubated for 12 h. After the incubation period, the monolayer cultures were checked for cross contamination and light microscopic examination revealed the presence of adherent liver cells in microtitre plate. The cell viability by MTT assay displays that the compound induced hepatotoxicity in a concentration dependent manner (Figure 5). Zebrafish challenged with 24, 48, and 128 µg/ml compound shows 94, 83, and 61% relative cell viability, respectively (Figure 5). In addition, the fluorescent microscopic image depicts the toxicity by discriminating dead cells and viable cells. The effective dose (48 µg/ml) was found to have more viable cells (Figure 6B) similar to control (Figure 6A) compared to high dose (128 µg/ml) (Figure 6C). The statistical significance by Student's *t*-test for 24 µg/ml was $p = 0.0337$, 48 µg/ml; $p = 0.0040$ and 128 µg/ml; $p = 0.0003$.

TABLE 2 | Optimization of infectious dose of *K. pneumoniae* in zebrafish (planktonic cells).

Dose of challenge	Type of infection	<i>Klebsiella</i> burden in muscle cultured on UTI agar medium	Survival rate (%)				
			24 hpi	48 hpi	72 hpi	96 hpi	120 hpi
10 ⁴ cfu/ml	Sub-clinical	1 ± 0.5 × 10 ⁵ cfu/ml	100	100	100	100	100
10 ⁶ cfu/ml	Sub-clinical	2 ± 0.4 × 10 ⁶ cfu/ml	100	100	100	100	100
10 ⁸ cfu/ml	Sub-clinical	2.6 ± 0.3 × 10 ⁶ cfu/ml	100	100	100	100	100
10 ¹⁰ cfu/ml	Sub-clinical	2.8 ± 0.3 × 10 ⁶ cfu/ml	100	100	100	100	100
10 ¹² cfu/ml	Clinical	3.8 ± 0.6 × 10 ⁶ cfu/ml	90	50	35	30	0
10 ¹⁴ cfu/ml	Clinical	3.2 ± 0.7 × 10 ⁷ cfu/ml	0	0	0	0	0

Experiment was repeated thrice on three independent days, n = 5.

TABLE 3 | Effect of biofilms of *K. pneumoniae* in zebrafish (Biofilm).

Bacterial inoculum	<i>Klebsiella</i> burden in muscle	Survival rate (%)				
		24 hpi	48 hpi	72 hpi	96 hpi	120 hpi
10 ⁸ cfu/ml	2.3 ± 0.5 × 10 ⁶ cfu/ml	100	100	100	100	100
10 ¹⁰ cfu/ml	2.8 ± 0.4 × 10 ⁶ cfu/ml	100	100	100	100	100
10 ¹² cfu/ml	3.9 ± 0.7 × 10 ⁶ cfu/ml	85	50	40	35	0

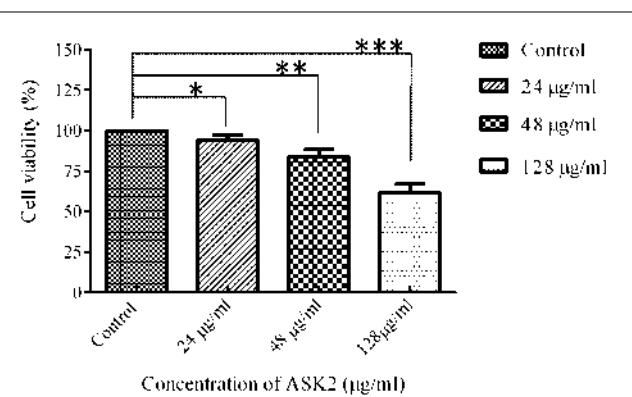
Experiment was repeated one time on three independent days, n = 5.

Histopathology

A histopathological analysis of muscle after injection of the ASK2 compound was performed to check for toxicity of the compound. As can be seen from **Figure 6**, exposure of zebrafish to ASK2 compound at a concentration of 48 µg/ml (**Figure 6E**) did not produce any adverse change in the muscle, the site of injection of the compound. The muscle showed normal morphology as that observed with the control fish (**Figure 6D**). However, in the case of zebrafish that were exposed to the compound at a higher dose, 128 µg/ml (**Figure 6F**), there were focal points of cell infiltration into the muscle but the overall morphology of the muscle in this case also appeared to be normal. This suggests that the compound ASK2 is not toxic at the effective dose of 48 µg/ml, used to treat *Klebsiella* infection.

Efficacy of Compound to Treat *Klebsiella* Infection

Injection of 10¹² cfu/ml of *Klebsiella* inoculum to the healthy adult fish showed clinical infection and 100% mortality within 120 h. Whereas, 90% of survival rate were observed for the treatment group after being treated with 48 µg/ml of ASK2 compound. Also, there was visible reduction in *Klebsiella* burden

**FIGURE 5 | MTT assay of zebrafish liver cells in ex vivo conditions.**

Student's t-test was performed to each treated group comparing with control. 24 µg/ml (*p = 0.0337), 48 µg/ml (**p = 0.0040), 128 µg/ml (**p = 0.0003).

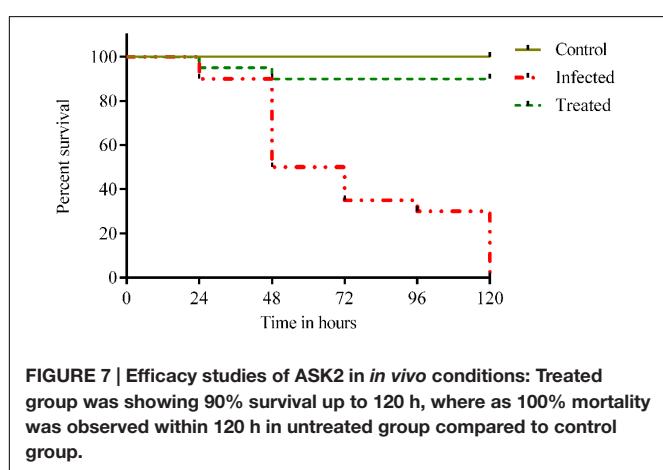
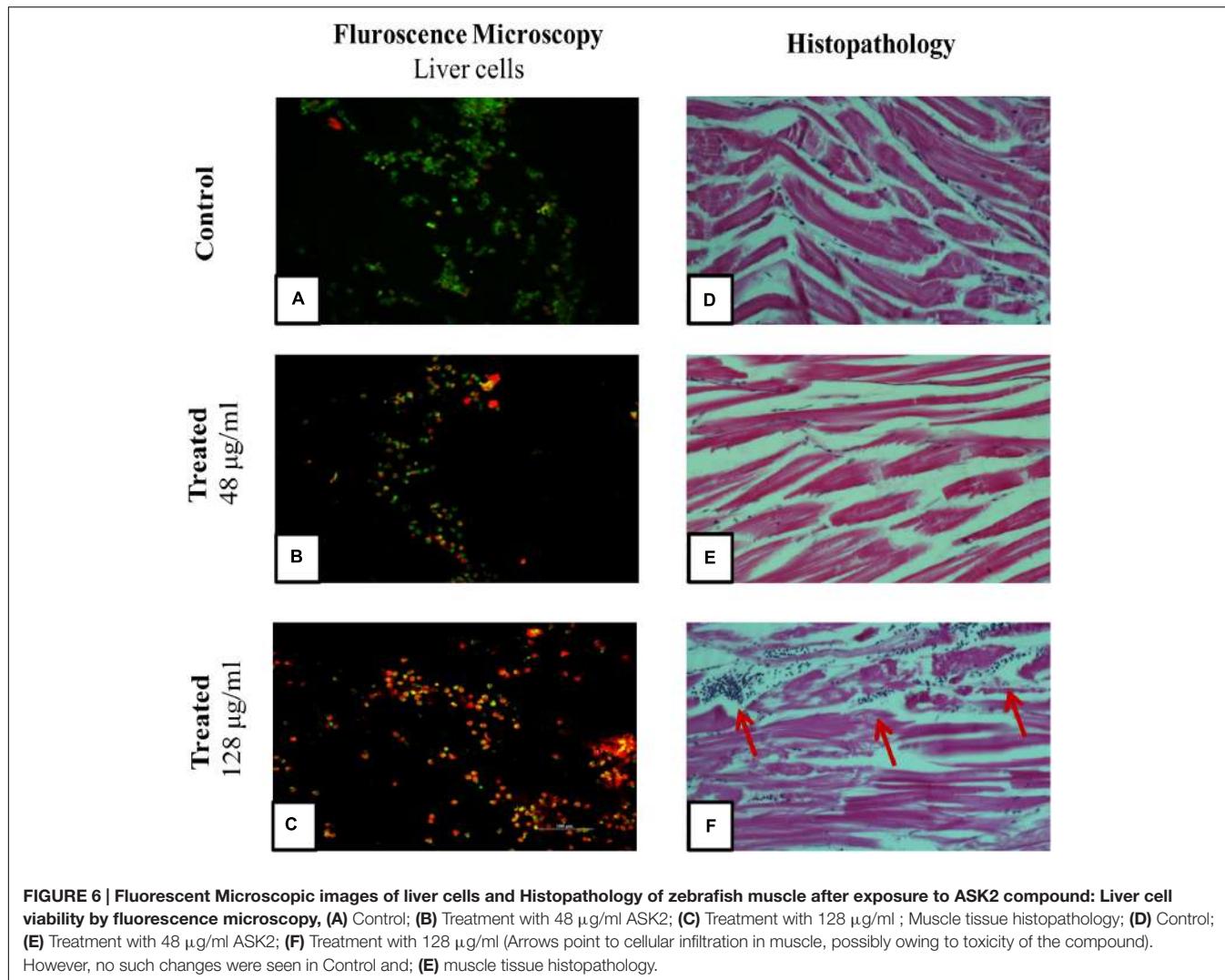
TABLE 4 | In vivo toxicity of different concentrations of compound (n = 5).

Concentration of compound (µg/ml)	Survival rate (%)				
	24 h	48 h	72 h	96 h	120 h
8	100	100	100	100	100
16	100	100	100	100	100
32	100	100	100	100	100
64	100	60	60	60	60
128	20	0	0	0	0

after 24 hpi in zebrafish treated with effective dose of ASK2 compound (**Figure 7**). The significant increase in survival rate at intermediate concentration clearly indicates that ASK2 exerts a potential therapeutic effect by controlling *K. pneumoniae* proliferation.

DISCUSSION

As the burden of MDR *K. pneumoniae* is growing rapidly, discovery of new drugs is an indispensable process to combat those emerging resistant pathogens (Bérdy, 2012). Actinomycetes are still the promising candidates for the discovery of novel antibiotics which are being isolated from wide range of natural habitats (Tiwari and Gupta, 2012). Exploring the untapped region for collection of rare and novel microbial species is



challenging for the exploration of novel drugs (Cragg and Newman, 2013). However, we believe that potential antibiotics producers are still hidden in very familiar sources like soil,

plants, marine plant and animals etc. Rhizosphere soil of medicinal plants are one such striking source of diverse microbial community (Köberl et al., 2013). In the present study, 51 different actinomycetes spp. were isolated from rhizosphere soil of five different medicinal plants. *Streptomyces* sp. ASK2 isolated from *Solanum trilobatum* was found to have a potential inhibitory activity against MDR *K. pneumoniae*. *Solanum trilobatum* is an important plant in medicine with wide applications including treatment of respiratory diseases, tuberculosis, cardiac and liver infection. It is also familiar for anti-inflammatory, anti-oxidant and immunostimulatory properties (Priya and Chellaram, 2014; Venugopal et al., 2014).

Streptomyces rimosus is well-known for the synthesis of oxytetracycline, possessing broad spectrum antibacterial activity and some bacterial strains have developed resistance (Chopra and Roberts, 2001). However, the usage of oxytetracycline for humans is prohibited as it is associated with adverse toxic effects. Hence it is now generally used to treat rickettsiae and mycoplasma infection in animals (Stuen and Longbottom, 2011).

The significance of toxicological screening are well recognized as an important step in drug development process, hence several zebrafish models and assays have been developed to study drug toxicology this decade (Bhusnure, 2015). In the current report, we have modeled *K. pneumoniae* infection in adult zebrafish, to study toxicity and efficacy of ASK2 isolated from *Streptomyces* sp. ASK2.

Since there are no reports on *K. pneumoniae* zebrafish infection model, we decided to optimize infectious dose, which are quite varied with strain, host susceptibility and even the route of transmission. Sub-clinical and clinical infections were established with infectious dose of 10^4 – 10^{10} and 10^{12} – 10^{14} cfu/ml, respectively (Table 2). A similar kind of observation was recorded for *E. faecalis* in a dose-dependent manner in zebrafish larvae. In general, most of the studies with zebrafish embryo and early larvae used bacterial density of 10^3 – 10^8 cfu/ml to study host pathogen interactions and to predict drug safety. However, the infectious dose and pathogenicity are varied with pathogens belonging to same species of different strains. For instance, in *Galleria mellonella* infection model, Wand et al. (2013), demonstrated that different strains of *K. pneumoniae* showed different virulent expression. And another study revealed that different strain of same species can exhibit different degree of pathogenicity *in vivo* model (Lavender et al., 2004).

Though several zebrafish infectious models have been developed (Neely et al., 2002), the effect of drug to treat biofilm related infections are very limited using zebrafish (Chu et al., 2014). Infact, many studies have suggested that biofilm formation is an important phase in the infective process. Therefore, we investigated the significant role of planktonic and biofilm state of *K. pneumoniae* to cause infection. In our study, *K. pneumoniae* biofilm being formed *in vitro* were analyzed for its virulence in zebrafish. We observed a similar kind of pathological consequences in zebrafish planktonic and biofilm model. The infectious dose, severity of illness, survival rates and bacterial counts in muscle homogenates were similar for biofilm and planktonic zebrafish infection models (Tables 2, 3). One possible reason for similar pathological observation is the gene expression for biofilm formation on solid support is different from those required to establish *in vivo* (Lavender et al., 2004). Many studies have explored biofilm formation as a virulence phenotype in majority of the bacteria. For example, in a study with *Streptococcus suis* zebrafish model, *S. suis* biofilm had LD50 values greater than the LD50 values of planktonic cells, the virulence of biofilm cells were weaker than planktonic cells (Wang et al., 2011). However, in our case, the pathological consequences are similar for planktonic and biofilms. Our result suggests that biofilm state of the pathogens is not exclusively required for virulence expression. However, screening of biofilm establishment in zebrafish and validating with different strains of *K. pneumoniae* are essential to confirm our findings which is our lab's future focus.

Once the infectious doses were optimized, we investigated the efficacy of ASK2 compound and toxicity in adult zebrafish, because drug induced liver injury is a major toxicological

problem. The similarity of zebrafish liver functions and drug metabolism to that of humans, zebrafish has been used as good model for drug toxicity (Triebeskorn et al., 2004; Zodrow et al., 2004; Devi et al., 2015; Ayaz Ahmed et al., 2016; Lawrence et al., 2016). The most commonly used drug induced hepatotoxicity assay in laboratory animals includes serum enzyme assay and histological assay (McGrath and Li, 2008). The present study assessed the liver injury by measuring MTT reduction to determine the cell viability for *ex vivo* culture of liver. There were no obvious changes in liver cell viability and metabolic activity, during *ex vivo* culture of liver from healthy adult zebrafish. The cytotoxic effect of ASK2 was observed in a dose dependent manner in zebrafish. The effective dose (48 µg/ml) of ASK2 displayed 83% liver cell viability, similarly no mortality were recorded during *in vivo* toxicity study. However, 61% liver damage within 6 h was recorded for treatment group with high dose (128 µg/ml), that is at eightfold of MIC and at the same point, 100% mortality were recorded within 24 h. The results of *ex vivo* culture of liver toxicity are highly co-related with *in vivo* toxicity and histopathology analysis, suggesting that *ex vivo* culture of zebrafish liver are a highly reliable and reproducible method for the assessment of drug induced toxicity. In an earlier study, zebrafish brain was similarly cultured *ex vivo* to study the effects of ethanol and acetaldehyde (Zenki et al., 2014).

The efficiency of drug to treat infected zebrafish showed that ASK2 compound was highly effective in killing and controlling *K. pneumoniae* proliferation. The obvious reduction of 10^{12} to 10^4 cfu/ml bacterial burden was recorded with an improvement of 90% survival rate. The efficiency of the compound was highly comparable with untreated group which showed 100% mortality at 120 hpi (Figure 7). Hence, ASK2 compound isolated from *Streptomyces* sp. ASK2 was shown to be effective for the management of MDR *K. pneumoniae* infection in zebrafish model.

CONCLUSION

The continuous use of similar kind of drugs for treatment and overuse of antibiotics in both human and livestock induces different drug resistance mechanisms in bacteria. Since there are no novel antibiotics in drug development in the present century for carbapenemase producing enterobacteriaceae, the existing antibiotics have re-emerged for current medications. Hence in response to address these issues the present research has come up with a potential anti-MDR *K. pneumoniae* molecule from *Streptomyces* sp. ASK2. The antibacterial compound ASK2 reported in the present study was shown to control MDR *K. pneumoniae* infection in zebrafish infection model with a significant improvement in fish survival rate. Furthermore, the *in vivo* toxicity, MTT assay of *ex vivo* liver culture, and histopathology results strongly support the non-toxic property of ASK2 at effective dose. These findings support the potentiality of ASK2 to treat MDR *K. pneumoniae* infection in zebrafish model. Further investigations on complete structural elucidation and extensive evaluation of chronic toxicity may lead to a novel drug candidate to combat the persistent MDR *K. pneumoniae*.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00614/full#supplementary-material>

IMAGE S1 | Isolation of bioactive molecule by Multi step reverse phase HPLC purification: (A)

HPLC purification: (A) Crude extract: *active peak at RT 4.57 min with 23% purity (B) Re-passage of *active peak eluted at RT 4.4 min with 86% purity; (C) Re-passage of *active peak eluted at RT 2.23 min with 94% purity.

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Exploring the Antimicrobial and Antitumor Potentials of *Streptomyces* sp. AGM12-1 Isolated from Egyptian Soil

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The occurrence of extensive antibiotics resistant bacteria increased the demands for mining out new sources of antimicrobial agents. Actinomycetes, especially *Streptomyces* sp. have grasped considerable attention worldwide due to production of many useful bioactive metabolites. In the present study, a total of 52 actinomycetes were isolated from agricultural soil samples in Beni-Suef, Egypt. All isolates were characterized based on colony morphology, mycelium coloration, and pigment diffusion. They were screened for their capabilities to show antimicrobial activities against different indicator microorganisms, and only 20 isolates have shown significant antimicrobial activities against at least one of the tested indicator microorganisms. The isolate AGM12-1 was active against all tested microorganisms and showed a marked antitumor activity with IC₅₀ 3.3 and 1.1 µg/ml against HCT-116 and HepG-2 cell lines respectively. It was genetically characterized as *Streptomyces* sp. with the presence of PKS II biosynthetic gene cluster. Mannitol, ammonium sulfate, pH 7, 2% inoculum size and incubation for 11 days at 30°C were the optimum conditions that used to maximize the production and hence allowed purification of one active antimicrobial compound to homogeneity using high performance liquid chromatography with a molecular mass of m/z 488.05. Nuclear magnetic resonance structural elucidation showed that this compound was a diketopiperazine derivative.

Keywords: actinomycetes, antimicrobial, antitumor, diketopiperazine, *Streptomyces* sp., *Streptomyces vinaceusdrappus*

INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria with fungal morphology. They are characterized by a complicated life cycle belonging to the phylum *Actinobacteria* (Dilip et al., 2013). They are widely distributed in terrestrial ecosystems, especially in soil, where they play a pivotal role in recycling of industrial wastes and biomaterials by decomposing complex polymeric structures in dead plants, animals, and fungal materials (Goodfellow and Williams, 1983).

Actinobacteria, especially *Streptomyces* sp. are known as noble factories for the production of many biologically active compounds that are useful as antibacterials, antifungals, antivirals, antithrombotics, immunomodifiers, anti-tumor drugs, and enzyme inhibitors in many fields especially in medicine (Sacramento et al., 2004; Atta, 2009; Fukuchi et al., 2009; Olano et al., 2009; Ser et al., 2015). Due to the emergence of multi-resistant microorganisms to almost all available antibiotics, many researchers are focused now on discovering novel antimicrobials from many natural resources such as those produced by actinomycetes especially those isolated from many undiscoverable or poorly explored environments (Undabarrena et al., 2016). These antimicrobials are produced by many metabolic pathways, mainly organized by polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Undabarrena et al., 2016). Incidence and presence of these biosynthetic genes in actinobacteria are obviously high (Donadio et al., 2007).

Egyptian soil reservoir is considered as a poorly investigated source for actinobacteria, and very few reports were published (Hozzein and Goodfellow, 2007; Awad et al., 2009; Abd-Alla et al., 2013; Rifaat et al., 2013). In these perspectives, the present study aimed to isolate and characterize different actinomycetes from soil niche in Beni-Suef governorate, Egypt. Actinomycetes were screened for their capabilities to produce antimicrobial and antitumor active metabolites. The bioactive compound from the most potent isolate was further purified and characterized.

MATERIALS AND METHODS

Collection of Samples and Isolation of Actinomycetes

A number of 13 agricultural soil samples were collected aseptically from four different sites located in Egypt (Beni-Suef City, Beba City, Ehnasia City, and El-Fayum roads) from the upper 15 cm layer of soil using sterile plastic bags and transported to the laboratory for further isolation steps. Isolation of actinomycetes was managed using soil dilution plate technique (Williams et al., 1983) on starch nitrate agar (SNA) and tryptone soya agar (TSA) supplemented with Rifampicin (10 µg/ml) and Nystatin (50 µg/ml) to inhibit any bacterial or fungal contaminants. Briefly, 1 g of each soil sample was diluted with 9 ml of 0.9% saline, homogenized and then a serial dilution up to 10^{-4} was carried out. A 100 µl from 10^{-2} , 10^{-3} , and 10^{-4} dilutions were spread on SNA and TSA, and incubated at 30°C for 7 days. Suspected colonies of actinomycetes were characterized morphologically (Aghamirian and Ghiasian, 2009; Reddy et al., 2011), re-purified using streak plate method, and then stored in 40% glycerol at -80°C.

Screening of Actinomycetes for their Antimicrobial Activities

Antimicrobial activities of pure isolates were determined by agar diffusion method (Williams and Davies, 1965) and double layer agar method (modified spot on lawn technique) (Thakur

et al., 2007; Dundar et al., 2015), against different indicator microorganisms; *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (clinical sample), *Candida albicans* (clinical sample), and *Sarcina lutea* (environmental sample). In double layer agar method, all pure isolates were spot inoculated on SNA and incubated at 30°C for 5 days and then 5 ml of molten TSA seeded with 100 µl overnight culture of indicator microorganisms were poured on spotted plates and incubated at 37°C for 24 h. Antimicrobial activities represented in zones of inhibitions were examined.

Fermentation and Extraction of Secondary Metabolites and Total Proteins

The most potent actinomycetes, showing significant antimicrobial activities, were undergoing fermentation. Briefly, actinomycetes were sub-cultured in tryptone soya broth (TSB) for 5 days and 2% of starting inoculum was used to inoculate 1 L of International Streptomyces Project 4 (ISP4) broth in a 2 L Erlenmeyer flask and then incubated on rotary shaker incubator (200 RPM) at 30°C for 7 days. The cell-free supernatant from each flask was collected after centrifugation at 13,000 g for 20 min and divided into two portions; one used to extract total secondary metabolites, and the other used to extract total proteins. For extraction of total metabolites, a 1:1 v/v ethyl acetate was added to the cell-free culture supernatant and shacked vigorously for 1 h. The organic phase was separated and evaporated to dryness using a rotary evaporator (Romankova et al., 1971; Selvameenal et al., 2009). Total extract residues were weighed and dissolved in 5 ml ethyl acetate and kept in a refrigerator at 4°C. For extraction of total proteins, an ammonium sulfate was added to the cell-free culture supernatant in a concentration of 40%. Protein pellets were collected by centrifugation at 14,000 g for 30 min at 4°C and then dissolved in 5 ml distilled water, and kept in a refrigerator at 4°C.

In vitro Anti-tumor Cytotoxicity

Both ethyl acetate extracts and total proteins of the most potent actinomycetes isolates with significant antimicrobial activities were evaluated for their cytotoxicity using tissue culture technique. HepG2 (hepatocellular carcinoma cell line) and HCT 116 (human colon carcinoma) were obtained from the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Cells were maintained in DMEM medium with 10% fetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂ till the cytotoxicity bioassay was carried out. The potential cytotoxicity of four samples was tested using the method of Skehan et al. (1990). Briefly, 100 cells/well were plated onto 96-well dishes overnight before the treatment with the tested compounds to allow the attachment of cells to the wall of the plate. Different concentrations of each tested compound (0, 6.25, 12.5, 25, 50, 100 µg/ml) were added to the cell monolayer and triple wells were used for each individual dose. Monolayer cells were incubated with the tested agent(s) for 48 h at 37°C and 5% CO₂. At the end of the incubation period, the cells were fixed and stained

with sulforhodamine B dissolved in acetic acid. Unbound stain was removed by washing four times with 1% acetic acid and the protein-bound dye was extracted with Tris-EDTA buffer. The absorbance was measured in an ELISA reader. The relation between surviving fraction and compound concentration was plotted to get the survival curve of each tumor cell line and the IC₅₀. The concentration of an agent that causes a 50% growth inhibition, for each tested agent using each cell line was obtained from the survival curve (Skehan et al., 1990).

Phenotypic Characterization of AGM12-1 Isolate

The physiological, biochemical, and cultural characteristics of the talented isolate AGM12-1 which showed broad antimicrobial and cytotoxic activities were examined in detail. The growth capability, pigment production, and color of both aerial and substrate mycelium was determined using different growth media; ISP-3, ISP-4, ISP-5, Czapek Dox, Sato, nutrient agar and SNA. The color was determined visually by making a comparison with chips from the ISCC-NBS centroid color charts (Williams et al., 1983). The types of spore-bearing hyphae and spores chain morphology were determined using direct microscopical examination and the shape of the spore surface was observed using scanning electron microscope (SEM). Production of catalase, lecithinase, protease, lipase, pectinase,

amylase, hydrogen sulfide, nitrate reductase, urease, gelatinase, and melanin besides screening for utilization of different nitrogen sources (peptone, protease peptone, potassium nitrate, yeast extract, ammonium sulfate), different carbon sources (starch, glucose, sucrose, fructose, mannitol) and ability to grow at a wide range of pH from 5 to 11 were carried out according to Williams et al. (1983).

Genomic DNA Extraction and Purification

Genomic DNA extraction was done according to Sinha et al. (2004) and Aly et al. (2016) with some modifications. Briefly, a 1.5 ml of culture was centrifuged for 10 min at 3,000 g, the supernatant was discarded and the pellets were resuspended in 200 μ l spheroblast buffer (10% sucrose, 25 mM Tris pH 8.4, 25 mM EDTA pH 8.0, 2 mg/mL lysozyme and 0.4 mg/ml RNase A), vortexes and incubated at 37°C for 10 min until cell lysis occurred. Then, 50 μ l of 5% SDS (lysis buffer 1) and 5 M NaCl (lysis buffer 2) were added, mixed and incubated at 65°C for 5 min. A 100 μ l neutralizing buffer (60 ml 5 M Potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml dH₂O) was then added and put on ice for 5 min before centrifugation at 18,000 g at 4°C for 15 min. The supernatant (approximately 400 μ l) was transferred to a new tube, mixed with equal volume of isopropanol, left 5 min at room temperature

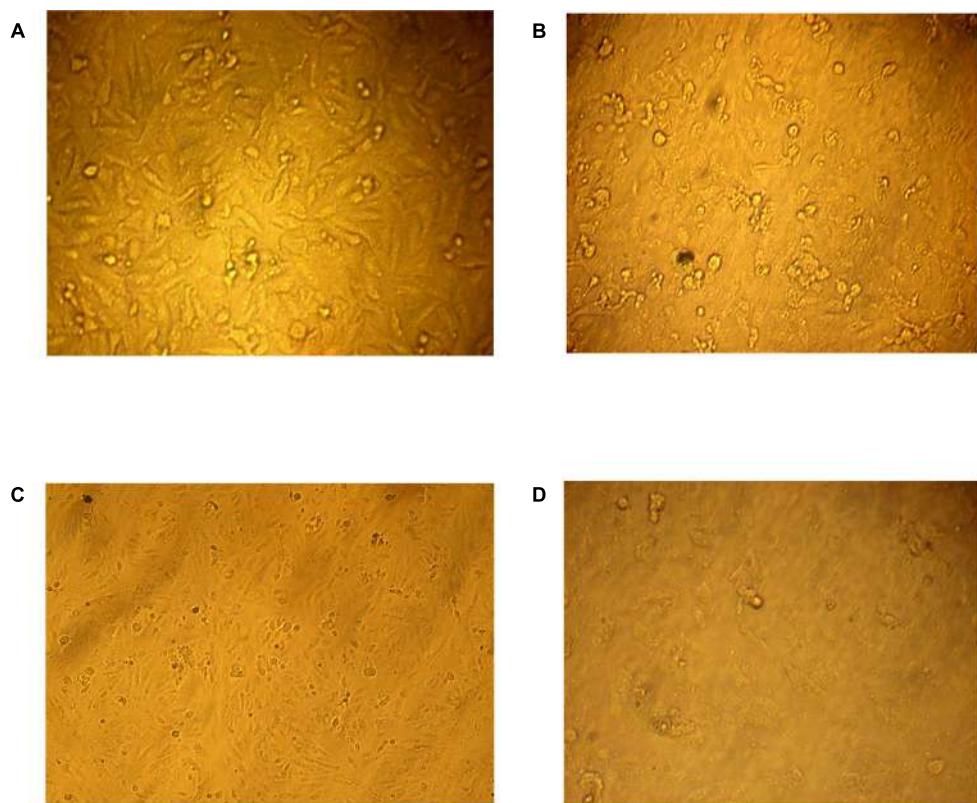
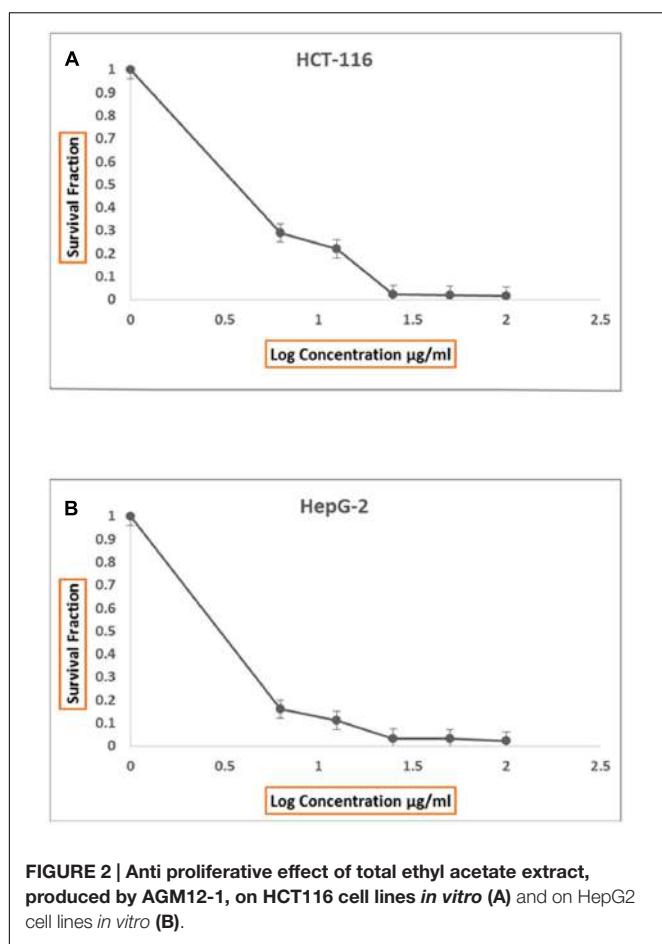


FIGURE 1 | *In vitro* anti-tumor cytotoxicity bioassay. (A) Normal human liver cancer cell line (HepG2) while the effect of three tested concentrations 25, 50, 100 μ g/ml of the AGM12-1 extract on the survival percent of HepG-2 are illustrated in (B–D), respectively.



and centrifuged at 18,000 g at room temperature for 15 min to precipitate the DNA. The resulting pellet was washed with 70% ethanol by centrifugation at 18,000 g at room temperature for 5 min. The final pellet was air-dried and resuspended in 50 μl 1x TE buffer pH 8 and stored in the refrigerator at 4°C.

PCR Amplification and Sequencing of 16S rRNA Gene

The primers used for amplification of the 16S rRNA gene were 11F: 5'-TAACACATGCAAGTCGAACG-3' (Birri et al., 2013; Hong-Thao et al., 2016) and 12R: 5'-AGGGTTGCGCTCGTTG-3' (Stackebrandt and Charfreitag, 1990; Isik et al., 2014). PCR was carried out in 50 μl reaction volume in sterile 200 μl PCR tube. The PCR reaction mixture consisted of 500 ng genomic DNA, 10 mM dNTPs mixture, 1 μl (20 uM of each primer), 2.5 units of Taq DNA polymerase enzyme and 10 μl 5x reaction buffer. The PCR program included template denaturation at 94°C (3 min), followed by 34 cycles of denaturing at 94°C (30 s), annealing at 56°C (30 s), and extension at 72°C (60 s), and followed by completion of DNA synthesis at 72°C (5 min). Primers were removed from the final PCR product prior to sequencing using QIAquick PCR purification kit (QIAGEN, Germany). The PCR product of interest was detected and purified

by agarose gel electrophoresis using 1% (w/v) agarose gels with reference to 1 kbp DNA ladder. DNA was sequenced using the ABI Prism BigDye terminator sequencing ready reaction kit version 3.1 and analyzed with the ABI Prism 3100 generic analyzer.

Sequence Manipulation and Phylogenetic Analysis

The BLAST facility¹ was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using MEGA7 software (Tamura et al., 2007).

PCR Screening for Antibiotic Biosynthetic Gene Clusters

The genomic DNA of AGM12-1 isolate was screened for the presence of the biosynthetic genes involved in the production of type I polyketide synthase (PKS I), type II polyketide synthase (PKS II), NRPS and glycopeptide antibiotics. This was achieved by PCR amplification of these genes using the following primers; PKS/K1 F: 5'-TSAAGTCSAACATCCGBCA-3' and PKS/M6 R: 5'-CGCAGGTTSCSGTACCAAGTA-3' to amplify the PKS I gene with expected product size of 1200–1400 bp (Passari et al., 2015), ARO-PKS-F: 5'-GGCAGCGGITTGGC GGITTCAG-3' and ARO-PKS-R: 5'-CGITGTTIACIGCG TAGAACACCAGGCG-3' to amplify the PKS II gene with expected product size of 492–630 bp (Wood et al., 2007), NRPS/A3 F: 5'-GCSTACSYSATSTACACSTCSGG-3' and NRPS/A7 R: 5'-SASGTCVCCSGTSGCGTAS-3' to amplify the NRPS gene with expected product size of 700 bp (Passari et al., 2015), and finally oxyB F: 5'-CTGGTCGGCACCTGATGGAC-3' and oxyB R: 5'-CAGGTACCGGATCAGCTCGTC-3' to amplify the glycopeptide antibiotic gene with expected product size of 696 bp (Wood et al., 2007). The PCR program included template denaturation at 95°C (5 min), followed by 40 cycles of denaturing at 95°C (30 s), annealing for PKS I, PKS II, NRPS and glycopeptide primers at 55, 64, 59, and 60°C, respectively (60 s), extension at 72°C (2 min), and followed by completion of DNA synthesis at 72°C (10 min) (Baker et al., 2003).

Optimization of Antimicrobial Production

In order to maximize the production of secondary metabolites by the isolate AGM12-1, the effect of different carbon sources; fructose, glucose, mannitol, starch, and sucrose (20 g/l) in basal nitrate salt medium were studied (Selvin et al., 2009). The effect of different nitrogen sources; peptone, yeast extract, ammonium sulfate, protease peptone, and KNO_3 (2 g/l) in the basal starch salt medium were also studied. The most effective carbon and nitrogen sources were further used in different concentrations at (1, 1.5, 2, 2.5, 3 g/100 ml) and (0.1, 0.15, 0.2, 0.25, 0.3 g/100 ml) respectively. The effects of cultural conditions like different incubation time (2–14 days), different starting pH (5, 6, 7, 8, 9, 10, and 11), and different starting inocula (0.01, 0.1, 2, 5, 10, and 15%) were also

¹www.ncbi.nlm.nih.gov/blast

TABLE 1 | Morphological characters and growth of *Streptomyces* sp. AGM12-1.

Growth media	Growth	Color of aerial mycelium	Color of substrate mycelium	Diffused pigments	Form of spore chain
Sato medium	Abundant	Light brown	Light yellowish brown	–ve	Rectus
Starch nitrate medium	Good	Medium gray	Light grayish-reddish brown	–ve	Rectus
Czapek dox medium	Good	Light gray	Light yellowish brown	–ve	Rectus
Nutrient agar	Abundant	Medium gray	Dark grayish yellow	–ve	Rectus
ISP 3	Good	Light grayish-yellowish brown	Grayish yellow	–ve	Rectus
ISP 4	Good	Light gray	Light yellowish brown	–ve	Rectus
ISP 5	Good	Grayish-yellowish brown	Pale yellow	–ve	Rectus

examined (Kadiri and Yarla, 2016). The antimicrobial activity assay using cup technique against sensitive indicator *Sarcina lutea* was managed after each experiment, and zones of inhibitions were measured after incubation of plates at 37°C for 24 h.

Fermentation Using Optimized Conditions and Extraction of the Antimicrobial Compound

To extract putative antimicrobial compound, a 20 ml of 5 days sub-cultured broth was inoculated in 2 L Erlenmeyer flasks containing 1 L of liquid mannitol – ammonium sulfate medium (tow flasks). These flasks were incubated in a rotary shaker (160 RPM) at 30°C for 11 days. A 2-L total volume was filtered through Whatman No. 1 filter. After filtration, the total culture filtrate was extracted with ethyl acetate in a ratio of (1:1 v/v) and shaken vigorously in a separating funnel. Then, the organic layer was collected and the solvent extracts were concentrated to dryness using rotary evaporator and tested for their antimicrobial activity against various indicator microorganisms.

Purification by HPLC

The total ethyl acetate extract of AGM12-1 was concentrated and chromatographed via high performance liquid chromatography (HPLC) (Dionex Ultimate 3000 model HPLC system at the faculty of pharmacy, Beni-Suef University) using a Nucleosil C18 column. Elution was carried out using flow-rate 3 ml/min of 10–100% acetonitrile in water with total run time 25 min. A total of 23 fractions, 3 ml each, were collected. Fractions were concentrated, dried, weighed then dissolved in DMSO and tested for their antimicrobial activities by spotting on the lawn of *Sarcina lutea*, and tested for their minimum inhibitory concentrations by broth micro-dilution method against various indicator microorganisms.

Spectroscopic Characterization

The LC-Mass spectrum in positive and negative ion mode was determined at the faculty of postgraduate studies of advanced science, Beni-Suef University, Egypt and the nuclear magnetic resonance (NMR) spectrum was determined at the faculty of pharmacy, Beni-Suef University, Egypt (El-Hawary et al., 2016).

TABLE 2 | Physiological and biochemical characteristics of AGM12-1 isolate.

Experiment	Reaction by AGM12-1
The enzymatic activity	
Catalase activity	+
Gelatinase activity	+++
H ₂ S production	–
Urea decomposition	++
Amylase activity	+++
Pectinolytic activity	+++
Lethinase activity	–
Lipase activity	–
Nitrate reductase activity	–
Carbon source utilization	
Starch utilization	+++
Glucose utilization	++
Sucrose utilization	+
Fructose utilization	++
Mannitol utilization	+++
Nitrogen source utilization	
Peptone utilization	++
Protease peptone utilization	++
Potassium nitrate utilization	+
Yeast extract utilization	+
Ammonium sulfate utilization	+++
Growth at different pH	
pH 5	+
pH 6	+
pH 7	+++
pH 8	++
pH 9	++
pH 10	+
pH 11	+
Melanin production	
–, negative; +, weakly positive; ++, moderately positive; +++, highly positive. Bolded symbols mean: Main experimental category.	

RESULTS AND DISCUSSION

The emergence of extensive antibiotics resistant bacteria increased the demands for finding out new sources of antimicrobial

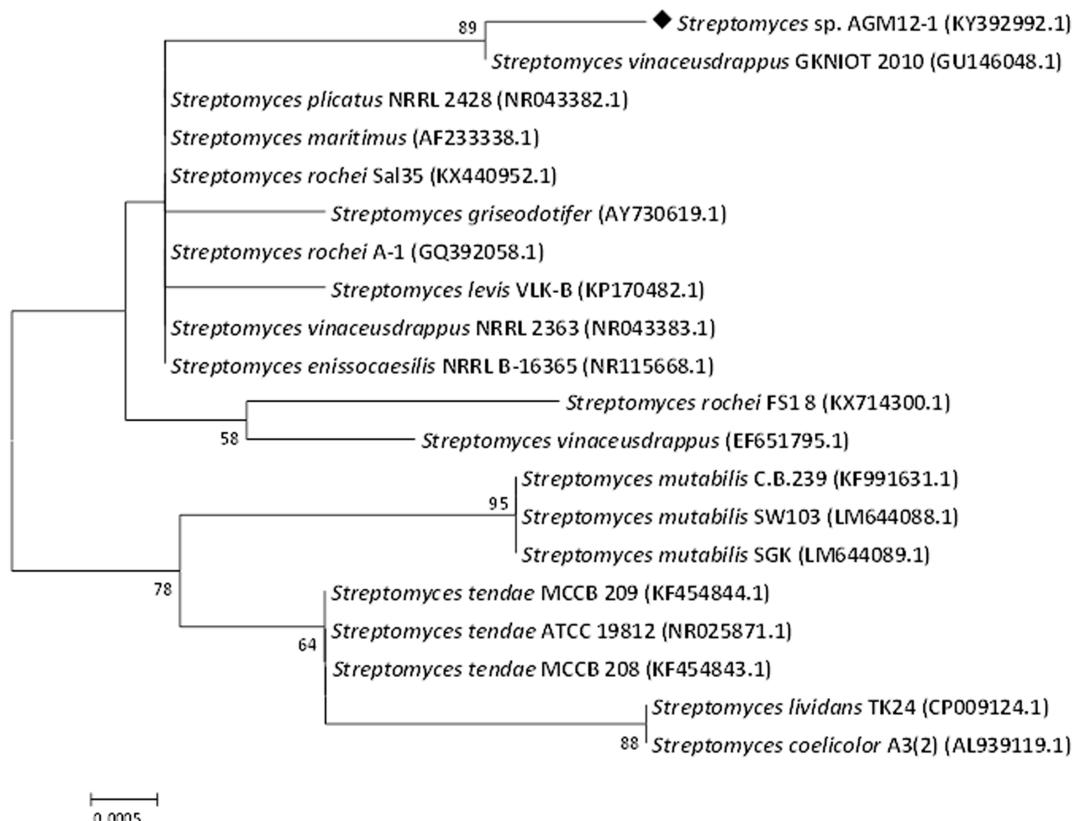


FIGURE 3 | Phylogenetic tree of AGM12-1 isolate based on partial 16S rRNA gene sequences. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Numbers at nodes indicate percentages of 1000 bootstrap re-samplings, only values above 50% are shown. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 827 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

agent. Actinomycetes, especially *Streptomyces* sp., have grasped considerable attention worldwide due to the production of many useful bioactive metabolites. Isolation of these species from poorly explored habitats could increase the possibility to discover novel microbial products with new types of activities (Ser et al., 2016b; Sharma et al., 2016).

Isolation of Actinomycetes and Screening for their Antimicrobial Activities

A total of 52 actinomycetes were isolated from agricultural soil samples collected from different locations in Beni-Suef Governorate, Egypt. Soil niches were reported to be rich in many significant actinomycetes (Savic et al., 2007; Tan et al., 2015). All isolates were characterized based on colony morphology, mycelium coloration, and pigment diffusion. The protein and organic extracts of each strain were screened for antimicrobial activity against different indicator microorganisms. The organic extracts of 20 (38.46%) out of 52 actinomycetes isolates showed antimicrobial activity against at least one of the tested indicator microorganisms (Supplementary Tables 1a,b).

This percentage was not surprising because it was reported many times before that incidence of actinomycetes with antimicrobial activities from the soil niche was relevant high (Thakur et al., 2007; Bizuye et al., 2013). In this study, four isolates were able to exhibit promising broad spectrum activity against all tested indicator microorganisms especially AGM12-1 isolate. So, these talented isolates were undergoing batch fermentation plus extraction of their secondary metabolites for further assessments.

In vitro Anti-tumor Cytotoxicity

Incessant efforts have been directed at the search for more effective anti-tumors from natural resources which could be settled into new therapeutic drugs (Ser et al., 2016a). In this study, both of protein and organic extracts were screened for anti-tumor cytotoxicity against human liver cancer cell line (HepG2) and human colon carcinoma (HCT116). The organic extracts of 20 isolates showed anti-tumor toxicity against both cell lines. The organic extract of AGM12-1 isolate showed substantial anti-tumor activity (Figure 1) where the survival fractions were significantly decreased as the concentration increased (Figures 2A,B). The IC₅₀ values were reached for all examined

TABLE 3 | Different growth conditions of *Streptomyces* sp. AGM12-1 and their impacts on antimicrobial agent production measured by inhibition zones against *Sarcina lutea*.

Different growth conditions	Inhibition zone in millimeter
Starch	19
Glucose	15
Sucrose	14
Fructose	18
Mannitol	20
Potassium nitrate	19
Peptone	24
Yeast extract	22
Ammonium sulfate	26
Protease peptone	24
Mannitol 1%	26
Mannitol 1.5%	26
Mannitol 2%	28
Mannitol 2.5%	29
Mannitol 3%	28
Ammonium sulfate 0.1%	25
Ammonium sulfate 0.15%	27
Ammonium sulfate 0.2%	29
Ammonium sulfate 0.25%	26
Ammonium sulfate 0.3%	25
pH 5	14
pH 6	15.5
pH 7	22
pH 8	19
pH 9	17
pH 10	16
pH 11	11
Starting inoculum 0.01%	NA
Starting inoculum 0.1%	NA
Starting inoculum 2%	22
Starting inoculum 5%	15.5
Starting inoculum 10%	11
Starting inoculum 15%	11
Incubation period 2nd day	NA
Incubation period 3rd day	12
Incubation period 4th day	15
Incubation period 5th day	18
Incubation period 6th day	19
Incubation period 7th day	19
Incubation period 8th day	20
Incubation period 9th day	21
Incubation period 10th day	22
Incubation period 11th day	25
Incubation period 12th day	23
Incubation period 13th day	22
Incubation period 14th day	21

extracts using the tested concentrations for both cell lines. The ethyl acetate extract from isolate AGM12-1 exhibited the most potent effect against both cell lines with IC₅₀ 3.3 and 1.1 µg/ml against HCT 116 and HepG-2 respectively. Moreover, all tested

compounds showed higher cytotoxicity against HepG 2 cell line compared to HCT 116.

Phenotypic Characterization of AGM12-1 Isolate

The light microscopic observation of AGM12-1 isolate on ISP4 media showed a straight chain section with no fragmentation of the aerial mycelium. Also SEM observation showed a crimped spore surface with aerial and vegetative hyphae which were well-developed and not fragmented. The other morphological characters, after growing in different growth media, are summarized in Table 1. The physiological and biochemical characters denoted in the production of different enzymes, utilization of different nitrogen and carbon sources, and ability to grow at a wide range of pH are illustrated in Table 2. Based on these morphological and biochemical characteristics of AGM 12-1, it was presumptively identified as a member of *Streptomyces* sp. according to Williams et al. (1983).

Genotypic Characterization of AGM12-1 Isolate and Screening for Antibiotic Biosynthetic Gene Clusters

The partial 16S rRNA gene sequencing revealed a 99% similarity with *Streptomyces vinaceusdrappus* according to NCBI GenBank. The resulted sequence was aligned to 19 of the closely related *Streptomyces* sp. by retrieving their sequences from the NCBI GenBank database and assembled in MEGA7 software for phylogenetic analysis using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method. The obtained phylogenetic tree (Figure 3) confirmed the similarity of the AGM12-1 isolate to *Streptomyces vinaceusdrappus* with a similarity matrix bootstrap value of 89. The GenBank accession number for the partial 16S rRNA gene sequence of AGM12-1 strain is KY392992.

Screening of *Streptomyces* sp. AGM12-1 for the presence of biosynthetic genes involved in the production of glycopeptide antibiotics (OXY B), NRPS, type I polyketide synthase (PKS I) and type II polyketide synthase (ARO-PKS II) revealed the presence of type II polyketide synthase system which is mostly responsible for the synthesis of aromatic polyketides. In another study (Busti et al., 2006), they reported the presence of antibacterial activated genes NRPSs, type I and type II PKSs in their isolate.

Optimizing the Production of Antimicrobial Secondary Metabolites

The optimum growth conditions for production of antimicrobial and antitumor agents from *Streptomyces* sp. AGM12-1 were screened and illustrated in Table 3. It was found that the maximum productivity was achieved after using mannitol and ammonium sulfate at concentrations of 2.5 and 0.2%, respectively. Other factors like pH 7, starting inoculum 2% and incubation for 11 days at 30°C were found to produce a high yield of antimicrobial and antitumor substance. In other studies (Sujatha et al., 2005), it was reported that the glucose and ammonium nitrate in synthetic media were the optimum

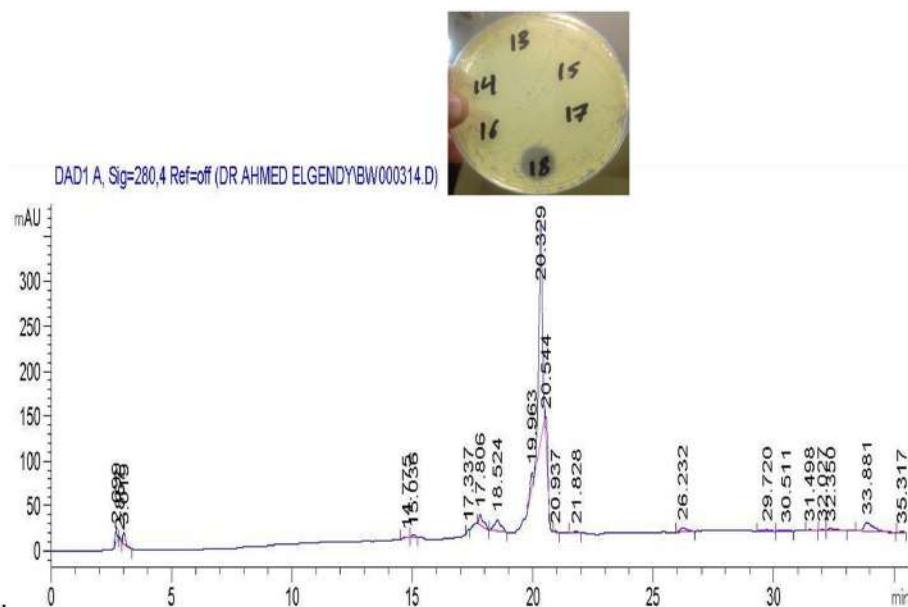


FIGURE 4 | High performance liquid chromatography (HPLC) chromatograms of a re-purified sample using a Nucleosil C18 column showing the active compound eluted at 20.32 min (fraction tube number 18).

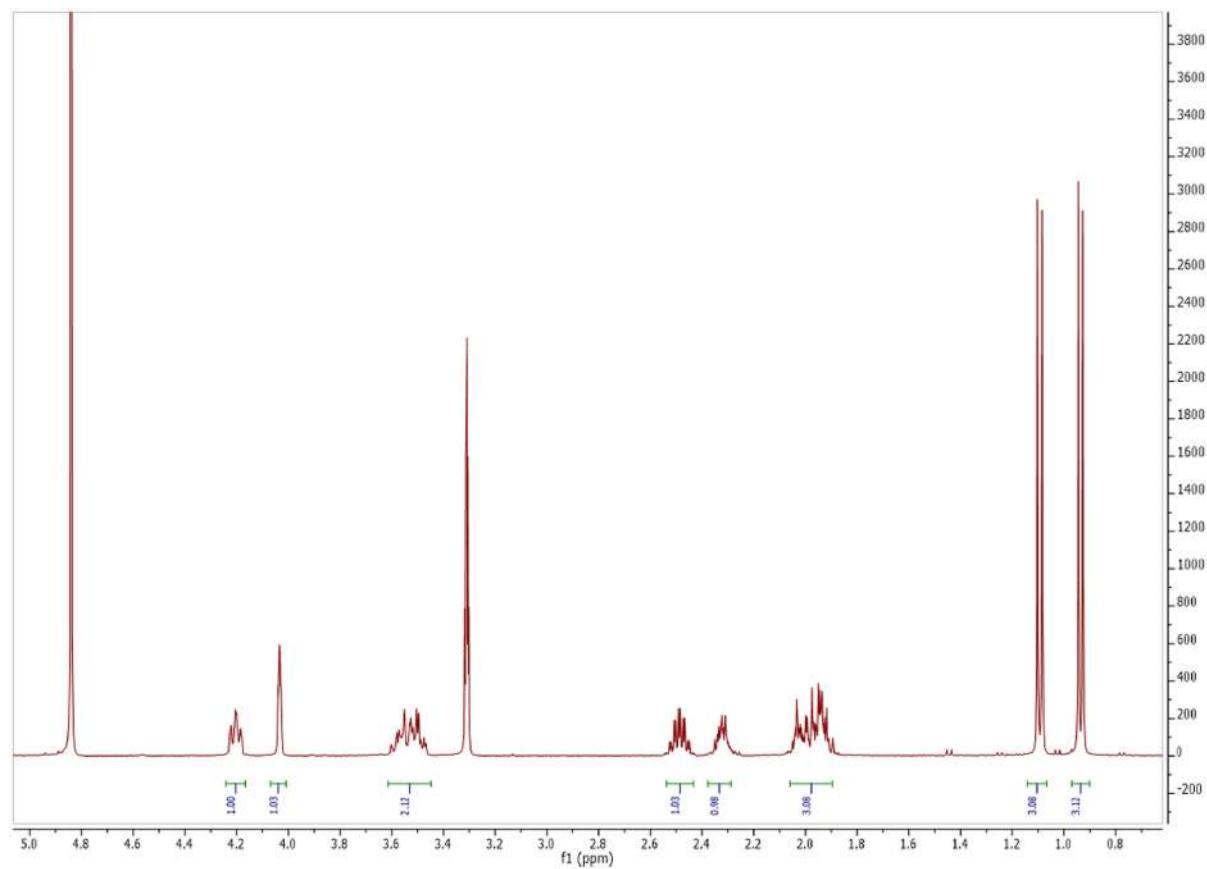


FIGURE 5 | Nuclear magnetic resonance (NMR) spectrum of antimicrobial and antitumor agent produced by *Streptomyces* sp. AGM12-1.

carbon and nitrogen sources to obtain a high yield of antibiotic. Also (Kadiri and Yarla, 2016) reported that the arabinose and dextrose were the best carbon sources and L-asparagine was the best nitrogen source in their study. Pandey et al. (2005) tested a number of carbon and nitrogen compounds for their effect on the production of an antibacterial antibiotic by *Streptomyces kanamyceticus* M27. It was found dextrose as the most suitable carbon source while maltose, sucrose, and soluble starch gave moderate yield. $(\text{NH}_4)\text{H}_2\text{PO}_4$ and yeast extract were adequate nitrogen sources for antibiotic production. It was found that media with alkaline pH gave high antibiotic yield.

Purification of the Antimicrobial Compound and MIC Determination

The active metabolites were extracted with ethyl acetate at the level of (1:1 v/v) and the separation of the antimicrobial compound was carried out by HPLC in which 23 fractions were collected manually and tested for their antimicrobial activities (Supplementary Figure 1). The active fractions were re-chromatographed till showing one pure compound at 20 min (fraction number 18) as seen in **Figure 4**. MIC values, ranging from 50 to 0.77 $\mu\text{g}/\text{ml}$, were tested against all indicator microorganisms by broth-micro dilution method. Lowest MIC was recorded against *Sarcina lutea* (6.25 $\mu\text{g}/\text{ml}$) while largest MIC was recorded against *Candida albicans* and *Escherichia coli* ATCC 8739 (25 $\mu\text{g}/\text{ml}$). For both *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis*, the recorded MIC was 12.5 $\mu\text{g}/\text{ml}$.

Spectroscopic Characteristics

Based on $^1\text{HNMR}$ spectrum (**Figure 5**), molecular mass (m/z 488.05) and comparing the results with previously published data, the isolated compound was identified as Cyclo (S-Pro-S-Val) (**Figure 6**) (Jayatilake et al., 1996). This compound is related to the diketopiperazine family that has important biological activities as inhibition of plasminogen activator inhibitor-1 (PAI-1) (Einholt et al., 2003) and alteration of cardiovascular and blood-clotting functions (Martins and Carvalho, 2007). They also have activities as antitumor, antiviral, antifungal, antibacterial, and antihyperglycaemic (Martins and Carvalho, 2007).

Diketopeprazine is a huge family with variable bioactivities and about 200 articles had isolated many of these derivatives. For examples, Wang et al. (2013) reported that five new diketopiperazine derivatives were isolated from the marine-derived actinomycetes "*Streptomyces* sp. FXJ7.328." In another study, researchers reported that five diketopiperazines derivatives were isolated from deep-sea bacterium *Streptomyces fungicidicus* with a novel antifoulants activity (Li et al., 2006). Also, *Streptomyces globisporus* 1912, a producer of the antitumor antibiotic landomycin E, forms new low molecular signaling molecule *N*-methyl phenylalanyl-dehydronbutyryne diketopiperazine (Matselyukh et al., 2012).

CONCLUSION

Actinomycetes, especially streptomycetes, still an important source for bioactive compounds that are used for treating

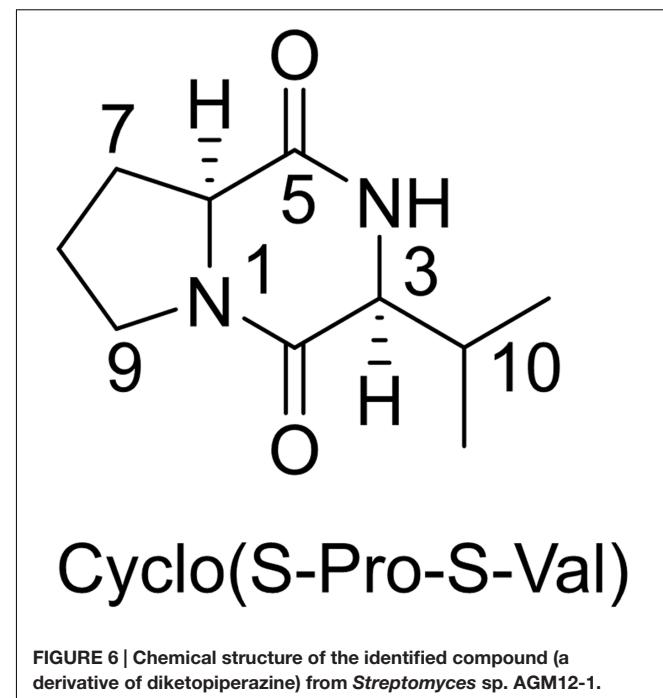


FIGURE 6 | Chemical structure of the identified compound (a derivative of diketopiperazine) from *Streptomyces* sp. AGM12-1.

infections, cancer, and many other diseases. The derivative of diketopiperazine produced by *Streptomyces* sp. AGM12-1, isolated from Beni Suef Governorate, Egypt, demonstrated obvious inhibitory effects against both Gram-positive and Gram-negative bacteria beside an antifungal activity. Also, an antitumor toxicity against human liver and colon cell lines; HepG 2 and HCT 116 was reported. To our knowledge, this is the first time to characterize a diketopiperazine derivative as a secondary metabolite recovered from *Streptomyces* sp. in Egypt.

AUTHOR CONTRIBUTIONS

MA and AE-G performed the microbiological and molecular biology experiments. RA worked on antitumor activity assay. HH manipulated the NMR data and elucidated the final molecular structure. HE-K and AM put the study design. AE-G drafted the manuscript and all authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00438/full#supplementary-material>

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Exploring the Diversity and Antimicrobial Potential of Marine Actinobacteria from the Comau Fjord in Northern Patagonia, Chile

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Bioprospecting natural products in marine bacteria from fjord environments are attractive due to their unique geographical features. Although, *Actinobacteria* are well known for producing a myriad of bioactive compounds, investigations regarding fjord-derived marine *Actinobacteria* are scarce. In this study, the diversity and biotechnological potential of *Actinobacteria* isolated from marine sediments within the Comau fjord, in Northern Chilean Patagonia, were assessed by culture-based approaches. The 16S rRNA gene sequences revealed that members phylogenetically related to the *Micrococcaceae*, *Dermabacteraceae*, *Brevibacteriaceae*, *Corynebacteriaceae*, *Microbacteriaceae*, *Dietziaceae*, *Nocardiaceae*, and *Streptomycetaceae* families were present at the Comau fjord. A high diversity of cultivable *Actinobacteria* (10 genera) was retrieved by using only five different isolation media. Four isolates belonging to *Arthrobacter*, *Brevibacterium*, *Corynebacterium* and *Kocuria* genera showed 16S rRNA gene identity <98.7% suggesting that they are novel species. Physiological features such as salt tolerance, artificial sea water requirement, growth temperature, pigmentation and antimicrobial activity were evaluated. *Arthrobacter*, *Brachybacterium*, *Curtobacterium*, *Rhodococcus*, and *Streptomyces* isolates showed strong inhibition against both Gram-negative *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella enterica* and Gram-positive *Staphylococcus aureus*, *Listeria monocytogenes*. Antimicrobial activities in *Brachybacterium*, *Curtobacterium*, and *Rhodococcus* have been scarcely reported, suggesting that non-mycelial strains are a suitable source of bioactive compounds. In addition, all strains bear at least one of the biosynthetic genes coding for NRPS (91%), PKS I (18%), and PKS II (73%). Our results indicate that the Comau fjord is a promising source of novel *Actinobacteria* with biotechnological potential for producing biologically active compounds.

Keywords: cultivable actinobacteria, antimicrobial activity, Comau fjord, marine sediments, Northern Patagonia

INTRODUCTION

The increased prevalence of multi-drug resistance pathogens along with the rapid development of cross resistances with new antibiotics is the driving force in the identification and production of novel therapeutic agents (Livermore, 2009). All classes of antibiotics have seen emergence of resistance compromising their use; hence there is an urgent need for new bioactive compounds (Genilloud, 2014). The traditional approach consisting of isolation and cultivation of new microorganisms of underexplored habitats is still rewarding (Axenov-Gribanov et al., 2016), and has brought to the identification, production and commercialization of most of the antibiotics (Newman and Cragg, 2012). Despite the chemically synthetic efforts, natural environments are still the main source for the discovery of novel antibiotics (Fenical and Jensen, 2006; Bull and Stach, 2007). Although, the diversity of life in terrestrial environments is well reported, the highest biodiversity is in the world's oceans (Donia and Hamann, 2003). Oceans are strongly complex habitats in terms of pressure, salinity and temperature variations (Fenical, 1993), therefore marine microorganisms have to develop physiological traits including chemically complex biosynthesized metabolites to ensure their survival in this highly dynamic habitat. Research has taken advantage from these unique molecules to discover novel bioactive compounds with antibacterial, antifungal and/or antitumor properties, and apply them in current clinical challenges (Gulder and Moore, 2010).

In this scenario, bacteria from the phylum *Actinobacteria* are a prominent source of biologically active natural compounds, since they are well known for their capacity to biosynthesize versatile secondary metabolites (Katz and Baltz, 2016). *Actinobacteria* are one of the major phyla of the domain *Bacteria* (Goodfellow and Fiedler, 2010). It encompasses high GC-content Gram-positive bacteria that includes 17 orders (Gao and Gupta, 2005; Sen et al., 2014). Surprisingly, the class *Actinobacteria* contains both the most deadly bacterial pathogen (i.e., *Mycobacterium* genus) and the microorganisms that are the most important for antibiotic production (i.e., *Streptomyces* genus) (Doroghazi and Metcalf, 2013). *Streptomyces* are responsible for two-thirds of all known antibiotics. In addition, several other important biologically-active compounds have been found, including antitumoral, antifungal, herbicidal, and antiparasitic compounds (Bérdy, 2005). Due to the extensive sampling of soil *Streptomyces*, the rate of discovery of novel metabolites is decreasing (Fenical, 1993), which is the reason why bioprospecting efforts are currently being developed in marine underexplored ecosystems.

Marine environments are an established ecological niche for actinobacteria (Das et al., 2006; Ward and Bora, 2006). Cultivable actinobacteria from marine habitats have been characterized from mangrove forests (Hong et al., 2009; Baskaran et al., 2011; Lee et al., 2014a,b; Ser et al., 2015, 2016), marine sponges (Kim et al., 2005; Montalvo et al., 2005; Zhang et al., 2006; Jiang et al., 2007; Sun et al., 2015), corals (Hodges et al., 2012; Kuang et al., 2015; Mahmoud and Kalendar, 2016; Pham et al., 2016), sea cucumbers (Kurahashi et al., 2010), pufferfishes (Wu et al., 2005), and seaweed (Lee et al., 2008). Notably, actinobacteria are predominant in marine sediments (Mincer et al., 2002;

Magarvey et al., 2004; Jensen et al., 2005; Bredholdt et al., 2007; Gontang et al., 2007; León et al., 2007; Maldonado et al., 2008; Duncan et al., 2014; Yuan et al., 2014) and also in deep sea sediments (Colquhoun et al., 1998; Pathom-Aree et al., 2006). Marine actinobacteria have been described as an emerging source for novel bioactive molecules (Lam, 2006; Joint et al., 2010; Subramani and Aalbersberg, 2012; Zotchev, 2012). The majority of these secondary metabolites are produced by polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) metabolic pathways (Salomon et al., 2004). Notably, it is reported that actinobacteria have a higher number of these biosynthetic genes (Donadio et al., 2007).

The extensive coast of Chile is a promising biome to explore marine actinobacterial communities, and in this context, the bioprospecting of sediments of a marine protected area, the Comau fjord, in the Chilean Northern Patagonia was proposed. The Comau fjord is a pristine area unique by its geological nature. It is comparatively smaller than other fjords in Chile, and also one of the deepest (Ugalde et al., 2013); characterized by steep slopes, with surrounding mountains that have a height of up to 2000 m with a dense extratropical rainforest covering from the sea to the top (Lagger et al., 2009). The aim of this study was to isolate marine actinobacteria from this unique ecosystem. The cultivable diversity of actinobacterial strains along with their environmental adaptation traits was investigated, and their ability to produce antibacterial activity against model strains was assessed.

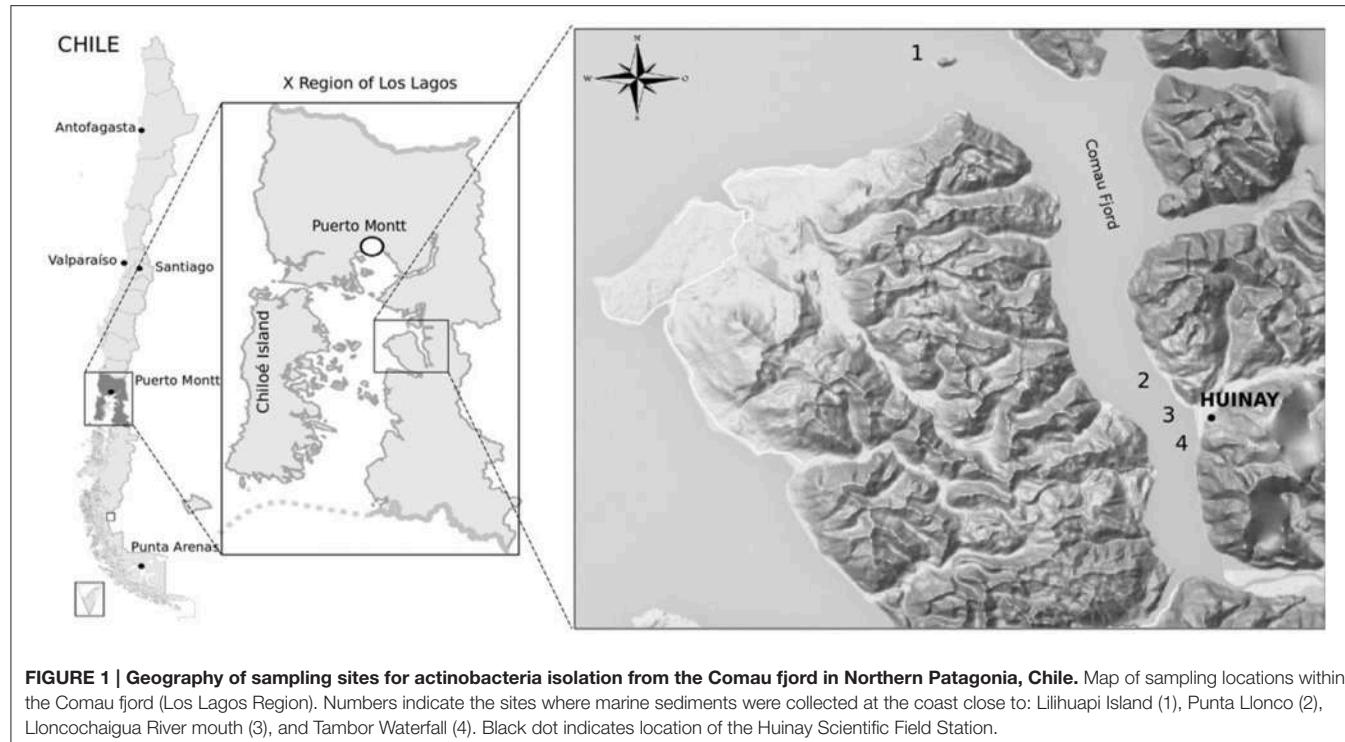
MATERIALS AND METHODS

Environmental Samples

Sampling was performed in the Marine Protected Area of Huinay in January 2013, located in the Commune of Hualaihué, in the Los Lagos Region, Chile. Samples were collected from marine sediments within the Comau Fjord in the Northern Patagonia. Four different coastal locations were sampled in front of Liliuapi Island ($42^{\circ}20'$, $634'S$; $72^{\circ}27'$, $429'W$), Tambor Waterfall ($42^{\circ}24'$, $161'S$; $72^{\circ}25'$, $235'W$), Punta Llonco ($42^{\circ}22'$, $32'S$; $72^{\circ}25'$, $4'W$), and in front of Lloncochaigua River mouth ($42^{\circ}22'$, $37'S$; $72^{\circ}27'$, $25'W$) (Figure 1). Underwater samples were collected by Huinay Scientific Field Station scuba divers, dispensing samples directly from marine sediments into sterile 50 mL tubes. Marine sediments were taken from subtidal zones at different depths, ranging from 0.25 to 26.2 m. Salinity was measured at each sampling site, and ranged from $5 \mu\text{g L}^{-1}$ in the coast in front of Lloncochaigua River mouth, where there is a meaningful input of fresh water, to $31 \mu\text{g L}^{-1}$ in the coast of Liliuapi Island, located further away from continental land. Samples were maintained on ice until transported to the laboratory, where they were stored at 4°C .

Isolation of Actinobacteria

Samples were both plated directly or serially diluted (10^{-4} and 10^{-6}) before plating on selective media for the isolation of actinobacteria. Five selective media were used as previously reported (Claverías et al., 2015): M1 Agar (Mincer et al., 2002), ISP2 and NaST21Cx Agar (Magarvey et al., 2004),



R2A Agar (Difco), and Marine Agar (MA) 2216 (Difco). All media were amended with nalidixic acid ($25 \mu\text{g mL}^{-1}$), as an inhibitor of primarily fast-growing Gram-negative bacteria, and cycloheximide ($100 \mu\text{g mL}^{-1}$) for fungi inhibition [28]. All media with the exception of Marine Agar, were prepared with artificial sea water (ASW) (Kester et al., 1967). The agar media cultures were incubated at 30°C until visible colonies were observed, up to 1–2 months. For isolation purposes, colonies were individually streaked out onto Tryptic Soy Agar medium (TSA) prepared with ASW (TSA-ASW) and eventually transferred on new plates until pure cultures were obtained. Isolated bacteria were stored at -20 and -80°C , in 20% glycerol, TSB medium and ASW for maintenance.

Detection and Identification of Actinobacteria

A PCR-assay was conducted as a screening method for detecting actinobacterial strains among the isolates with primers targeting the V3–V5 regions of the 16S rRNA gene of actinobacteria (S-C-Act-0235-a-S-20 and S-C-Act-0878-A-19) (Stach et al., 2003). DNA extractions were performed, using a lysis method by culture boiling suspensions of bacterial cells (Moore et al., 2004). Each PCR reaction contained 1 μL of genomic DNA, 12.5 μL of GoTaq Green Master Mix (Promega) and 0.6 μM of each primer in a final reaction volume of 25 μL . The reaction started with an initial denaturation, at 95°C for 5 min, followed by 35 cycles of DNA denaturation, at 95°C for 1 min, primer-annealing, at 70°C for 1 min and extension cycle, at 72°C for 1.5 min, with a final extension at 72°C for 10 min (Claverías et al., 2015). PCR-amplons were visualized in 2% agarose gel electrophoresis

and subsequently revealed with SYBR Green staining (E-gel, Invitrogen).

Positive isolates were selected for 16S rRNA gene amplification, using universal primers 27F and 1492R (Lane, 1991). The reaction mix (50 μL) contained 1 μL of genomic DNA, 25 μL of GoTaq Green Master Mix (Promega) and 0.2 μM of each primer. The reaction started with an initial DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer-annealing at 55°C for 1 min and primer-extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. PCR products were sent to Macrogen Inc. (Seoul, Korea) for purification and sequencing using the conserved universal primer 800R. Retrieved sequences were manually edited and BLAST nucleotide analyses were performed with the National Center for Biotechnology Information server (NCBI) and actinobacteria were initially identified up to the genus level.

Antimicrobial Activity Tests

Bioprospecting for antimicrobial activity was initially performed using the cross-streak method as described (Haber and Ilan, 2014), with slight modifications (Claverías et al., 2015). Fresh cultures of the isolated actinobacterial strains were inoculated as a line in the middle of an agar medium plate and incubated at 30°C until notable growth was observed (7 days for mycelial strains and 5 days for non-mycelial strains). Strains were grown on TSA-ASW and ISP2-ASW media. Five reference bacteria were the target of inhibition tests: *Staphylococcus aureus* NBRC 100910^T (STAU); *Listeria monocytogenes* 07PF0776 (LIMO); *Salmonella enterica* subsp. *enterica* LT2^T (SAEN); *Escherichia*

coli FAP1 (ESCO) and *Pseudomonas aeruginosa* DSM50071^T (PSAU). Cultures were incubated at 37°C overnight and inhibition zones were ranked qualitatively as: –, no inhibition; +/–, attenuated growth of test strain in the area closest to the actinobacterial line; +, <50% growth inhibition (less than half of the bacterial line was inhibited); ++, 50% growth inhibition (half of the bacterial line was inhibited); +++, >50% growth inhibition (more than half of the bacterial line was inhibited). All experiments were performed in duplicate, using an internal control with one of the reference strains.

Further antimicrobial tests were performed with selected isolates *Streptomyces* sp. H-KF8, *Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Kocuria* sp. H-KB5 and *Rhodococcus* sp. H-CA8f. Strains were grown in a 50 mL liquid culture in ISP2-ASW medium for 10 days for non-mycelial strains and 15 days for the mycelial strain, with continuous shaking at 30°C. Crude extracts were obtained after solvent extraction using hexane, methanol and ethyl acetate in a 1:1 ratio (v/v) for two times. Evaporation of solvent was performed with speed vacuum, and extract was dissolved in 10% dimethyl sulphoxide (DMSO) until a final concentration of 5 mg mL⁻¹. Antimicrobial assays were evaluated using 10 µL of each extract, over LB agar plates spread with the bacterial test strains STAU, PSAU, SAEN, and ESCO. Plates were incubated overnight at 37°C and inhibitions zones were checked. ISP2 medium and 10% DMSO were used as negative controls.

Detection of PKS and NRPS Biosynthetic Genes

Amplification of biosynthetic genes was carried out by PCR, using degenerate primers targeting the ketosynthase domain in PKS type I with primers KS-F (5'CCSCAGSAGCGCSTS YTSCCTSGA3') and KS-R (5'GTSCCSGTSCCGTGSGYSTCSA3') (Gontang et al., 2010); and PKS type II with primers KSα (5'TSGRCTACRTCAACGGSCACGG3') and KSβ (5'TACSG TCS WTCGCCTGGTTC3') (Ayuso et al., 2005). The adenylation domain in NRPS systems was detected with primers A3F (5'GCSTACSYSATSTACACSTCSGG3') and A7R (5'SASGTCV CCCGTSCGGTAS3') (Ayuso-Sacido and Genilloud, 2005). PCR programs were performed as previously described (Ayuso et al., 2005; Ayuso-Sacido and Genilloud, 2005; Gontang et al., 2010). Products were visualized in 1% agarose gels electrophoresis, and stained with GelRed (Biotium). *Streptomyces violaceoruber* DSM 40783 was used as a control for all PCR reactions. Detection was determined as +, if the amplicon was located at the expected size (700 bp for PKS type I; 800–900 bp for PKS type II and 700–800 bp for NRPS); and –, if amplicon was absent or it was present at any other size.

Phylogenetic Analysis

Representative strains for each genus identified from partial 16S rRNA gene sequence analyses were selected for the nearly-complete sequencing of this gene, as previously described (Claverías et al., 2015). PCR products were quantified and sent to Macrogen Inc. (Seoul, Korea) for purification and sequencing, using primers 27F, 518F, 800R, and 1492R. Manual sequence edition, alignment, and contig assembling were performed using Vector NTI v10 software package (Invitrogen). Sequence contigs

were analyzed performing BLAST with NCBI to determine the closest type strain match using the 16S ribosomal RNA sequence of Bacteria and Archaea database. The Neighbor-Joining algorithm (Saitou and Nei, 1987) using MEGA software version 6.0 (Tamura et al., 2013) with bootstrap values based on 1000 replications (Felsenstein, 1985) was used to construct a phylogenetic tree based on the V1-V9 region of the 16S rRNA gene sequences. The 16S rRNA gene sequences were deposited in GenBank under the following accession numbers: *Arthrobacter* sp. H-JH3 (KT799841); *Brachybacterium* sp. H-CG1 (KT799842); *Brevibacterium* sp. H-BE7 (KT799843); *Corynebacterium* sp. H-EH3 (KT799844); *Curtobacterium* sp. H-ED12 (KT799845); *Kocuria* sp. H-KB5 (KT799846); *Dietzia* sp. H-KA4 (KT799847); *Micrococcus* sp. H-CD9b (KT799848); *Rhodococcus* sp. H-CA8f (KT799849); *Streptomyces* sp. H-KF8 (KT799850) and *Streptomyces* sp. H-CB3 (KT799851).

Phenotypic Characterization of Actinobacterial Strains

For the morphological and physiological characterization of the representative strains, colony pigmentation, spore formation, growth temperatures, ASW requirement and NaCl tolerance were evaluated. Optimal colony pigmentation was observed on TSA-ASW after a 3-month incubation at 4°C. To establish the effects of temperature on growth, 10 µL of actinobacterial cultures were streaked onto TSA-ASW plates, and incubated at 4, 20, 30, 37, and 45°C. For NaCl tolerance, LB agar with 0, 1, 3.5, 5.0, 7.0, 10, and 20% (w/v) NaCl was prepared. As described previously, 10 µL of the actinobacterial cultures were streaked onto LB agar plates and incubated at 30°C. To detect the requirement of seawater on growth, ISP2 was prepared as follows: medium with Milli-Q H₂O; medium with ASW; and medium with Milli-Q H₂O supplemented with 3.5% (w/v) NaCl (equivalent to ASW NaCl concentration). Incubation times were from 10 days (for non-mycelial strains) to 14 days (for mycelial stains) at 30°C. The reference time for growth was that on which growth was observed on control plates. Results were interpreted as: +, if the strain tested was able to grow on medium-ASW but did not grow on medium/Milli-Q H₂O and on medium/Milli-Q H₂O supplemented with 3.5% NaCl; and –, if the strain tested was able to grow on all three media.

Resistance to Model Antibiotics

Representative strains of each genus were grown to exponential phase (turbidity at 600 nm of 0.3) and plated on Mueller-Hinton agar plates for antibiotic susceptibility testing. Antibiotic discs for Gram-positive bacteria (Valtek) were placed above and inhibition grown zones as diameters were measured and compared with values obtained from the Clinical and Laboratory Standards Institute (CLSI) from year 2016 to determine susceptibility (S), or resistance (R) of each antibiotic tested.

RESULTS

Isolation and Identification of Actinobacteria

Eleven marine sediment samples were collected from four different sites in Comau fjord, Northern Patagonia, Chile

(Figure 1). Altogether 25 marine actinobacteria were isolated. Their distribution according to the sampling site was: 40% from Lilihuapi island coast, 28% from Punta Llonco, and 16% from Loncohaigua river mouth and Tambor waterfall, each. The majority (80%) of the isolates were from sediments situated approximately 10 m deep. Only occasional isolates were obtained from deeper sediments or from the shallow locations. The *Actinobacteria* isolated belong to three suborders: *Streptomycineae*, *Micrococcineae*, and *Corynebacterineae*; comprising eight different families. Relative abundances of the strains according to the genera isolated (**Figure 2A**) indicated that most abundant genera were *Kocuria* and *Brachybacterium*. The selective media had a major influence on the number of isolates obtained (**Figure 2B**). M1-ASW medium was the most effective regarding the number and diversity of isolates recovered. Interestingly, strains of *Brachybacterium*, *Brevibacterium*, *Micrococcus*, and *Rhodococcus* genera were isolated exclusively with this medium (**Figure 2B**).

Antimicrobial Activity Assays

Our first approach was to screen all actinobacterial strains for antimicrobial activity, using the cross-streak method, against five reference strains: STAU, LIMO, PSAU, SAEN, and ESCO (**Figure 3A**). Actinobacterial strains showed antimicrobial activity, presenting a broad spectrum of inhibition although with different inhibition patterns (**Table 1**). Inhibition of reference strains largely depended on the media where actinobacterial strains were cultivated, proving TSA-ASW to be generally better for antimicrobial activity than ISP2-ASW medium. *Arthrobacter*, *Brachybacterium*, *Curtobacterium*, and *Rhodococcus* isolates showed potent antimicrobial bioactivity to more than one target (**Table 1**). Regarding the Gram-negative bacteria tested, TSA-ASW-grown actinobacterial strains were able to inhibit ESCO (84%) and PSAU (24%); whereas ISP2-ASW-grown isolates inhibited up to 76 and 48%, respectively. Concerning the Gram-positive reference strains, 64% of the TSA-ASW-grown

actinobacterial strains inhibited both LIMO and STAU; whereas ISP2-ASW-grown strains, 56% showed inhibition for LIMO and 36% for STAU (**Figure 3B**).

Notably, 67% of the antimicrobial activities observed with the cross-streak method were retrieved with various solvent extractions from actinobacterial liquid cultures (**Table 2**). Ethyl acetate was more effective in extracting active compounds, as crude extracts from *Rhodococcus* sp. H-CA8f, *Kocuria* sp. H-KB5 and *Brevibacterium* sp. H-BE7 presented antimicrobial activity. On the other hand, antimicrobial activity from *Arthrobacter* sp. H-JH3 was effectively extracted from the cell pellet using methanol. Crude extracts from *Rhodococcus* sp. H-CA8f showed an antimicrobial effect against all bacteria tested, confirming results obtained from the cross-streak method.

Detection of PKS and NRPS Biosynthetic Genes

The presence of biosynthetic PKS (type I and II) and NRPS genes were detected by PCR in representative actinobacterial isolates (**Table 3**). Interestingly, most isolates bear at least one biosynthetic gene of PKS or NRPS. Among them, NRPS was the predominant gene observed (91%), followed by PKS type II (73%). Only 18% of actinobacterial isolates showed the presence of PKS type I gene.

Phylogenetic Analysis

For phylogenetic analysis, the 16S rRNA gene was sequenced for selected actinobacterial isolates, representatives of each genus retrieved in sediment samples from Comau fjord. A dendrogram of the estimated phylogenetic relationships is presented in **Figure 4** and the sequence similarities of selected actinobacterial strains to type strains of related species are given in **Table 3**. Four of the actinobacterial isolates are below the 98.7% sequence identity threshold and therefore may be potential candidates of new taxons. These isolates belong to *Arthrobacter* and *Kocuria* genera (*Micrococcaceae*

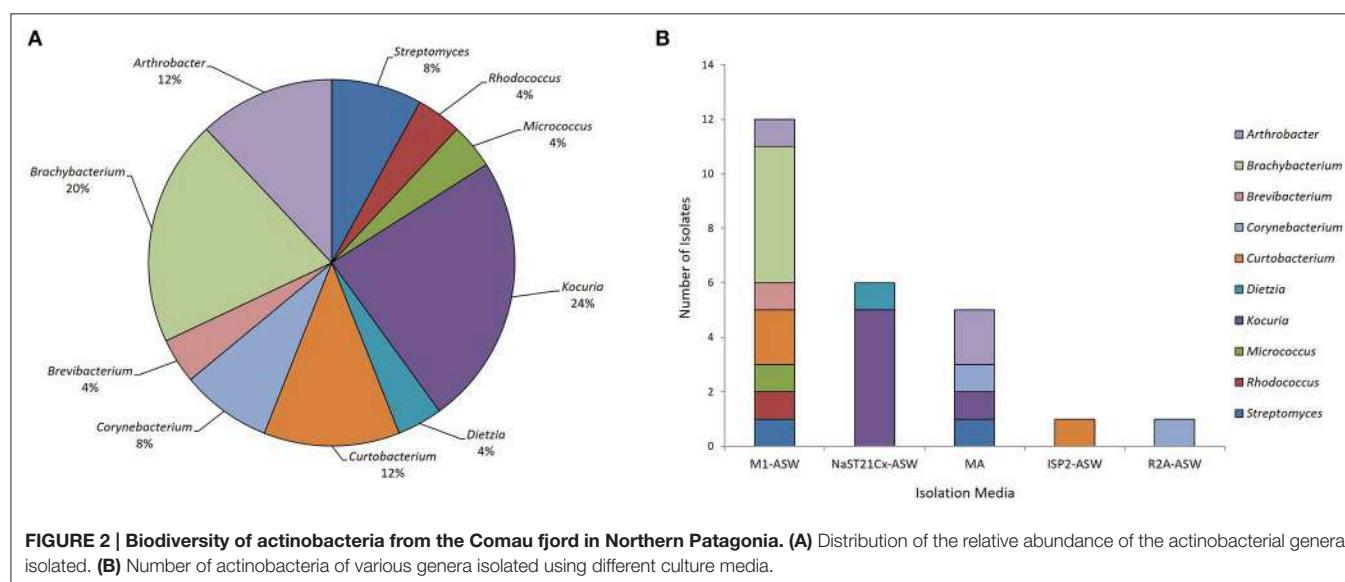


FIGURE 2 | Biodiversity of actinobacteria from the Comau fjord in Northern Patagonia. (A) Distribution of the relative abundance of the actinobacterial genera isolated. **(B)** Number of actinobacteria of various genera isolated using different culture media.

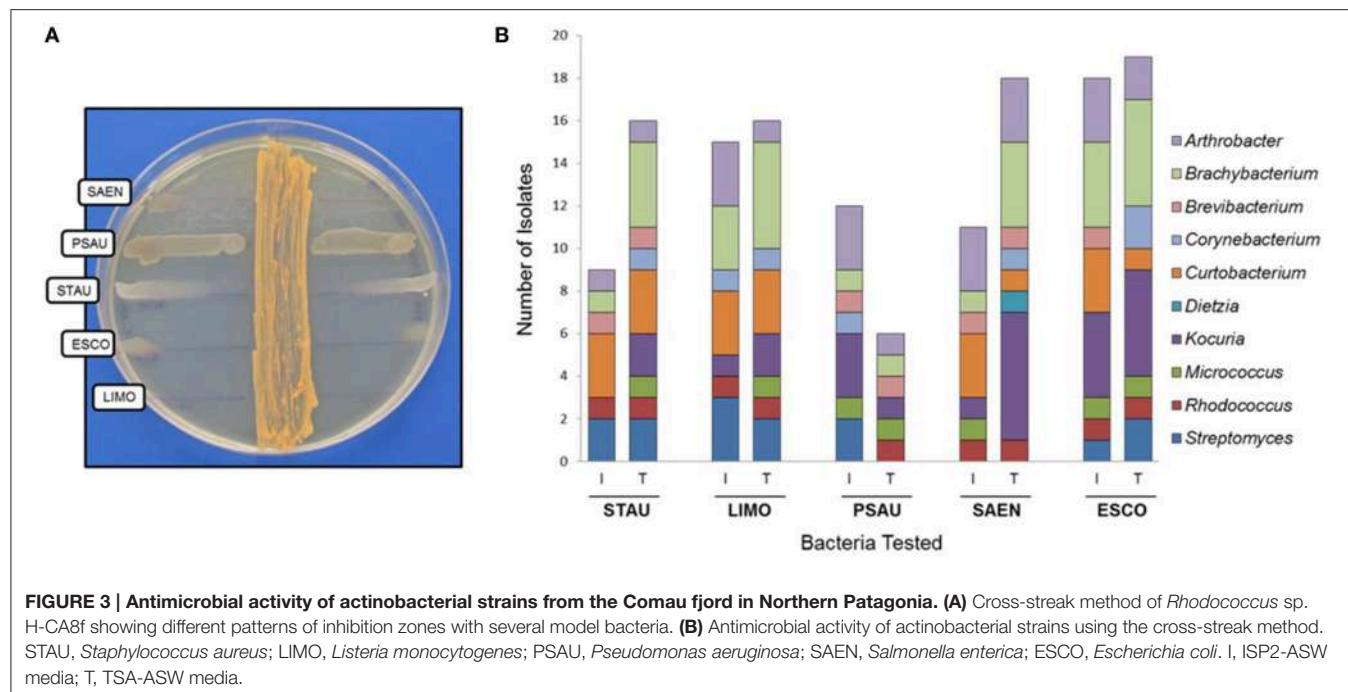


TABLE 1 | Antimicrobial activity of actinobacterial strains against model pathogens using the cross-streak method.

Strain	Genus	STAU		LIMO		PSAU		SAEN		ESCO	
		ISP2	TSA								
H-CA8b	<i>Arthrobacter</i>	+/-	+/-	+++	+++	+++	+++	+/-	+/-	++	++
H-JH1	<i>Arthrobacter</i>	-	-	++	-	+	-	+/-	+/-	+	-
H-JH3	<i>Arthrobacter</i>	-	-	++	-	+	-	+/-	+/-	+	+/-
H-CA4	<i>Brachybacterium</i>	-	-	+++	+	-	++	-	+/-	+/-	++
H-CD1	<i>Brachybacterium</i>	-	+/-	-	+++	-	-	-	+/-	+/-	+/-
H-CE9	<i>Brachybacterium</i>	-	+/-	+	++	-	-	+	+	++	+
H-CF1	<i>Brachybacterium</i>	++	++	+	++	-	-	-	+/-	-	+/-
H-CG1	<i>Brachybacterium</i>	-	+/-	-	+++	+/-	-	-	-	+/-	+/-
H-BE7	<i>Brevibacterium</i>	+	+/-	-	-	+	+/-	++	+	+	-
H-EH3	<i>Corynebacterium</i>	-	+/-	+/-	+++	-	-	-	-	-	+/-
H-KF5	<i>Corynebacterium</i>	-	-	-	-	+/-	-	-	+/-	-	+/-
H-BE10	<i>Curtobacterium</i>	+++	+++	+/-	+++	-	-	++	+++	+/-	++
H-CD9a	<i>Curtobacterium</i>	+	+	+/-	+	-	-	+/-	-	+/-	+/-
H-ED12	<i>Curtobacterium</i>	++	++	+	++	-	-	+/-	-	+/-	+/-
H-KA4	<i>Dietzia</i>	-	-	-	-	-	-	-	+/-	-	-
H-KA9	<i>Kocuria</i>	-	-	-	-	+/-	-	-	+/-	-	+/-
H-KA10	<i>Kocuria</i>	-	+/-	-	+/-	-	-	-	+++	+/-	+/-
H-KB1	<i>Kocuria</i>	-	-	-	-	-	-	-	+/-	+/-	+/-
H-KB5	<i>Kocuria</i>	-	-	-	-	+/-	-	-	+/-	+/-	-
H-KB6	<i>Kocuria</i>	-	+/-	+/-	+++	+/-	+/-	+	+/-	+/-	+
H-JH7	<i>Kocuria</i>	-	-	-	-	-	-	-	+	-	+/-
H-CD9b	<i>Micrococcus</i>	-	+/-	-	+++	+	+/-	+/-	-	+/-	+/-
H-CA8f	<i>Rhodococcus</i>	++	++	+++	+++	-	+++	+++	+++	+++	+++
H-CB3	<i>Streptomyces</i>	+++	+++	+/-	+/-	+/-	-	-	-	+	++
H-KF8	<i>Streptomyces</i>	+++	+++	+/-	+/-	+/-	-	-	-	+	+

-, no inhibition; +/-, attenuated growth; +, <50% growth inhibition; ++, 50% growth inhibition; +++, >50% growth inhibition. Both media were prepared with ASW.

TABLE 2 | Antimicrobial activities of crude extracts using various solvents for selected actinobacterial isolates grown in ISP2-ASW medium.

Strain	Solvent	Bacterial Test Strain			
		STAU	PSAU	SAEN	ESCO
H-KF8	Hexane	–	–	–	–
	Ethyl acetate	–	–	–	–
	Methanol	+	–	–	+
H-CA8f	Hexane	–	–	–	–
	Ethyl acetate	+	+	+	+
	Methanol	–	–	–	–
H-KB5	Hexane	–	–	–	–
	Ethyl acetate	–	+	+	+
	Methanol	–	–	–	–
H-JH3	Hexane	–	–	–	–
	Ethyl acetate	–	–	–	–
	Methanol	–	–	+	+
H-BE7	Hexane	–	–	–	–
	Ethyl acetate	–	–	+	–
	Methanol	–	–	–	–

family), *Brevibacterium* genus (*Brevibacteriaceae* family), and *Corynebacterium* genus (*Corynebacteriaceae* family) (Table 3). Interestingly, the psychrotolerant isolate *Kocuria* sp. H-KB5 has a 96.97% sequence identity with the type strain *K. polaris* CMS 76 or^T, a strain isolated from an Antarctic cyanobacterial mat sample (Reddy et al., 2003). Moreover, strain H-KB5 forms a separate branch within the *Kocuria* group in the phylogenetic tree (Figure 4). This isolate will be further characterized in a polyphasic approach to determine its taxonomic position.

Phenotypic Characterization of Isolated Actinobacterial Strains

The Comau fjord is characterized by defined zoning patterns of strong vertical and horizontal salinity gradients. The first 15 m underwater are influenced by waters of low salinity (~1.0%). Below this depth, a halocline is found that produces a constant water salinity of 3.2% (Castillo et al., 2012). In order to analyze how the salinity affects the growth of the actinobacterial isolates, NaCl tolerance was determined for each strain (Table 3). 82% of the representative isolates were able to grow in the presence of 1.0, 3.5, 5.0, and 7.0% (w/v) NaCl (Figure 5). 45% of the strains, belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Curtobacterium*, and *Kocuria* genera, were able to grow in presence of 10% (w/v) NaCl (Table 3). None of the isolated actinobacteria was able to grow with 20% w/v NaCl.

To study adaptation to marine environments, actinobacterial strains were tested for ASW requirement. Most strains (73%), belonging to *Arthrobacter*, *Brachybacterium*, *Corynebacterium*, *Dietzia*, *Kocuria*, *Rhodococcus*, and *Streptomyces* genera were positively influenced by sea water as they required ASW for growth, suggesting marine adaptation. Interestingly, strain

Brevibacterium sp. H-BE7, showed improved growth with both ASW and 3.5% NaCl, rather than with Milli-Q H₂O and 0% NaCl, suggesting a specific salt requirement confirmed by its growth in 10% (w/v) NaCl (Figures 5B–D).

As the Comau fjord deep-waters reach temperatures below 10°C, actinobacterial strains were tested for growth at different temperatures. Notably, 73% of strains belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Dietzia*, and *Rhodococcus*, and to a lesser extent, *Streptomyces*, were able to grow at 4°C (Figure 6). Moreover, pigmentation of the colonies was more intense after growth at 4°C, in comparison to 30°C (Figures 6B–D). Colony pigmentation of all representative actinobacteria was visualized macroscopically and detailed in Table 3.

Resistance to Model Antibiotics

Antibiogram experiments demonstrated that all isolated actinobacterial strains are resistant to at least one of the antibiotics tested. Furthermore, these isolates showed resistance to several antibiotics of different classes. Interestingly, strains H-JH3, H-BE7, H-KA4, H-CD9, H-CG1, H-ED12, and H-CA8f showed resistances to ≥6 antibiotics, wherein resistance to tetracycline, ciprofloxacin and oxacillin were observed for all the actinobacterial strains. Strain H-KA4 and H- ED12 showed resistance to all antibiotics tested, whereas strain H-BE7 was susceptible only for sulfonamides (Table 4).

DISCUSSION

Marine actinomycetes isolated from the National Marine Protected Area of Huinay at the Comau fjord in Northern Patagonia were studied, along with their physiological and taxonomic properties, and their potential to produce antimicrobial compounds. Patagonian fjords are largely unexplored, and may provide a rich source of microorganisms producing novel anti-infective compounds. This is the first bioprospection report of cultivable actinobacteria in this unique ecosystem, where 25 actinobacteria were isolated and characterized. Two studies report the isolation of marine actinobacteria from sediments of Chile's vast coast; one from Chiloé Island (Hong et al., 2010) and a recent study performed in Valparaíso Central Bay (Claverías et al., 2015). Only a metagenomic study has been carried out with a microbial mat located in the Comau fjord, revealing that 1% of community reads was represented by the phylum *Actinobacteria* (Ugalde et al., 2013).

In this study, a lower abundance of actinobacteria associated to marine sediments was observed compared to Valparaíso Bay where actinobacterial strains belonging to 18 genera were isolated, using the same cultivating conditions (Claverías et al., 2015). Although, members of the *Rhodococcus* and *Dietzia* genera were successfully isolated from the Comau fjord, they were less represented (8%) than in Valparaíso Bay (33%). The lower actinobacterial abundance in Comau fjord could be due to the lower content of organic matter in this microhabitat that can range between 0.5 and 3.4% of organic carbon content for Northern Chilean Patagonian fjords (Sepúlveda et al., 2011).

TABLE 3 | Biogeographic and physiological characteristics of representative actinobacterial strains.

Strain	Closest Type Strain (Accession N°) (% Identity)	Biogeographic Characteristics				Physiological Characteristics				Biosynthetic genes		
		Sampling Site	Depth (m)	Salinity (ppt)	Sediment characteristics	Temperature (°C)	Salinity (% NaCl)	ASW Requirement	Pigmentation	PKS I	PKS II	NRPS
H-JH3	<i>Arthrobacter oxydans</i> DSM 20119 ^T (X83408) (98.26)	Lilhuapi Island	11.3	28.5	Shells and sponges	4–37	0–10	+	Bright cream	–	–	+
H-CG1	<i>Brachybacterium paracondoleratum</i> JCM 17781 ^T (AB645761) (99.16)	Tambor Waterfall	6.1	30.5	Hard sediment	4–37	0–10	+	Bright yellow	+	+	+
H-BE7	<i>Brevibacterium oceani</i> BBH7 ^T (AM158906) (97.94)	Punta Llonco	25.1	29.5	Good water visibility	4–37	0–10	–	Bright orange	+	+	+
H-EH3	<i>Corynebacterium pilbarensense</i> IMIB WACC-658 ^T (FN4295567) (98.10)	Loncochagua River	0.25	5	Low tide	20–37	7	+	Bright cream	–	+	+
H-ED12	<i>Curtobacterium oceanosedimentum</i> ATCC 31317 ^T (GU289547) (99.02)	Punta Llonco	25.1	29.5	Good water visibility	20–45	0–10	–	Pale cream	–	+	+
H-KB5	<i>Kocuria polaris</i> CMS 760r ^T (NR028924) (96.97)	Loncochagua River	0.25	5	Low tide	4–37	0–10	+	Bright pink	–	+	+
H-KA4	<i>Dietzia natronilimnaea</i> DSM 444860 ^T (FJ468329) (99.06)	Tambor Waterfall	15.6	28.5	Sand, mussels and sea urchins	4–37	0–7	+	Intense orange	–	+	+
H-jCD9b	<i>Micrococcus luteus</i> NCTC 2665 ^T (CP001628) (99.15)	Punta Llonco	14.5	29	Shells, poor water visibility	20–37	0–7	–	Light yellow	–	–	–
H-CA8f	<i>Rhodococcus jianlingiae</i> dJ-6-2 ^T (DQ185597) (98.84)	Lilhuapi Island	22.9	31	Shells and old nets	4–30	0	+	Light pink	–	+	+
H-KF8	<i>Streptomyces prasinus</i> NRRL B-2712 ^T (DQ026658) (99.92)	Punta Llonco	14.5	29	Shells, poor water visibility	4–37	0–7	+	White mycelium	–	+	+
H-CB3	<i>Streptomyces prasinus</i> NRRL B-2712 ^T (DQ026658) (99.86)	Tambor Waterfall	15.6	28.5	Sand, mussels and sea urchins	4–37	0–7	+	White mycelium	–	–	+

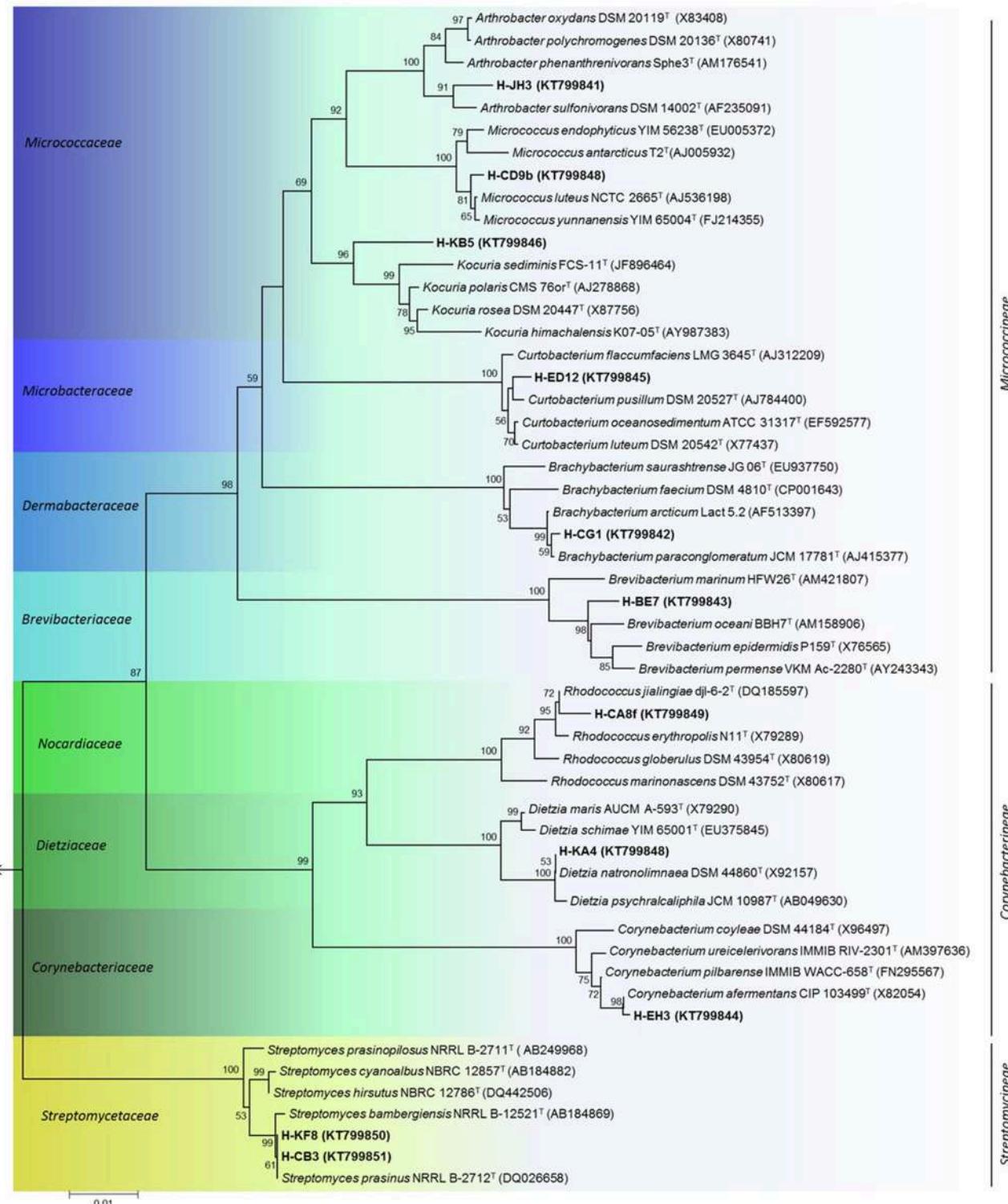


FIGURE 4 | Phylogenetic tree of representative actinobacterial strains isolated from the Comau fjord in Northern Patagonia, Chile. Neighbour-joining tree of 16S rRNA gene showing the three suborders within the phylum Actinobacteria. Node numbers represent the percentage of bootstrap replicates (1000 resampling) which supported the proposed branching order shown at consistent nodes (values below 50% were not shown). Gene sequence positions 55–1410 were considered, according to the *Escherichia coli* K12 (AP012306) 16S rRNA gene sequence numbering. Arrow points to the outgroup *E. coli* K12. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Scale bar corresponds to 0.01 substitutions per nucleotide positions.

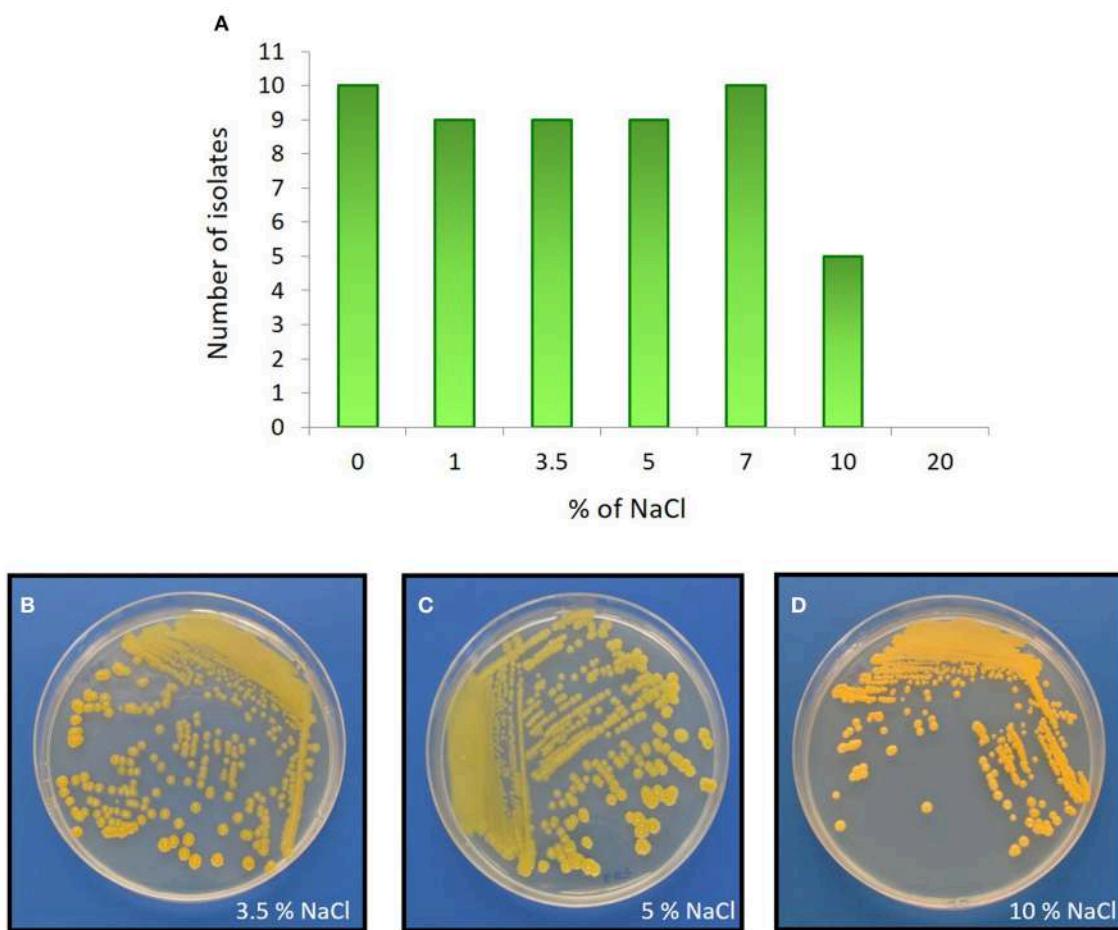


FIGURE 5 | NaCl effect on actinobacterial growth. Upper panel: (A) Distribution of actinobacterial isolates and their ability to grow in LB medium with various percentages of NaCl. Bottom panel: As an example, the halophilic *Brevibacterium* sp. H-BE7 grown in LB medium containing: (B) 3.5%; (C) 5%; and (D) 10% NaCl concentrations.

Gram-positive bacteria are more commonly observed in organic rich habitats (Fenical, 1993). Water samples from Valparaíso Bay are influenced by contamination with polycyclic aromatic hydrocarbons as well as with heavy metals (Campos et al., 1987; Palma-Fleming et al., 2008; Fuentes et al., 2015). It can also be influenced by hydrographic features such as seasonal upwelling which can supply nutrients to shallow waters (Capone and Hutchins, 2013). In contrast, the Comau fjord has a high precipitation rate that provides a fresh water input (Silva, 2006) which can affect microorganisms in marine sediments. The four sites from Comau fjord have minimal anthropogenic intervention, thereof changes in microbial communities are given almost exclusively by natural processes.

Despite the fact that a relatively low number of actinobacterial strains were retrieved from Comau fjord, a rather high cultivable biodiversity (10 genera) was observed using 5 isolation media. In comparison, the actinobacteria isolated using 11 selective media from the Trondheim fjord (Norway) belonged to 12 genera (Bredholdt et al., 2007). Also, in a culture-dependent study using sediments collected near Chiloé Island, Chile,

five genera were retrieved using 7 media, being dominant the *Micromonospora* genus (Hong et al., 2010). Although, no *Micromonospora* members have been isolated in this work, this could be due to the different isolation media used. In this report, 24% of isolates were obtained from NaST21Cx medium, which is derived from ST21Cx medium by elimination of yeast extract and replacement of artificial sea water (Magarvey et al., 2004). It has been reported that media composed of relatively simple nutrients yielded more cultured actinobacteria in diverse environments (Zhang et al., 2006; Gontang et al., 2007; Qin et al., 2012). This is consistent with the negligible amount of nutrients that are actually available for marine actinobacteria within hostile ocean ecosystems (Das et al., 2006). This is the case for our study since more isolates were obtained with media containing low nutrients or complex carbon sources rather than common media constituents such as peptone and simple sugars, which are proposed to be unrealistic marine nutrients (Kurtböke et al., 2015). In this study, the major abundance of actinobacteria was found in deeper samples, which is in accordance with that observed in the Trondheim fjord (Hakvåg

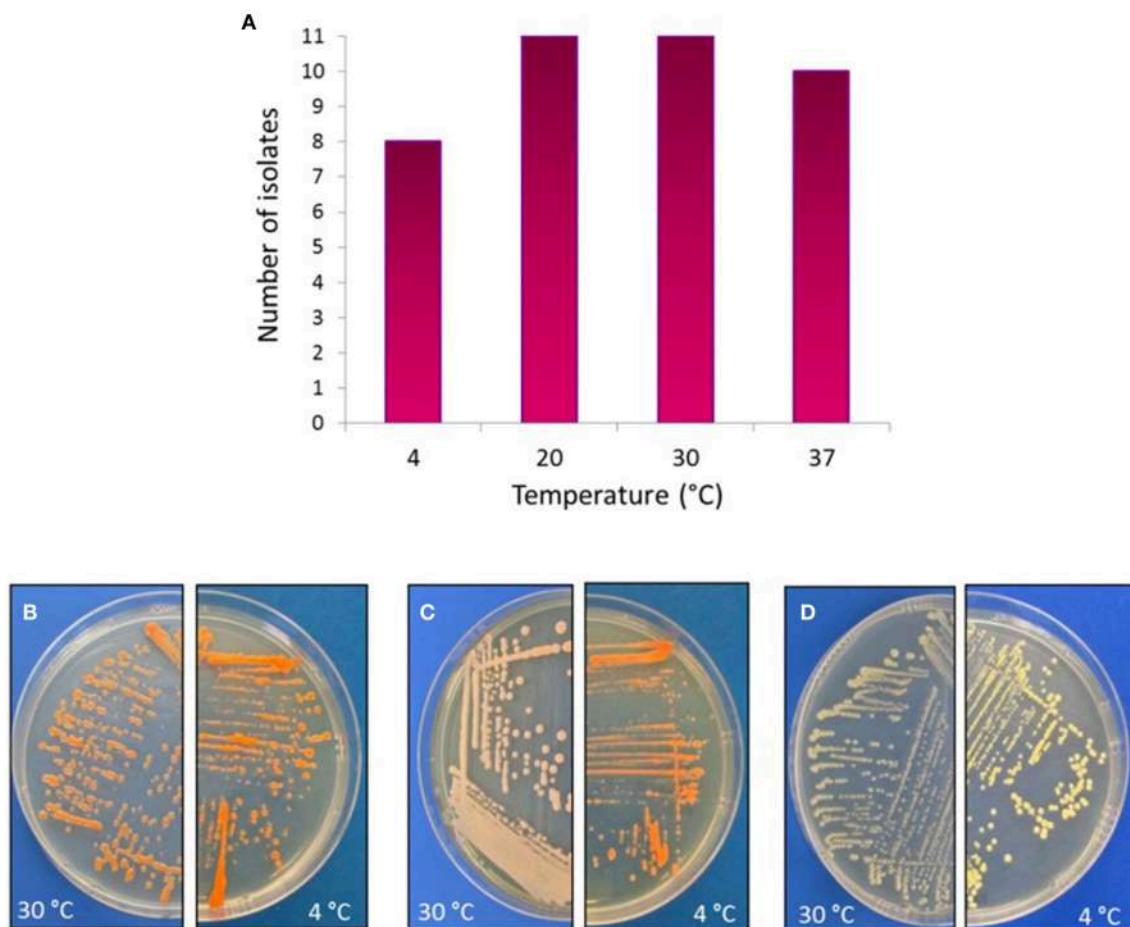


FIGURE 6 | Temperature effect on actinobacterial growth. Upper panel: (A) Distribution of actinobacterial isolates and their ability to grow in different temperatures. Bottom panel: As an example, actinobacterial strains grown in TSA-ASW medium at either 30°C (left) or 4°C (right), showing differences in pigmentations. (B) *Dietzia* sp. H-KA4; (C) *Kocuria* sp. H-KB5; (D) *Brachybacterium* sp. H-CG1.

TABLE 4 | Antibiotic resistance of selected actinobacterial strains.

Antibiotic	Class	Huinay Isolates										
		H-JH3	H-CG1	H-BE7	H-EH3	H-ED12	H-KB5	H-KA4	H-CD9	H-CA8f	H-KF8	H-CB3
Penicillin (10 UOF)	β-Lactam	R	R	R	NG	R	R	R	R	R	R	S
Chloramphenicol (30 µg)	Other	S	S	R		R	S	R	R	R	R	R
Tetracycline (30 µg)	Poliketide	R	R	R		R	R	R	R	R	R	R
Oxacyllin (1 µg)	β-Lactam	R	R	R		R	R	R	R	R	R	R
Erythromycin (15 µg)	Macrolide	R	S	R		R	S	R	S	S	R	S
Clindamycin (2 µg)	Lincosamides	R	S	R		R	S	R	S	R	S	S
Sulfa-Trimethoprim (25 µg)	Sulfonamide	R	S	S		R	S	R	S	R	S	S
Ciprofloxacin (5 µg)	Fluoro-quinolone	R	R	R		R	R	R	R	R	R	R
Cefazolin (30 µg)	Cephalosporin (1st)	R	R	R		R	R	R	R	R	S	S
Gentamicin (10 µg)	Aminoglycoside	S	R	R		R	S	R	R	R	S	S

NG, No growth; R, Resistant; S, Susceptible.

et al., 2008). Moreover, an elevated number (73%) of isolates showed an ASW requirement for growth. Evidence of isolation of seawater-dependent actinobacteria from marine sediments has

been reported (Mincer et al., 2002; Maldonado et al., 2005). The fact that growth of some isolates is positively influenced by sea water can be an indicator that suggests they might be well

adapted to the marine environments (Bredholdt et al., 2007; Penn and Jensen, 2012; Yuan et al., 2014). Nevertheless, since isolates obtained from Comau fjord can also grow without NaCl, they represent novel moderate halotolerant features in actinobacteria from this pristine sampling zone. This is consistent with the fact that these isolates have to overcome the dynamics of strong salinity gradients observed within the Comau fjord.

Reports of marine actinomycetes as a source of novel secondary bioactive metabolites have been extensively recognized (Haefner, 2003; Knight et al., 2003; Fiedler et al., 2005; Fenical and Jensen, 2006; Zhang et al., 2006; Gulder and Moore, 2010; Kurtböke et al., 2015). Two screenings for antimicrobial activities were pursued in this report, and notably, inhibition of the growth of at least one of the model bacteria was observed. It is noteworthy to highlight that antimicrobial activities from non-mycelial strain (e.g., *Rhodococcus* sp. H-CA8f) outcompete the activities of mycelial-type strains. To our knowledge, this is the first report of strong antibacterial activities associated to a *Rhodococcus* isolated from marine sediments. The *Rhodococcus* strain isolated in this study has a strong activity (>50% growth inhibition) against *E. coli*, *S. enterica*, *P. aeruginosa*, and *L. monocytogenes*; whereas a *Rhodococcus* strain isolated from Valparaíso Bay sediments (Claverías et al., 2015) had only a modest activity against *E. coli*. Antimicrobial activity from marine-derived isolates, but not necessarily from sediments, includes a *Rhodococcus* isolated from South China Sea corals that presented activity against *B. subtilis*, *B. thuringiensis*, and *E. coli* (Zhang et al., 2013), whereas *Rhodococcus* strains isolated from corals of the Arabian Gulf showed activity against *S. aureus* (Mahmoud and Kalendar, 2016). In this study, antimicrobial activity of *Arthrobacter* sp. H-JH3 against *S. enterica* and *E. coli* is highlighted by its novelty. In this line, there are reports about antarctic *Arthrobacter* strains isolated from sponges that were able to inhibit the growth of *Burkholderia cepacia* complex by the production of volatile organic compounds (Fondi et al., 2012; Orlandini et al., 2014). Also, antimicrobial activity against *Vibrio anguillarum* and *S. aureus* was detected from samples collected from the Arctic Ocean (Wietz et al., 2012). Interestingly, this is the first report indicating growth inhibition of Gram-negative strains by a *Brevibacterium* isolate. Only a bacteriocin able to inhibit *L. monocytogenes*, but inactive against Gram-negative was reported for this genus (Motta and Brandelli, 2002). In contrast, antimicrobial activity against *S. enterica* was observed in crude extracts, suggesting a different mode of action.

It has been reported that most natural products with interesting biological activities are synthesized by PKS (type I or type II), NRPS, and even PKS-NRPS hybrid pathways (Fischbach and Walsh, 2006). Some pharmacologically commercial examples include the polyketide antibiotic erythromycin (Staunton and Wilkinson, 1997) and the non-ribosomal peptide antibiotic of the cephalosporin family (Aharonowitz and Cohen, 1992). In this report, a PCR-based screening was pursued for the detection of these biosynthetic genes in actinobacterial isolates, in order to explore the potential to produce secondary metabolites with biotechnological applications. Notably, 91% of the isolates tested showed the presence of at least one of the three biosynthetic genes, which confirms that these metabolic pathways are

widely distributed among this phylum (Donadio et al., 2007). As molecular methods for analyzing these genes are useful for screening of isolates for prediction of potential bioactive molecule production (Hodges et al., 2012), future efforts will be focused in sequencing these biosynthetic genes, to gain knowledge of the novelty of the bioproducts in which they are involved in.

The marine habitat sampled in the Northern Patagonia of Chile was a promising scenario to search for novel actinobacterial strains. In this study, four putative new species are proposed: *Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Corynebacterium* sp. H-EH3 and *Kocuria* sp. H-KB5, based on numerical thresholds related to 16S rRNA gene sequences (Rosselló-Móra and Amann, 2015). In addition, representatives of *Micrococcineae*, *Corynebacterineae*, and *Streptomycineae* suborders were isolated. Interestingly, actinobacterial isolates showed sequence similarity with strains reported from colder habitats. 73% of the isolates belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Dietzia*, *Rhodococcus*, and *Streptomyces* genera were able to grow at 4°C, suggesting a psychrotolerant adaptation which is in accordance with the water body temperature range of the Comau fjord (Lagger et al., 2009; Sobarzo, 2009), sustaining a thermohaline circulation (Bustamante, 2009). A difference in colony pigmentation was observed at low temperatures. Pigments can be enhanced under specific conditions such as climate stress, since they are part of the non-enzymatic antioxidant mechanisms in cell defense to prevent oxidative damage (Correa-Llantén et al., 2012). Another role of pigments in response to cold is to decrease the membrane fluidity to counterbalance the effects of fatty acids in Antarctic bacteria (Chattopadhyay, 2006). Pigments can also contribute to antibacterial activity, positioning them as interesting biotechnological candidates for food, cosmetic and textile industries (Rashid et al., 2014; Leiva et al., 2015).

Comparison with 16S ribosomal RNA sequences Bacteria and Archaea NCBI database, reveals only two closest type strains of marine origin: *Brevibacterium oceani* BBH7^T isolated from deep sea sediment of the Indian Ocean (Bhadra et al., 2008) and *Curtobacterium oceanosedimentum* ATCC 31317^T isolated from Irish sea marine sediments (Kim et al., 2009). In contrast, when sequences are compared with NCBI nucleotide collection database, actinobacterial isolates showed more similarity with polar marine isolates. This is the case for the psychrotolerant *Arthrobacter* sp. H-JH3, which showed a 98.82% identity with *A. scleromae* Asd M4-11 (Vardhan Reddy et al., 2009), a bacterium isolated from a melt water stream of an Arctic glacier. The psychrotolerant *Brachybacterium* sp. H-CG1 showed a high similarity (99.16%) with *B. articum* Lact 5.2 (Acc. Number: AF434185, unpublished), a bacterium isolated from a sea-ice sample from the permanently cold fjord of Wijde fjord, Spitzbergen, in the Arctic Ocean. Another interesting relation is given for strain H-CD9b from the genus *Micrococcus*, which has a 99.15% of sequence identity with the type strain *M. luteus* NCTC 2665^T (Rokem et al., 2011) that is a soil metal resistant bacterium, and a slightly more sequence identity (99.43%) with *Micrococcus* sp. strain MOLA4 (Acc. Number: CP001628, unpublished) a bacterium isolated from sea water of

North Western Mediterranean Sea. Also, strain H-CA8f, showed a higher sequence similarity (98.91%) to *Rhodococcus* sp. TMT4-41 isolated from a glacier in China (Acc. Number: JX949806, unpublished) than to its closest type strain *R. jialingiae* djl-6-2^T (Wang et al., 2010).

Antibiogram experiments demonstrated that, in general, actinobacterial strains showed resistance. Interestingly, *Curtobacterium* sp. H-ED12, *Dietzia* sp. H-KA4 and *Brevibacterium* sp. H-BE7 showed resistance to almost all antibiotics tested, possibly due to the presence of multiple biosynthetic clusters, involving different classes of antibiotic compounds. Strains H-BE7 and H-ED12 inhibited both Gram-positive and Gram-negative model bacteria, suggesting different modes of action of the antibacterial molecules produced by this strain. Thus, it seems plausible that biosynthetic pathways involving metabolites of similar nature could be present in these isolates. A typical cluster of secondary metabolism includes genes for multi-domains enzymes that carry out the synthesis of different bioactive metabolites and when this metabolite has an antimicrobial activity, it is coupled to its corresponding resistance gene (Zotchev, 2014).

To our knowledge, this is the first report of the isolation and ecophysiological characterization of actinobacteria from sediments of a Patagonian fjord. This single survey uncovered a broad cultivable diversity which provides the basis for the bioprospection of bioactive compounds. The isolation

of novel actinobacterial species and the evidence that most of our isolates produced antibiotic activities supports our approach.

AUTHOR CONTRIBUTIONS

AU conceived and designed the experiments, performed the experiments, analyzed the data, prepared the manuscript. FB designed the experiments. FC performed the experiments. MG performed the sampling and experiments. EM performed the sampling and edited the manuscript. MS performed the sampling, prepared and edited the manuscript. BC performed the sampling, conceived and designed the experiments, analyzed the data, prepared and edited the manuscript.

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A Phenotypic and Genotypic Analysis of the Antimicrobial Potential of Cultivable *Streptomyces* Isolated from Cave Moonmilk Deposits

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Moonmilk speleothems of limestone caves host a rich microbiome, among which Actinobacteria represent one of the most abundant phyla. Ancient medical texts reported that moonmilk had therapeutic properties, thereby suggesting that its filamentous endemic actinobacterial population might be a source of natural products useful in human treatment. In this work, a screening approach was undertaken in order to isolate cultivable Actinobacteria from moonmilk of the Grotte des Collemboles in Belgium, to evaluate their taxonomic profile, and to assess their potential in biosynthesis of antimicrobials. Phylogenetic analysis revealed that all 78 isolates were exclusively affiliated to the genus *Streptomyces* and clustered into 31 distinct phylotypes displaying various pigmentation patterns and morphological features. Phylotype representatives were tested for antibacterial and antifungal activities and their genomes were mined for secondary metabolite biosynthetic genes coding for non-ribosomal peptide synthetases (NRPSs), and polyketide synthases (PKS). The moonmilk *Streptomyces* collection was found to display strong inhibitory activities against a wide range of reference organisms, as 94, 71, and 94% of the isolates inhibited or impaired the growth of Gram-positive, Gram-negative bacteria, and fungi, respectively. Interestingly, 90% of the cave strains induced strong growth suppression against the multi-drug resistant *Rasamsonia argillacea*, a causative agent of invasive mycosis in cystic fibrosis and chronic granulomatous diseases. No correlation was observed between the global antimicrobial activity of an individual strain and the number of NRPS and PKS genes predicted in its genome, suggesting that approaches for awakening cryptic metabolites biosynthesis should be applied to isolates with no antimicrobial phenotype. Overall, our work supports the common belief that moonmilk might effectively treat various infectious

diseases thanks to the presence of a highly diverse population of prolific antimicrobial producing *Streptomyces*, and thus may indeed constitute a promising reservoir of potentially novel active natural compounds.

Keywords: geomicrobiology, secondary metabolism, MLSA phylogeny, cryptic antibiotics, genome mining

INTRODUCTION

Members of the phylum Actinobacteria can be found in all kinds of extreme environments (Saiz-Jimenez, 1999; Bull, 2010; Prieto-Davó et al., 2013; Zhang et al., 2014; Mohammadipanah and Wink, 2015; Shivlata and Satyanarayana, 2015). Their successful survival in severe conditions suggests broad adaptive abilities that might be directly related to their very diverse and specialized (secondary) metabolism. Natural small molecules, collectively termed the parvome (Davies and Ryan, 2012), apart from having essential ecological functions, possess a wide range of bioactivities, which are applicable for agro-industrial purposes and human/animal therapy (Hopwood, 2007). Since the metabolome of soil-dwelling Actinobacteria, especially of the members of the genus *Streptomyces*, has been widely exploited, leading to the multiple re-isolation of already known bioactive compounds, the attention has been refocused toward unexplored and extreme environments, which can potentially be a source of novel species and consequently of novel molecules of interest (Cheetham et al., 2013; Claverías et al., 2015; Mohammadipanah and Wink, 2015; Liao et al., 2016).

The geological isolation of caves from surface processes makes them a unique niche, not only to study microbial interactions and adaptations to extreme oligotrophy, but also to screen for potentially novel bioactive compounds. Although, the most common Actinobacteria reported from caves belong to *Pseudonocardiaceae* and *Nocardiaceae* families (Stomeo et al., 2008; Porca et al., 2012; Quintana et al., 2013; Riquelme et al., 2015), many investigations have identified cultivable members of the genus *Streptomyces*, which are the most prolific antimicrobial producers (Cañaverales et al., 1999; Groth et al., 1999; Cheetham et al., 2013; Nimaichand et al., 2015; Axenov-Gribanov et al., 2016). The presence of Actinobacteria as dominant members of microbial ecosystems in caves is puzzling, as these bacteria, particularly *Streptomyces* are often presented as major protagonists in the recycling of the residual plant biomass in nutrient-rich soil environments (Hodgson, 2000). Nonetheless, >99% of allochthonous carbon entering caves, primarily with drip water, contains soil-derived dissolved organic carbon in the form of partially degraded plant and fungal polymers, which can be catabolized by the enzymatic arsenals of *Streptomyces* (Saiz-Jimenez and Hermosin, 1999; Simon et al., 2007; Barton, 2015). Additionally, highly prolific and diversified secondary metabolism could be a driving force in the dominance of these species in oligotrophic environments, through which they could shape microbiomes thanks to their specialized metabolites, such as metal-chelators for acquiring trace metals, and antibiotics to prevent nutrient-exclusion by competitive species (Bhullar et al., 2012). Antibiotics do not exclusively prevent microbial growth, but are also known to act as

inter-/intraspecies communication (Sengupta et al., 2013), and as cues triggering adaptations, such as motility or biofilm formation (Linares et al., 2006), or can be used as alternative carbon and/or nitrogen sources (Dantas et al., 2008). Consequently, small bioactive molecules expressed under nutrient-starved conditions, could be used as weapons, as signals, or as nutrient sources.

Moonmilk is a comparatively rare speleothem in cave environments, where it forms as a thick calcite paste (similar in consistency to toothpaste) up to 10s-of-centimeters in thickness in passageways that receive significant airflow (Hill and Forti, 1997; Borsato et al., 2000). The exact process of moonmilk formation remains in debate; however, the consistency, the high abundance of filamentous bacterial cells and members of the Actinobacteria has led researchers to suggest a biogenic origin (Cañaverales et al., 2006; Rooney et al., 2010; Portillo and Gonzalez, 2011). Despite its relative scarcity in caves, moonmilk has had long scientific interest due to its historical use as a medical treatment. Interestingly, moonmilk was used in human and animal therapy since the Middle Ages (Reinbacher, 1994). Its curative properties could be associated with the presence of the numerous filamentous Actinobacteria, particularly *Streptomyces*, presumably producing bioactive molecules. Indeed, isolation of *Streptomyces* with antimicrobial and antifungal activities has been reported for moonmilk from the Bolshaya Oreshnaya Cave in Siberia (Axenov-Gribanov et al., 2016). The identification of potential novel compounds with broad-spectrum activities from *Streptomyces* found in this karstic secondary deposit supports the idea of moonmilk being a great target for bioactivity screening, although to date very few studies have examined cultivable moonmilk microbiome and the diversity of its metabolome as a possible reservoir of novel compounds. The fact that caves are unique and still highly under-explored environment increase the chances of finding novel organisms and consequently novel bioactive compounds that might be useful in the context of the global health problem of antibiotic resistance (Bush et al., 2011; Laxminarayan et al., 2013; Berendonk et al., 2015; Frère and Rigali, 2016).

In this work, we report the isolation and phylogenetic analysis of a collection of novel *Streptomyces* isolates from moonmilk deposits, and assess their potential as producers of compounds with antimicrobial and antifungal properties through *in vitro* screening and genome mining approaches.

MATERIALS AND METHODS

Site Description and Sampling

The cave called Grotte des Collemboles (Springtails' Cave) located in Comblain-au-Pont in Belgium is a shallow (<20 m), ~70 m long fissure cave, formed in the upper Viséan limestone

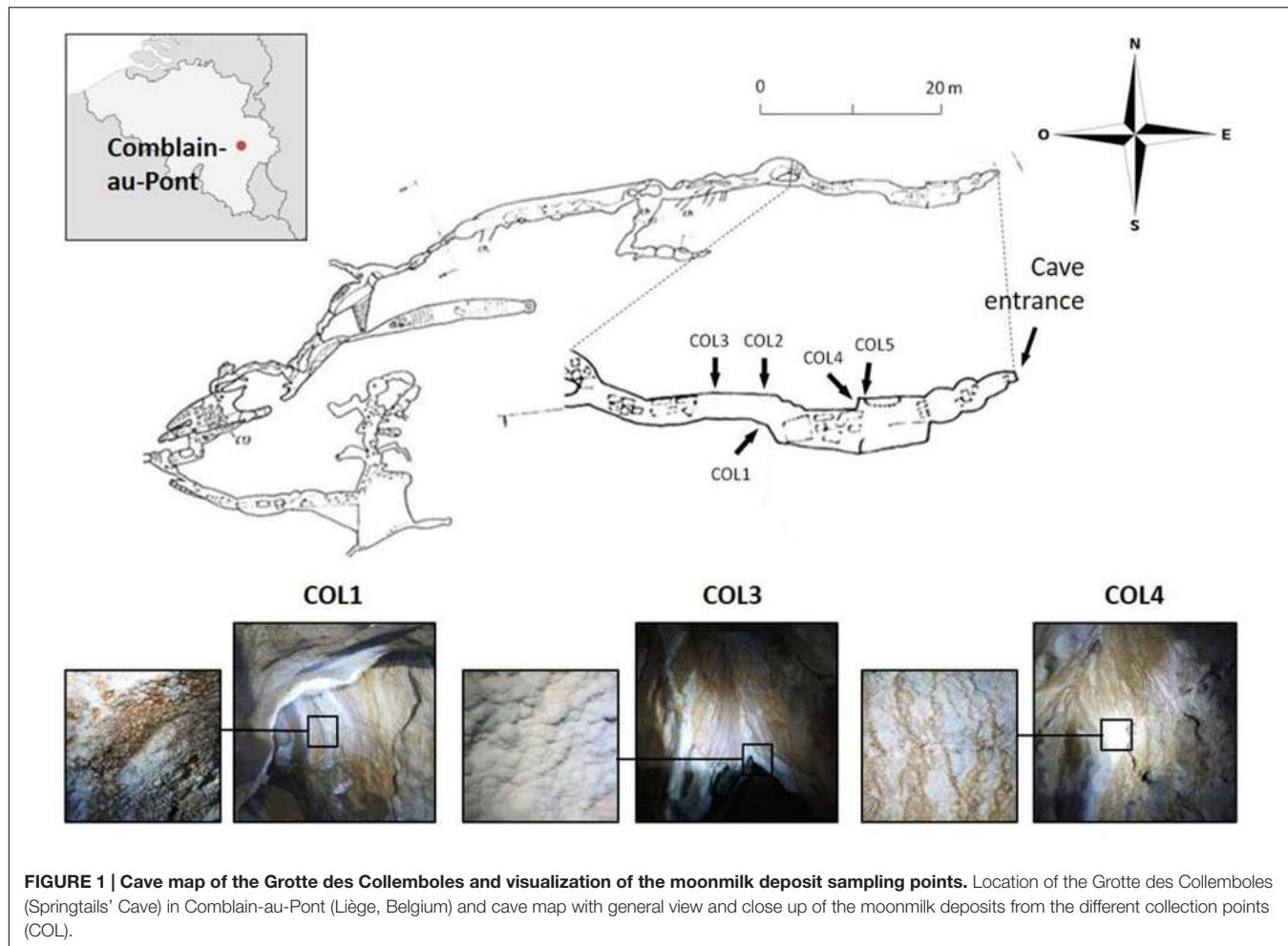


FIGURE 1 | Cave map of the Grotte des Collemboles and visualization of the moonmilk deposit sampling points. Location of the Grotte des Collemboles (Springtails' Cave) in Comblain-au-Pont (Liège, Belgium) and cave map with general view and close up of the moonmilk deposits from the different collection points (COL).

(Figure 1), with an average annual temperature of $\sim 11.5^{\circ}\text{C}$. Due to cave protection policies and location on private property, specific location details and access information is available to other researchers upon request. Within the cave, white to brown-orange (presumably from iron-oxide precipitates) moonmilk deposits are found on the walls within the first 20 m of the cave in the first narrow chamber located at the entrance of the cave as well as in the narrow passages leading deeper into the cave (Figure 1). Samples used in this work were aseptically collected in January 2012 from three moonmilk deposits (Figure 1). Soft moonmilk speleothem was scratched with sterile scalpels from the wall in the first chamber, adjacent to the cave entrance (COL4) and the walls in a narrow passage after the first chamber (COL1, COL3; Figure 1). Samples were collected into falcon tubes, transferred to the laboratory, freeze-dried on a VirTis Benchtop SLC Lyophilizer (SP Scientific, Warminster, PA, USA) and stored at -20°C .

Isolation of Cultivable Actinobacterial Species

Selective isolation of *Streptomyces* species from moonmilk was carried out by a serial dilution method as described previously (Maciejewska et al., 2015). 250 mg of lyophilized moonmilk

sample from each collection point was suspended in 0.25X strength Ringer's solution supplemented with 0.001% Tween 80. Resulting moonmilk suspensions were serially diluted in PBS and inoculated in duplicates on ISP media (Shirling and Gottlieb, 1966), starch nitrate (SN) medium (Gauze's medium No.1; Waksman and Lechevalier, 1961), B-4 agar (Boquet et al., 1973), and minimal medium (Kieser et al., 2000) with 1% chitin (MMch). Isolation media were supplemented with nalidixic acid (75 $\mu\text{g}/\text{ml}$) and nystatin (50 $\mu\text{g}/\text{ml}$) to suppress the growth of Gram-negative bacteria and fungi, respectively. After 1 month of incubation at 17°C , colony forming units (CFUs) were enumerated and 129 isolates were selected. After two rounds of subcultivation, 78 isolates were recovered as purified strains and subsequently preserved both on ISP2 slopes at 4°C and as 25% glycerol mycelium stock at -20°C .

DNA Extraction, Genome Sequencing, and Gene Selection from Moonmilk-Derived Isolates

In order to screen for the genes of interest, which would enable to identify moonmilk derived isolates, to perform phylogenetic analysis, as well as to investigate strains antimicrobial properties,

de novo genome sequencing was carried out. DNA from purified strains was extracted with GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions from liquid LB (Luria-Bertani; Difco, BD, Franklin Lakes, NJ, USA) cultures incubated at 28°C. The genomic libraries of moonmilk isolates were constructed using Nextera XT kit (Illumina, Inc., San Diego, CA, USA). Library concentrations and mean fragment lengths were measured by Qubit fluorometer (Invitrogen, Grand Island, NY, USA) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. *De novo* sequencing with 2 × 250 bp and 2 × 300 bp reads configuration was carried out on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) at the Luxembourg Institute of Science and Technology. Complete genomes were assembled from raw sequence data with SPAdes v.3.6.2 (Bankevich et al., 2012) using the "careful" option, and the quality of the assemblies was subsequently assessed with QUAST v2.3 (Gurevich et al., 2013).

To infer the evolutionary relationships between moonmilk strains and their closest relatives, as well as between cave isolates themselves, 16S rRNA-based phylogeny was combined with multilocus sequence analysis (MLSA). For this purpose, along with the 16S rRNA gene, five additional housekeeping genes were selected, namely *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB* (Han et al., 2012). In order to identify these genes within moonmilk genomes, the corresponding nucleotide sequences (16S rRNA) and protein translations (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) were retrieved from the NCBI web portal for three reference strains: *Streptomyces peucetius* AS 4.1799, *Streptomyces pristinaespiralis* ATCC 25486 and *Streptomyces venezuelae* ATCC 10712 (Supplementary Table S1). Core alignments were built using MAFFT v7.273 (Katoh and Standley, 2013) with default parameters, then enriched in the corresponding sequences from moonmilk genomes using the software "42" (D. Baurain, to be published elsewhere), which mines genomic contigs for orthologous genes and aligns the (translated) identified sequences on their closest relatives. Enriched alignments were then refined by hand using the ED program of the MUST software package (Philippe, 1993). Finally, protein sequences were turned into nucleotide sequences using the software "1331" (D. Baurain, to be published elsewhere), which uses a protein alignment as a guide to generate the corresponding nucleotide alignment from genomic contigs. The sequences of the five protein coding housekeeping genes for all the moonmilk isolates were deposited in GenBank and the corresponding accession numbers are given in Supplementary Table S2, while Table 1 and Supplementary Table S3 list the NCBI accession numbers of the 16S rRNA gene sequences.

In order to profile the potential of moonmilk isolates to biosynthesize secondary metabolites, the genes coding for type I, type II, and type III polyketide synthases (PKS-I, PKS-II, and PKS-III) and non-ribosomal peptide synthetases (NRPS) were recovered from their genomes using antiSMASH v3.0.4 (Weber et al., 2015). Due to the fragmented nature of the genome assemblies (ranging from 318 contigs longer

than 1 kb for MM23 to 1416 contigs for MM59) and to the large size of the modular NRPS and PKS-I genes (over 40 kb, Wang et al., 2014), the probability of finding complete coding sequences decreases together with the contig length. Therefore, counting the number of genes or clusters split across several shorter contigs would result in an overestimation of the total amount of such genes. To palliate the absence of fully assembled chromosomes while still collecting meaningful statistics, we decided to apply a cut-off on the length of the contigs selected for analysis (minimum length of 10 kb) and to consider NRPS and PKS-I gene sequences only when they displayed adenylation and acyltransferase domains, respectively. These domains, which are the highly selective gatekeeper enzymes for the incoming monomeric building blocks, are required for the initiation and elongation modules of the NRPS/PKS-I clusters. The number of predicted genes of each category for each individual phylotype is compared to their respective mean antimicrobial activities against Gram-positive, Gram-negative bacteria, and fungi. The accession numbers of NRPS and PKS-I/II/III genes are listed in Supplementary Table S4.

Phylogenetic Analysis

To carry out phylogenetic analysis, in each nucleotide alignment of the six selected housekeeping genes (see above), the sequences from the three reference strains used to mine the moonmilk genomes were removed. Then, positions with missing character states in >5 moonmilk isolates were removed. Finally, the trimmed alignments were concatenated into a single (MLSA) supermatrix of 10,632 nucleotides for 70 isolates using SCaFoS v1.30k (Roure et al., 2007). For 16S rRNA phylogenies, the closest relatives to the moonmilk isolates were recovered by BLAST searches using full-length 16S rRNA sequences, along with the *Streptomyces* isolates from a moonmilk deposit in Siberia (Axenov-Gribanov et al., 2016). For the eight isolates for which the genomes were not sequenced (MM9, MM32, MM39, MM55, MM73, MM88, MM90, MM93), nearly full-length 16S rRNA sequences were obtained using PCR primers and conditions as previously reported (Maciejewska et al., 2015). The 78-moonmilk strain alignment of the 16S rRNA was further processed using the software "two-scalp" (D. Baurain, to be published elsewhere) to integrate 35 additional sequences, corresponding to the 27 (non-redundant) best BLAST hits, 7 Siberian moonmilk sequences, and the sequence of *Saccharopolyspora erythraea*, used as the outgroup.

Both the MLSA supermatrix and the 16S rRNA alignment were submitted to phylogenetic inference using the rapid bootstrap analysis of RAxML v8.1.17 (Stamatakis, 2014; 100 pseudoreplicates) under the model GTR+I+Γ₄. The resulting MLSA and 16S rRNA trees were first formatted in FigTree v1.4.2¹ then further arranged using Inkscape v0.91². Patristic distances between moonmilk isolates were derived from the MLSA tree

¹<http://tree.bio.ed.ac.uk/software/figtree/>

²<https://inkscape.org/>

TABLE 1 | The closest relatives, phylogenetic affiliations, phylotype clustering, and isolation origin of the 31 representative moonmilk isolates.

Isolate	Closest relatives	16S rRNA identity % (gaps)	Accession number	Origin of the closest relatives	COL	Md	Phylotype 16S → MLSA
MM1	S. sp. CFMR 7 strain CFMR-7/S. <i>fulvissimus</i> DSM 40593	99.1 (4)/99.1 (6)	KU714864	Plant (rubber)/unknown	COL3	SN	I = I
MM3	Un. bacterium clone Md-54/Un. bacterium clone 10-355	99.8 (0)/99.8 (0)	KU714892	Soil/soil	COL3	SN	II = II
MM5	S. <i>scabiei</i> BCCO 10_524/S. <i>europaeiscabiei</i> 08-46-04-2 (#50)	99.7 (0)/99.4 (2)	KU714904	Both plant (potato)	COL3	SN	III = III
MM6	S. sp. Mg1/S. sp. SXY10	99.7 (0)/99.8 (0)	KU714910	Glacier soil (Alaska)/soil	COL3	SN	IV = IV
MM7	S. sp. NEAU-spg16/S. sp. A42	99.6 (0)/99.9 (0)	KU714915	Soil/soil	COL3	SN	V = V
MM10	S. sp. NEAU-QHHV11/S. sp. (Acc.Nr.D63866)	99.1 (4)/98.6 (7)	KU714865	Soil/soil	COL3	SN	VI = VI
MM12	S. <i>sanglieri</i> A14/S. sp. ME03-5656.2c	99.8 (0)/99.6 (0)	KU714878	Soil/plant (potato)	COL3	SN	VII = VII
MM13	S. <i>turgidiscabies</i> ATCC 700248/S. <i>turgidiscabies</i> WI04-05A	98.8 (4)/98.7 (4)	KU714882	Both plant (potato)	COL3	SN	VIII = VIII
MM14	S. <i>anulatus</i> strain 173826/S. <i>anulatus</i> strain 173541	100 (0)/100 (0)	KU714883	Both unknown	COL3	SN	IX = IX
MM17	S. sp. Mg1/S. sp. SXY10	99.7 (0)/99.8 (0)	KU714885	Glacier soil (Alaska)/soil	COL3	SN	IV → XXVI
MM19	S. sp. NEAU-spg16/S. sp. A42	99.6 (0)/99.9 (0)	KU714887	Soil/soil	COL3	SN	V → XXVII
MM21	Un. bacterium clone Md-54/Un. bacterium clone 10-355	99.7 (0)/99.7 (0)	KU714888	Soil/soil	COL3	SN	XI = XI
MM23	Un. bacterium clone Md-54/Un. bacterium clone 10-355	99.8 (0)/99.8 (0)	KU714890	Soil/soil	COL3	SN	II → XXVIII
MM24	S. sp. ME02-6979.3a/S. sp. 1C-HV8	98.3 (5)/98.4 (4)	KU714891	Plant (potato)/animals (ants)	COL3	SN	XII = XII
MM44	Un. bacterium clone Md-54/Un. bacterium clone 10-355	99.7 (0)/99.7 (0)	KU714900	Soil/soil	COL3	SN	XI → XXIX
MM48	S. sp. HBUM171258/S. sp. Mg1	99.9 (1)/99.6 (0)	KU714903	Unknown/glacier soil (Alaska)	COL3	MMch	XIII = XIII
MM59	S. sp. ID05-8D/S. sp. ID01-6.2a	99.5 (1)/99.4 (1)	KU714909	Both plant (potato)	COL3	MMch	III → XXX
MM68	S. <i>turgidiscabies</i> ATCC 700248/S. <i>turgidiscabies</i> WI04-05A	99.0 (2)/99.0 (2)	KU714913	Both plant (potato)	COL3	B-4	XIV = XIV
MM90	S. sp. AA58/S. sp. AS40	99.5 (4)/99.4 (5)	KU714925	Soil/soil	COL1	ISP4	XV → -
MM99	S. <i>fulvissimus</i> DSM 40593/S. sp. ME02-6987.2c	99.7 (2)/99.7 (2)	KU714928	Unknown/plant (potato)	COL1	ISP6	XVI = XVI
MM100	S. <i>sanglieri</i> A14/S. sp. ME03-5656.2c	99.9 (0)/99.5 (0)	KU714866	Soil/plant (potato)	COL1	B-4	XVII = XVII
MM104	S. <i>scopuliridis</i> strain SCSIO ZJ46/S. sp. AK02-1a	99.2 (0)/99.0 (0)	KU714869	Deep sea/plant (potato)	COL3	ISP6	XVIII = XVIII
MM105	S. <i>finlayi</i> strain CB00817/S. <i>olivoviridis</i> strain S3	99.4 (6)/99.3 (5)	KU714870	Soil/animals (earthworm)	COL3	ISP6	XIX = XIX
MM106	S. <i>rishiriensis</i> strain 1706/S. <i>fimbriatus</i> strain cfcc3155	99.0 (0)/98.8 (1)	KU714871	Soil/unknown	COL3	ISP1	XX = XX
MM107	S. <i>pristinaespiralis</i> strain HCCB 10218/S. sp. NEAU-bt10	98.8 (2)/98.8 (0)	KU714872	Soil/soil	COL3	ISP1	XXI = XXI
MM108	S. sp. SXY66/S. sp. 1H-TWYE2	100 (0)/99.3 (2)	KU714873	Soil/animals (ants)	COL3	ISP7	XXII = XXII
MM109	S. <i>lunaelactis</i> MM109 ^T /S. <i>lunaelactis</i> MM15	100 (0)/99.9 (0)	KM207217.2	Cave/cave	COL3	ISP7	X = X
MM111	S. sp. 1H-TWYE2/S. sp. SXY66	99.7 (0)/99.5 (2)	KU714875	Animals (ants)/soil	COL4	ISP6	XXIII = XXIII
MM117	S. sp. PAMC26508/S. <i>pratensis</i> ATCC 33331	99.7 (0)/99.7 (0)	KU714876	Antarctic lichen/soil	COL4	ISP7	XXIV = XXIV
MM122	S. sp. PAMC26508/S. <i>pratensis</i> ATCC 33331	100 (0)/100 (0)	KU714879	Antarctic lichen/soil	COL4	B-4	IX → XXXI
MM128	S. sp. ZLN234/S. sp. SXY66	99.9 (0)/99.0 (4)	KU714881	Glacier soil (Arctic)/soil	COL4	SN	XXV = XXV

B-4, B-4 agar medium; MMch, minimal medium with 1% chitin; ISP, International Streptomyces Project medium; SN, starch nitrate medium; COL, moonmilk collection site; Md, isolation medium; Un., uncultured. Symbols: -, isolates not included in the MLSA; T, Type strain (Maciejewska et al., 2015).

using the TREEPLOT program of the MUST software package (Philippe, 1993).

Antimicrobial Activity Screening

Antimicrobial activities of one representative of each phylotype deduced from the MLSA, together with MM90 (representing 16S-phylotype XV) were tested using the cross-streak method on two (antifungal test) to five (antibacterial test) different culture media: Mueller Hinton Agar (MHA) (Difco, BD, Franklin Lakes, NJ, USA), Tryptic Soy Agar (TSA) (tryptone 15 g, soybean meal 5 g, NaCl 5 g, agar 15 g; pH 7.3), ISP media No. 7 (Shirling and Gottlieb, 1966), starch nitrate (SN) medium (Waksman and Lechevalier, 1961), and minimal medium (Kieser et al., 2000) supplemented with 25 mM N-acetylglucosamine (MM + GlcNAc). Each moonmilk strain was independently inoculated from the mycelium stock as a single line in the center of the square plate and incubated for 7 days at 28°C, before being cross-streaked with bacterial or fungal reference strains.

Antibacterial activities were tested against a range of Gram-positive and Gram-negative bacteria, including *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Citrobacter freundii* (ATCC 43864), *Klebsiella pneumoniae* (ATCC 13883), *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* (ATCC 25923), and *Micrococcus luteus* (ATCC 9341). Each tested bacteria was cross-streaked perpendicular to the growth of the moonmilk isolate at the distance of 2 cm from one another from a suspension prepared according to EUCAST recommendations (EUCAST, 2015). Briefly, each inoculum was made from the overnight-grown plate of each pathogen in solid LB (Difco, BD, Franklin Lakes, NJ, USA) at 37°C by suspending several morphologically similar colonies in LB broth (Difco, BD, Franklin Lakes, NJ, USA) until the OD_{625 nm} reached 0.08–0.13, corresponding to the McFarland 0.5 standard. The reference bacterial strains were cross-streaked with a cotton swab, the plates incubated overnight at 37°C, and the activities determined by measurement of the inhibition zone (in cm).

Antifungal activities were tested against a range of pathogenic fungi including *Candida albicans* (ATCC 10231), *C. albicans* (azole-resistant routine strain from the National Reference Center for Mycoses, 13-160409-5014), referred as *C. albicans* 'R', *Aspergillus fumigatus* (Neqas 1210), *Rasamonia argillacea* (Neqas 1872), *Penicillium chrysogenum* (Neqas 2068), and *Trichophyton mentagrophytes* (Neqas 1208). Each fungal strain was suspended in water at the density equivalent to 0.5 McFarland, and the obtained fungal suspension was perpendicularly cross-streaked against moonmilk isolates with a distance of 4 cm from one another. The plates were incubated at 37°C, for up to 4 days, and the measurements of inhibition zones (in cm) were recorded every day.

MM99 Isolate Antifungal Agents Extraction and Purification by High Pressure Liquid Chromatography (HPLC)

Streptomyces sp. MM99 was inoculated on 15 Glucose Yeast and Malt medium (GYM; glucose 4 g; yeast extract 4 g; malt extract 10 g; casein enzymatic hydrolysate 1 g; NaCl 2 g; agar

20 g) plates and incubated for 10 days at 28°C. The agar was collected, poured into a flask with an equal volume of ethyl acetate (~300 ml) and agitated overnight at room temperature for metabolites extraction. Ethyl acetate was collected and pieces of agar were removed by centrifugation (25 min at 4000 rpm) before the solvent was evaporated on a rotary evaporator (IKA RV10 digital, VWR, Radnor, PA, USA). The dried crude extract was resuspended in 4 ml of pure methanol high pressure liquid chromatography (HPLC Barker UHPLC grade). Prior to fractionation by HPLC, the antifungal activity of the total extract was assessed by a disk diffusion assay on a yeast peptone dextrose (YPD; peptone 20 g; yeast extract 10 g; glucose 20 g; agar 15 g) agar plate inoculated with *Saccharomyces cerevisiae* (ATCC 9763; with a cotton swab dipped in a 0.25–0.27 OD₆₂₅ LB suspension). The full extract was then fractionated by HPLC (Waters, Milford, MA, USA) using a Waters 2695 Separations Module (Alliance) with a Waters 2998 Photodiode Array Detector coupled to a Waters Fraction Collector WFC III. The methanol extract was analyzed on a Nucleodur C18ec column (2.0 mm × 150 mm, 5 μm particle size, Macherey-Nagel) at a column temperature of 40°C. Extract separation was achieved by increasing the acetonitrile (Barker, HPLC far UV grade)/water (milliQ filtrated on 0.22 μm) + 0.05% trifluoroacetic acid (TFA, Sequencing grade; Thermo Fisher Scientific, San Jose, CA, USA), ratio (from 0 to 62.5% of acetonitrile during 30 min, then from 62.5 to 100% of acetonitrile during 8 min) at a 300 μl/min flow rate. Online UV absorption measurement was performed from 190 to 800 nm. Data were analyzed using Empower 3 software (Waters, Milford, MA, USA). The obtained extract fractions were subsequently tested for antifungal activities by a disk diffusion assay as described above.

Compound Identification by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

Fractions displaying antifungal activities revealed by the disk diffusion assay were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, compounds were separated by reverse-phase chromatography using Ultra Performance Liquid chromatography (UPLC IClass, Waters) using a Nucleodur C18ec column (2.0 mm × 150 mm, 5 μm particle size, Macherey-Nagel). Elution was achieved by increasing the acetonitrile/water (milliQ filtrated on 0.22 μm) + 0.05% trifluoroacetic acid ratio (from 0 to 62.5% during 30 min, then from 62.5 to 100% during 8 min) at a 300 μl/min flow rate. On-line UV absorption measurement was performed at 210 and 265 nm and the chromatography system was finally coupled to a Q Exactive Plus hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), operated in positive ion mode and programmed for data-dependent acquisitions. Survey scans were acquired at mass resolving power of 140,000 FWHM (full width at half maximum) from 100 to 1500 m/z (1×10^6 ions accumulation target). The five most intense ions were then selected to perform MS/MS experiments by Higher

Energy Collision Dissociation (HCD) fragmentations using stepped normalized collision energy (NCE; 21,2; 25; 28) within 2 amu isolation windows (resolution 17500, 1×10^5 ions accumulation target). A dynamic exclusion was enabled for 10 s. Data were analyzed using Xcalibur v2.2 (Thermo Fisher Scientific, San Jose, CA, USA). Commercial cycloheximide standard (Sigma-Aldrich, St. Louis, MO, USA) was used as a control.

RESULTS AND DISCUSSION

Isolation of Actinobacteria from Moonmilk Deposits

Cultivable actinobacterial species were isolated from the three separate moonmilk deposits within Grotte des Collemboles (**Figure 1**). Our cultivation-based screening on Actinobacteria-specific media revealed high population density across the three collection sites studied, with up to 10^4 CFUs/g (**Figure 2**). No microbial growth could be detected in the ISP5 medium (Glycerol Asparagine Agar), while the highest numbers of CFUs for each sampling point were recorded on the starch nitrate (SN) medium (5×10^4 and 1.3×10^4 CFUs/g in COL1 and COL4, respectively), and on the minimal medium supplemented with chitin (2.6×10^4 CFUs/g in COL3; **Figure 2**). Most of the viable isolates (>50%) were chosen from the starch nitrate medium (**Table 1**; Supplementary Table S3), in which the most distinct colony phenotypes were observed. From the 129 colonies initially selected, only 78 were recovered as pure isolates after three rounds of inoculation/cultivation, and were preserved for further studies. This important loss (40%) during the purification steps was expected, as living in an oligotrophic environment necessarily implies nutrient exchange between individuals of the microbiome, a cooperative strategy that was only possible in the non-diluted plates where colonies were originally picked. Inoculation of single colonies thus biased the selection of specimens able to feed only from nutrients present in the synthetic medium, whereas species most adapted to cooperative growth certainly represented a significant part of the isolates lost during the screening procedure.

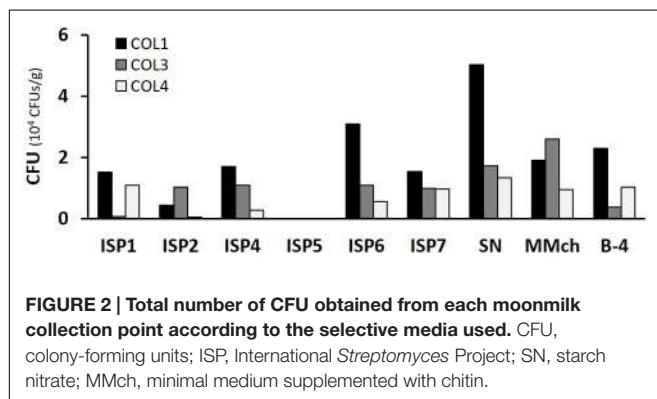


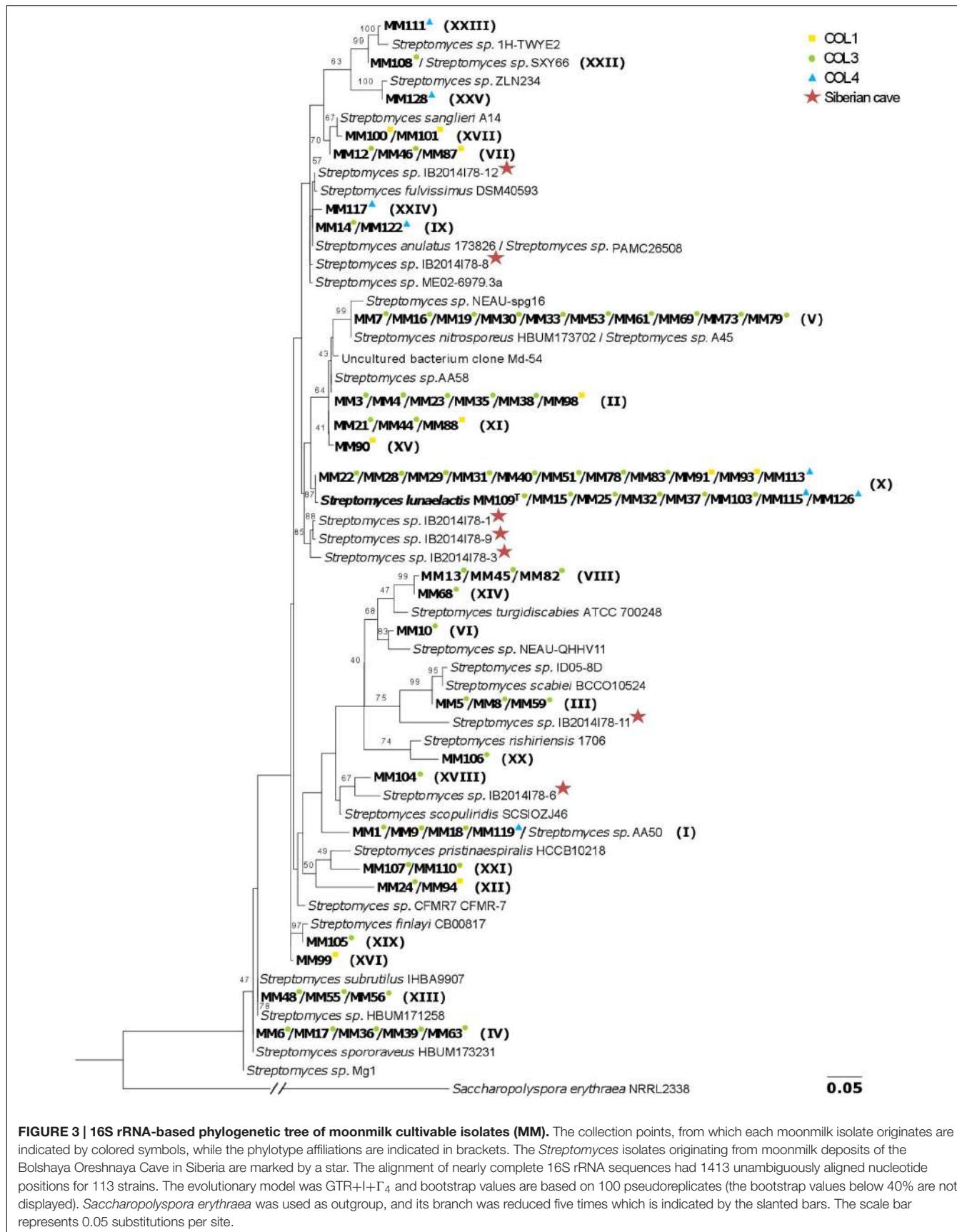
FIGURE 2 | Total number of CFU obtained from each moonmilk collection point according to the selective media used. CFU, colony-forming units; ISP, International Streptomyces Project; SN, starch nitrate; MMch, minimal medium supplemented with chitin.

Phylogeny of Cultivable Moonmilk-Derived *Streptomyces* Isolates

When cultivated in liquid cultures, all the isolates formed filamentous pellets, suggesting their affiliation to the genus *Streptomyces* or to other filamentous Actinobacteria (data not shown). BLAST search using either full or nearly full-length 16S rRNA gene sequences indeed revealed that the closest hits to each of the moonmilk-derived isolate were *Streptomyces* species displaying between 98.3 and 100% identity (**Table 1**; Supplementary Table S3). Cultivable members of this genus have been previously reported from moonmilk (Cañaverales et al., 1999; Axenov-Gribanov et al., 2016), however isolates belonging to other genera, such as *Amycolatopsis*, *Saccharothrix*, *Acinetobacter*, *Chryseomonas* (Cañaverales et al., 1999), *Arthrobacter* (Rooney et al., 2010), *Pseudonocardia*, *Propionibacterium* (Portillo and Gonzalez, 2011), and *Nocardia* (Axenov-Gribanov et al., 2016), have been found through both culture-dependent and independent approaches. It was therefore unexpected that only *Streptomyces* were isolated across a wide range of Actinobacteria-specific media. One reason for such biased recovery might be that *Streptomyces* are saprophytes accustomed to use a large variety of nutrient sources, and are thus better adapted to grow on the synthetic rich media used in our screening procedure. Secondly, among actinobacterial populations, *Streptomyces* are known for their more rapid growth in comparison to other genera, recognized as rare Actinobacteria, which are isolated much less frequently (Subramani and Aalbersberg, 2013). Therefore, in our *in vitro* culture conditions, *Streptomyces* have probably overtaken nutrients and space over other, slower growing, actinobacterial representatives.

Based on the 16S rRNA tree topology, we grouped the 78 moonmilk isolates into 25 phylogenetic clusters (**Figure 3**; **Table 1**; Supplementary Table S3). The largest cluster on the tree (**Figure 3**, phylotype X), delineating the recently described *Streptomyces lunaelactis* species, grouped six novel isolates in addition to the 13 strains reported previously (Maciejewska et al., 2015), which collectively originate from each of the studied sampling site. The clustering of isolates from multiple sampling sites in a single branch was also observed for six other phylotypes, namely phylotypes I, II, VII, IX, XI, and XII, which suggests a widespread occurrence of these isolates within the moonmilk deposits of the cave.

Among the closest environmental *Streptomyces* species deduced from the 16S rRNA BLAST searches, 36 (46%) have been isolated from soils, 12 (15%) from plants, while relatives associated with water ecosystems, lichens, and animals constituted 2 (3%), 2 (3%), and 1 (1%), respectively (**Table 1**; Supplementary Table S3). Finally, 19 isolates (24%) were found to be affiliated to the recently described moonmilk species, *S. lunaelactis* (99.9 or 100% 16S rRNA identity), with isolate MM109^T selected as the type strain (Maciejewska et al., 2015). The closest known relative of *S. lunaelactis* found through full-length 16S rRNA gene search, *Streptomyces globisporus*, further increases the number of relatives originating from soils to 55 (71%). The high sequence identity of 16S rRNA genes



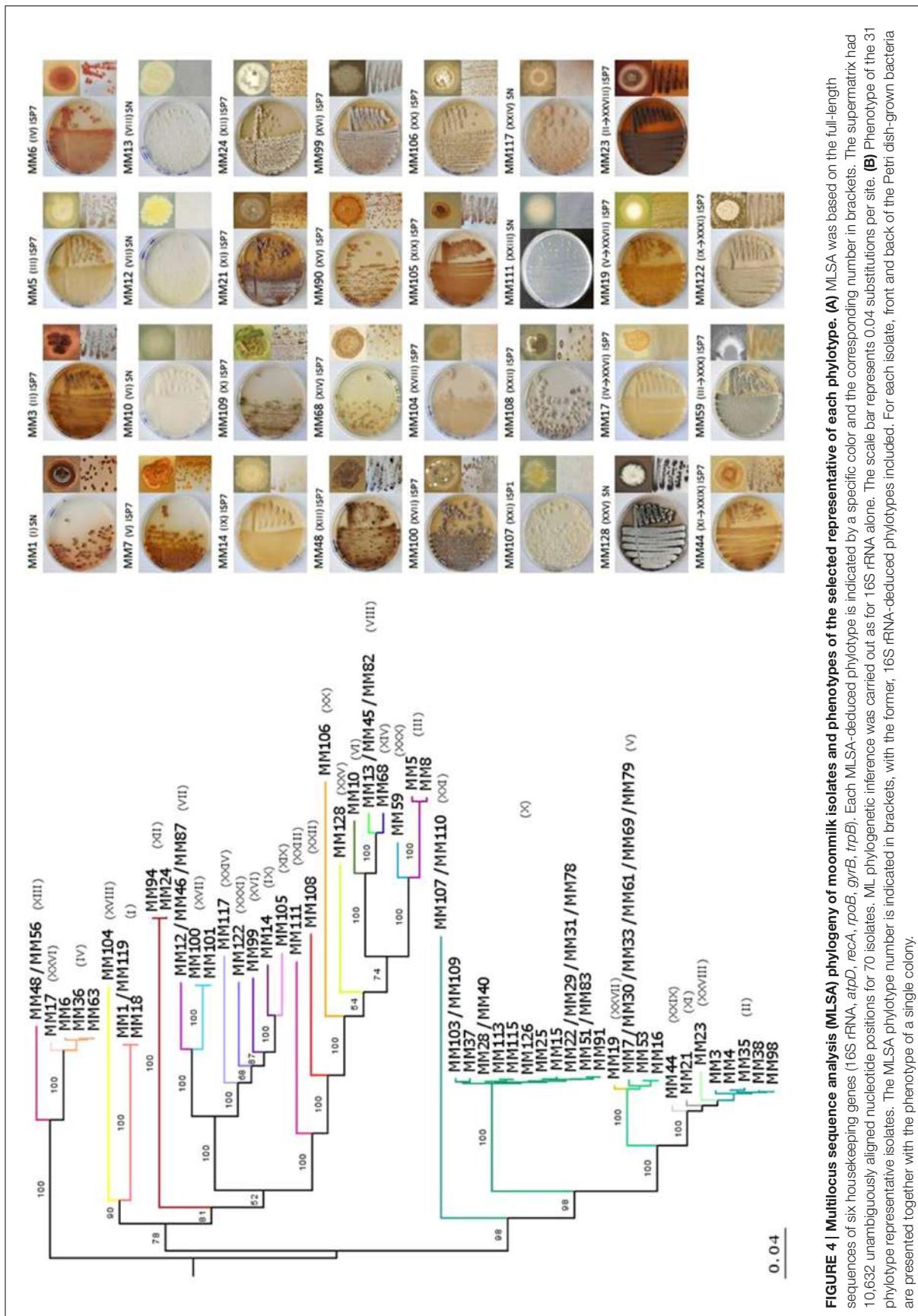


FIGURE 4 | Multilocus sequence analysis (MLSA) phylogeny of moonmilk isolates and phenotypes of the selected representative of each phylotype. (A) MLSA was based on the full-length sequences of six housekeeping genes (16S rRNA, *atpD*, *recA*, *rpoB*, *gyrB*, *trpB*). Each MLSA-deduced phylotype is indicated by a specific color and the corresponding number in brackets. The supermatrix 10/632 unambiguously aligned nucleotide positions for 70 isolates. ML phylogenetic inference was carried out as for 16S rRNA alone. The scale bar represents 0.04 substitutions per site. (B) Phenotype of phylotype representative isolates. The MLSA phylotype number is indicated in brackets, with the former, 16S rRNA-deduced phylotypes included. For each isolate, front and back of the Petri dish-grown bacteria are presented together with the phenotype of a single colony.

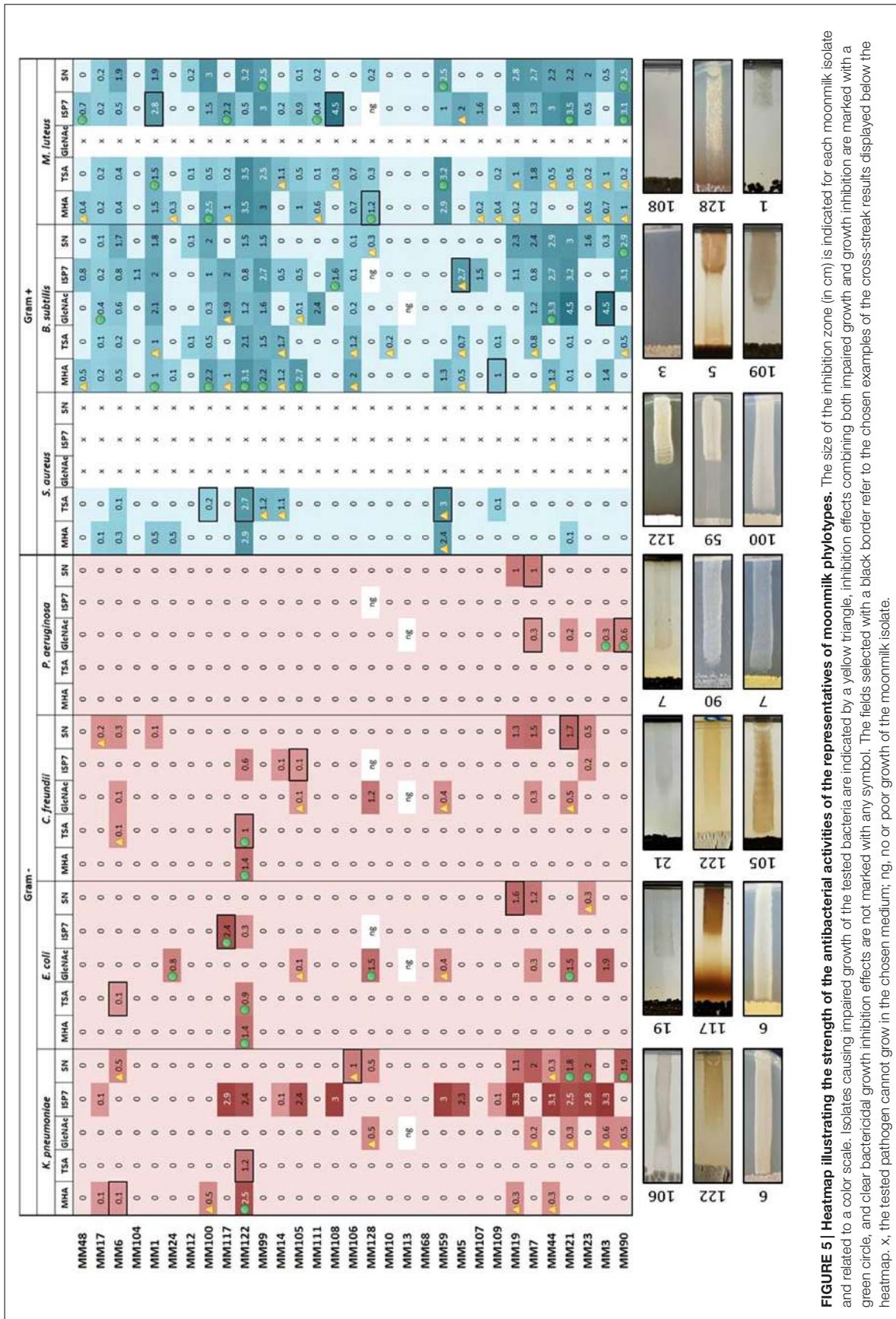


FIGURE 5 | Heatmap illustrating the strength of the antibacterial activities of the representatives of the moonmilk phyotypes. The size of the inhibition zone (in cm) is indicated for each moonmilk isolate and related to a color scale. Isolates causing impaired growth of the tested bacteria are indicated by a yellow triangle; inhibition effects combining both impaired growth and growth inhibition are marked with a green circle, and clear bactericidal growth inhibition effects are not marked with any symbol. The fields selected with a black border refer to the chosen examples of the cross-streak results displayed below the heatmap. X, the tested pathogen cannot grow in the chosen medium; ng, no or poor growth of the moonmilk isolate.

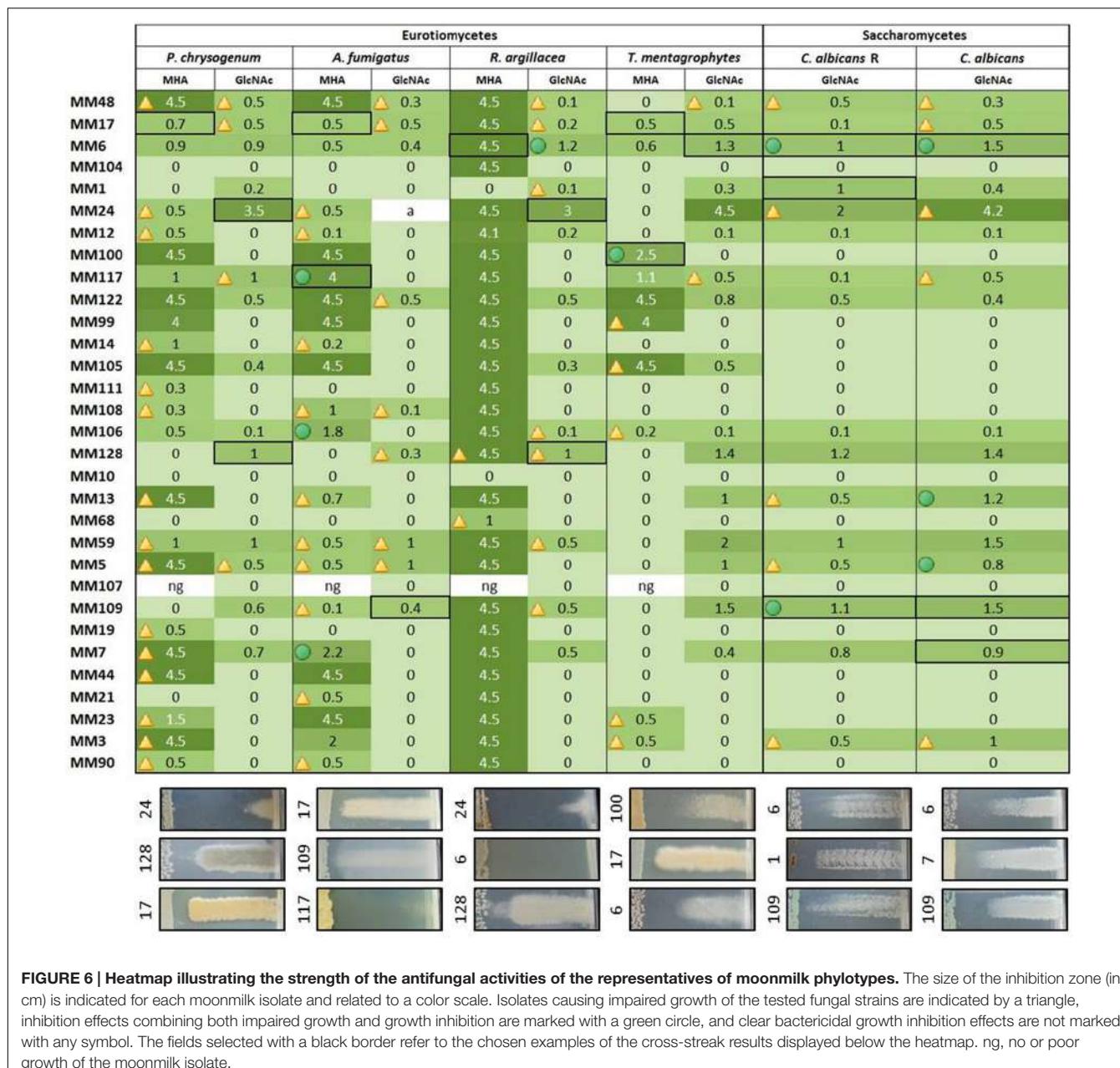


FIGURE 6 | Heatmap illustrating the strength of the antifungal activities of the representatives of moonmilk phylotypes. The size of the inhibition zone (in cm) is indicated for each moonmilk isolate and related to a color scale. Isolates causing impaired growth of the tested fungal strains are indicated by a triangle, inhibition effects combining both impaired growth and growth inhibition are marked with a green circle, and clear bactericidal growth inhibition effects are not marked with any symbol. The fields selected with a black border refer to the chosen examples of the cross-streak results displayed below the heatmap. ng, no or poor growth of the moonmilk isolate.

of moonmilk isolates to species from soil, plant, and water environments suggests that the moonmilk may have been seeded with Actinobacterial species brought into the cave from the surface by dripping water (Laiz et al., 1999; Legatzki et al., 2012; Yun et al., 2015). It should be noted that some of our phylotypes are phylogenetically closely related to *Streptomyces* isolates recently identified from moonmilk deposits of the Bolshaya Oreshnaya Cave in Siberia (Axenov-Gribanov et al., 2016). This is particularly the case for phylotypes IX, X, XVIII, and XXIV (Figure 3), however the comparison is restricted by the small size of the collection generated from the Siberian cave (limited to seven *Streptomyces* isolates). Nonetheless, this observation supports the idea that we may have identified at

least some bacterial species specifically associated with moonmilk speleothems.

As 16S rRNA-based phylogeny is known to be insufficient for discriminating between closely related *Streptomyces* species (Guo et al., 2008; Labeda, 2011; Rong and Huang, 2012) and, consequently, could underestimate the true diversity of our collection, a MLSA was additionally performed. Instead of partial gene fragments used previously (Maciejewska et al., 2015), we have retrieved nearly full-length sequences of the selected housekeeping genes (*atpD*, *rpoB*, *trpB*, *gyrB*, and *recA*) from the genomes of 70 out of 78 isolates. As expected, the phylogenetic resolution of the MLSA tree, based on a supermatrix of 10,632 unambiguously aligned nucleotides

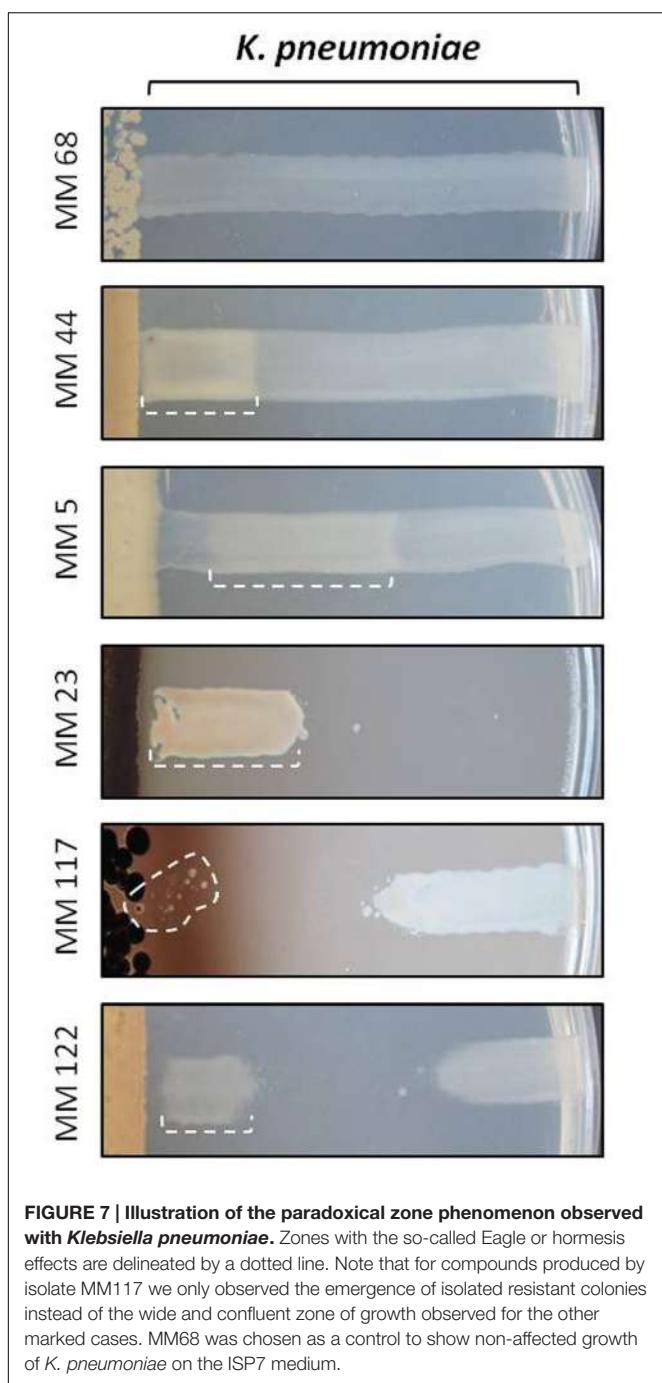


FIGURE 7 | Illustration of the paradoxical zone phenomenon observed with *Klebsiella pneumoniae*. Zones with the so-called Eagle or hormesis effects are delineated by a dotted line. Note that for compounds produced by isolate MM117 we only observed the emergence of isolated resistant colonies instead of the wide and confluent zone of growth observed for the other marked cases. MM68 was chosen as a control to show non-affected growth of *K. pneumoniae* on the ISP7 medium.

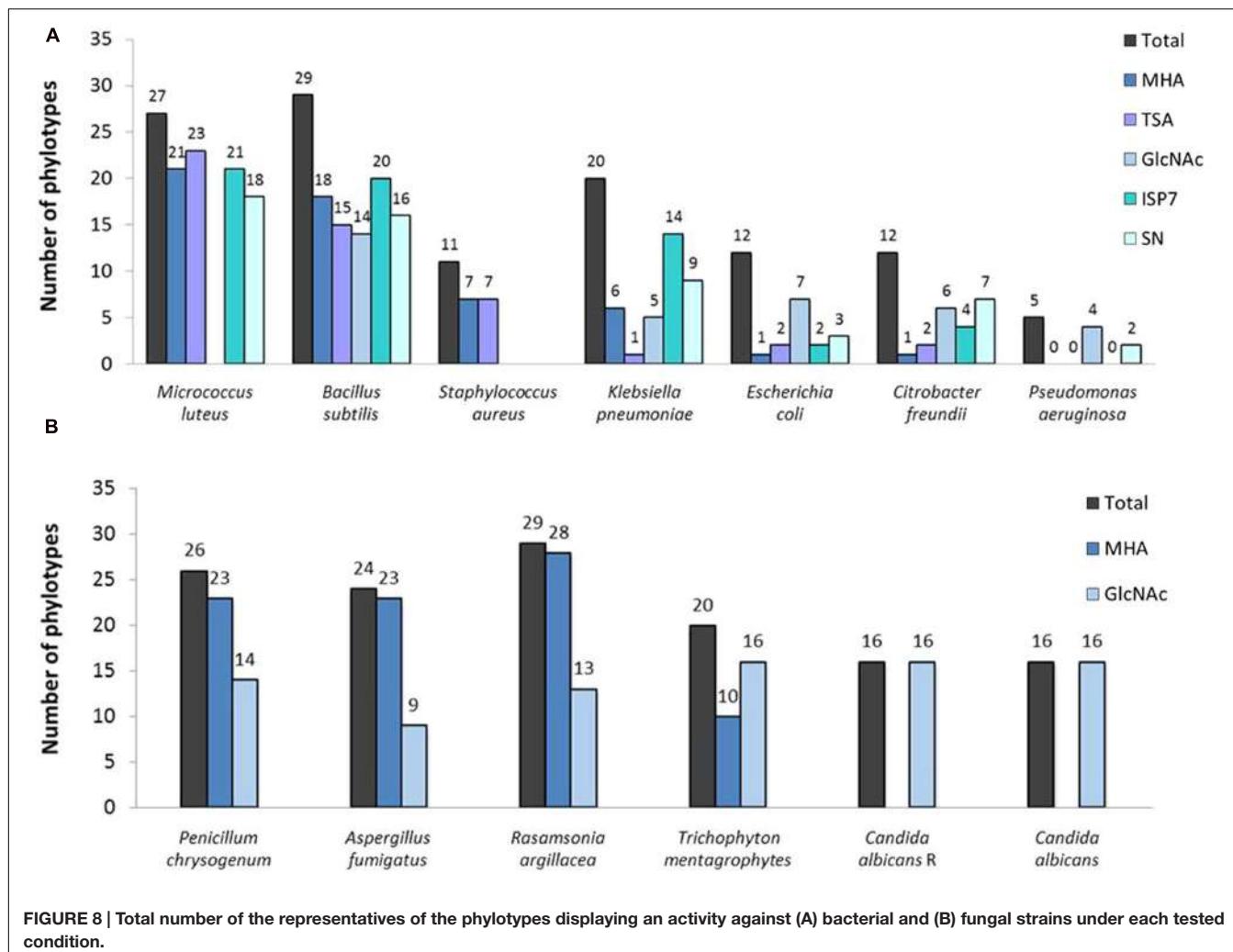
(including 16S rRNA), was much higher. Hence, some strains previously considered to belong to a single phylotype were separated into several sub-clusters (**Figure 4A**). To clearly delineate the phylotypes, the comparison of the pairwise patristic distances between isolates was taken into account together with the tree topology (Supplementary Figure S1; **Figure 4A**). The highest pairwise distance among *S. lunaelactis* strains (0.0096) was considered as the threshold for phylotype assignment since most of these isolates were previously reported to belong to a single species (Maciejewska et al.,

2015). Consequently, the number of phylogenetic clusters increased from 25, inferred from the 16S rRNA tree, to 31 based on MLSA. The new phylotypes differentiated through MLSA were represented by MM17 (IV→XXVI), MM19 (V→XXVII), MM23 (II→XXVIII), MM44 (XI→XXIX), MM59 (III→XXX), and MM122 (IX→XXXI) (**Table 1**). To assess whether the distinction of new phylotypes was supported by phenotypic traits, we compared pigment production and colony morphologies between representative isolates of MLSA phylotypes and representative isolates of their former 16S rRNA-based phylotypes. For instance, the separation of isolate MM17 (now phylotype XXVI) from isolates of phylotype IV (represented by MM6) was indeed additionally supported by the phenotype analysis, as unlike MM6 isolate, which produces a dense pink-reddish intracellular pigment, isolate MM17 remains unpigmented when grown on ISP7 (**Figure 4B**). Dissimilar phenotypes were also observed between representative isolates that formerly belonged to the same phylotype, i.e., MM7 and MM19, MM23 and MM3, MM44 and MM21, MM59 and MM5, and MM122 and MM14 (**Figure 4B**).

Antimicrobial Activity Screening

The potential for antimicrobial activity of the moonmilk-derived cultivable isolates was assessed for one representative of each of the 31 phylotypes deduced from the MLSA (see heatmaps in **Figures 5** and **6** for antibacterial and antifungal activities, respectively). Two categories of antimicrobial activities were recorded, i.e., (i) those that fully inhibited the growth (GI, growth inhibition) of the tested reference strains (see **Figure 5** MM122 against *S. aureus* as an example), and (ii) those that allowed only partial growth (IG, impaired growth, see **Figure 5** MM5 against *B. subtilis* as an example).

The screening for antibacterial properties was carried out under five different culture conditions, i.e., the two complex and nutrient rich media (MHA and TSA), the ISP7 and SN media from which most of our strains were isolated (see **Table 1**; Supplementary Table S3), and the minimal medium supplemented with GlcNAc, which is a known elicitor of antibiotics under poor culture conditions (Rigali et al., 2008; Świątek et al., 2012; Zhu et al., 2014). Results from the antibacterial cross-streak are presented in the heatmap of **Figure 5** (with particular cases highlighted in **Figure 7**), and summarized in **Figures 8A** and **9**. Overall, the moonmilk isolates expectedly displayed a much stronger antibacterial activity (GI and IG) against Gram-positive bacteria (94% of the phylotypes) than against Gram-negative bacteria (71% of the phylotypes). Indeed, 94% of the tested isolates were active against *B. subtilis*, 87% against *M. luteus*, and 36% against *S. aureus*, while 65% were found to show activity against *K. pneumoniae*, 39% against *E. coli*, 39% against *C. freundii* and only 16% against *P. aeruginosa* (**Figures 5** and **8A**). The most active isolate in terms of both strength and spectrum of targeted pathogens was MM122 (**Figures 5** and **9**). Interestingly, a group of four isolates (MM3, MM7, MM21, and MM90) similarly



triggered the production of compounds active against Gram-negative bacteria, particularly *P. aeruginosa* and *K. pneumoniae*, in the presence of GlcNAc (Figure 5). All these isolates branch together in the 16S rRNA and the MLSA-deduced phylogenetic trees (Figures 3 and 4A), which suggests that their similar response to the GlcNAc eliciting molecule should be induced by the same signaling pathway and involves similar or identical bioactive compounds. Those isolates together with other closely related moonmilk strains, namely MM19, MM44, and MM23, showed one of the strongest inhibitory profiles against a broad spectrum of pathogens and under most of the tested culture conditions. This suggests that they possibly might share a similar set of antibacterial secondary metabolite clusters. In contrast, MM10, MM13, and MM68, all representatives of closely related phylotypes, did not reveal any antibacterial activity; however, genome mining showed that those three isolates are predicted to possess in total 16, 19, and 13 biosynthetic cluster-associated genes (including NRPS, all types of PKS), respectively (Figure 9). This suggests that the absence of antibiotic activity would be rather caused by inappropriate culture conditions for eliciting the production

of cryptic antibiotics, rather than a lack of genetic material associated with secondary metabolism.

Another remarkable example evidencing that the antibiotic production highly depends on the culture medium composition is illustrated by the isolate MM108, which did not display any antibacterial activity unless grown on the ISP7 medium, where it induced a strong GI effect against *K. pneumoniae*, *B. subtilis*, and *M. luteus* (Figure 5).

Strikingly, the ISP7 medium also stimulated unusual growth phenotypes by *K. pneumoniae* to compounds produced by our moonmilk isolates. All different atypical cases observed for this pathogen are presented in Figure 7. For instance, beyond the classical GI and the IG effects (see Figure 5), we observed that *K. pneumoniae* would grow or be only slightly inhibited near the antimicrobial producing strain, whereas its growth was fully or partially inhibited (for example MM23 in Figure 7) or impaired (for example MM5 in Figure 7) at a higher distance. This non-linear response to a diffusion gradient of the secreted antibiotics has been described as the Eagle effect (Eagle and Musselman, 1948). According to this phenomenon, the activity of antimicrobial compounds is paradoxically higher

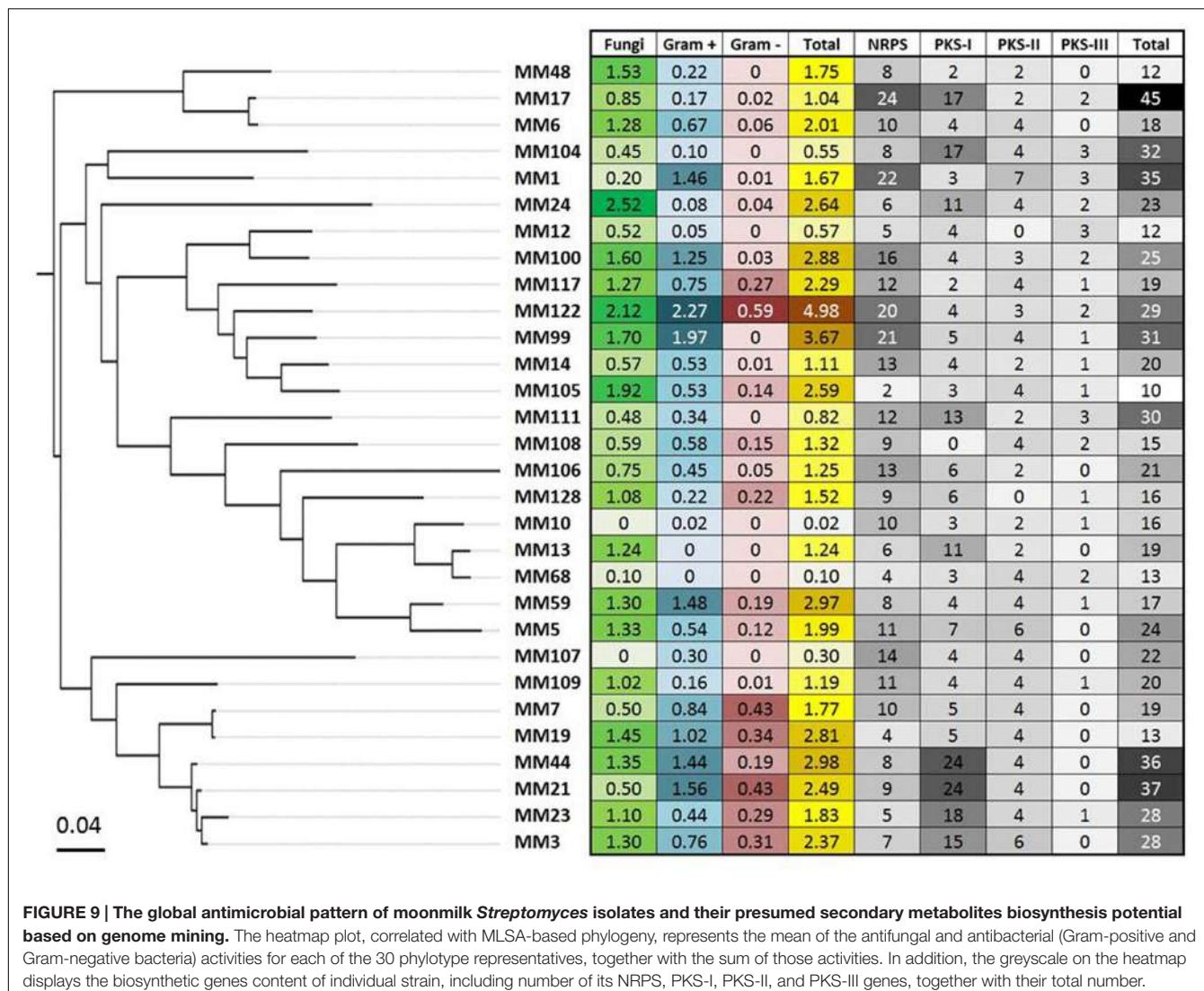


FIGURE 9 | The global antimicrobial pattern of moonmilk *Streptomyces* isolates and their presumed secondary metabolites biosynthesis potential based on genome mining. The heatmap plot, correlated with MLSA-based phylogeny, represents the mean of the antifungal and antibacterial (Gram-positive and Gram-negative bacteria) activities for each of the 30 phylotype representatives, together with the sum of those activities. In addition, the greyscale on the heatmap displays the biosynthetic genes content of individual strain, including number of its NRPS, PKS-I, PKS-II, and PKS-III genes, together with their total number.

at lower concentrations, whereas high concentrations allow growth within the vicinity of *Streptomyces*. The observed growth close to the antimicrobial-producing strain might be due to the onset of the resistance mechanisms, which were reported to be rapidly induced by some antibiotics in *K. pneumoniae* (Zhong et al., 2013; Adams-Sapper et al., 2015). Indeed, several resistant *K. pneumoniae* colonies were detected in the proximity to the moonmilk isolate MM117 (Figure 7). Another possible explanation might be that the high production of antimicrobials by certain moonmilk isolates on the ISP7 medium could lead to precipitation and consequently inactivation of the antibacterial agents active against *K. pneumoniae*. Finally, the observed atypical response might be explained by a certain form of the hormesis phenomenon, which states that ‘the dose makes the poison’ (Zhanel et al., 1992; Linares et al., 2006; Allen et al., 2010). Consequently, the produced molecules would effectively suppress the growth of the pathogen, but only at the appropriate concentration ranges (Migliore et al., 2013).

Our collection of cultivable moonmilk *Streptomyces* strains was further assessed for the production of compounds with antifungal activities (Figure 6). The corresponding screening was carried out under two different culture conditions, i.e., (i) the rich medium MHA and (ii) the minimal medium supplemented with GlcNAc. The latter medium was again selected as GlcNAc, being the monosaccharide composing chitin, exerts a strong carbon catabolite repression on the induction of the chitinolytic system in *Streptomyces* species (Saito et al., 2007; Colson et al., 2008). This prevents ‘false-positive’ growth inhibition of tested fungi due to enzymatic hydrolysis of their chitin-based cell wall. The results of antifungal screening are presented in Figure 6 and summarized in Figures 8B and 9. Overall, the antifungal activities were stronger when the *Streptomyces* strains were grown on the rich MHA medium (Figures 6 and 8B). Globally, 94% of the tested isolates showed inhibitory activities against fungal strains, with 84% being specifically active against *P. chrysogenum*, 77% against *A. fumigatus*, 94% against *R. argillacea*, 65% against *T. mentagrophytes*, and 52% active against both *C. albicans* and its

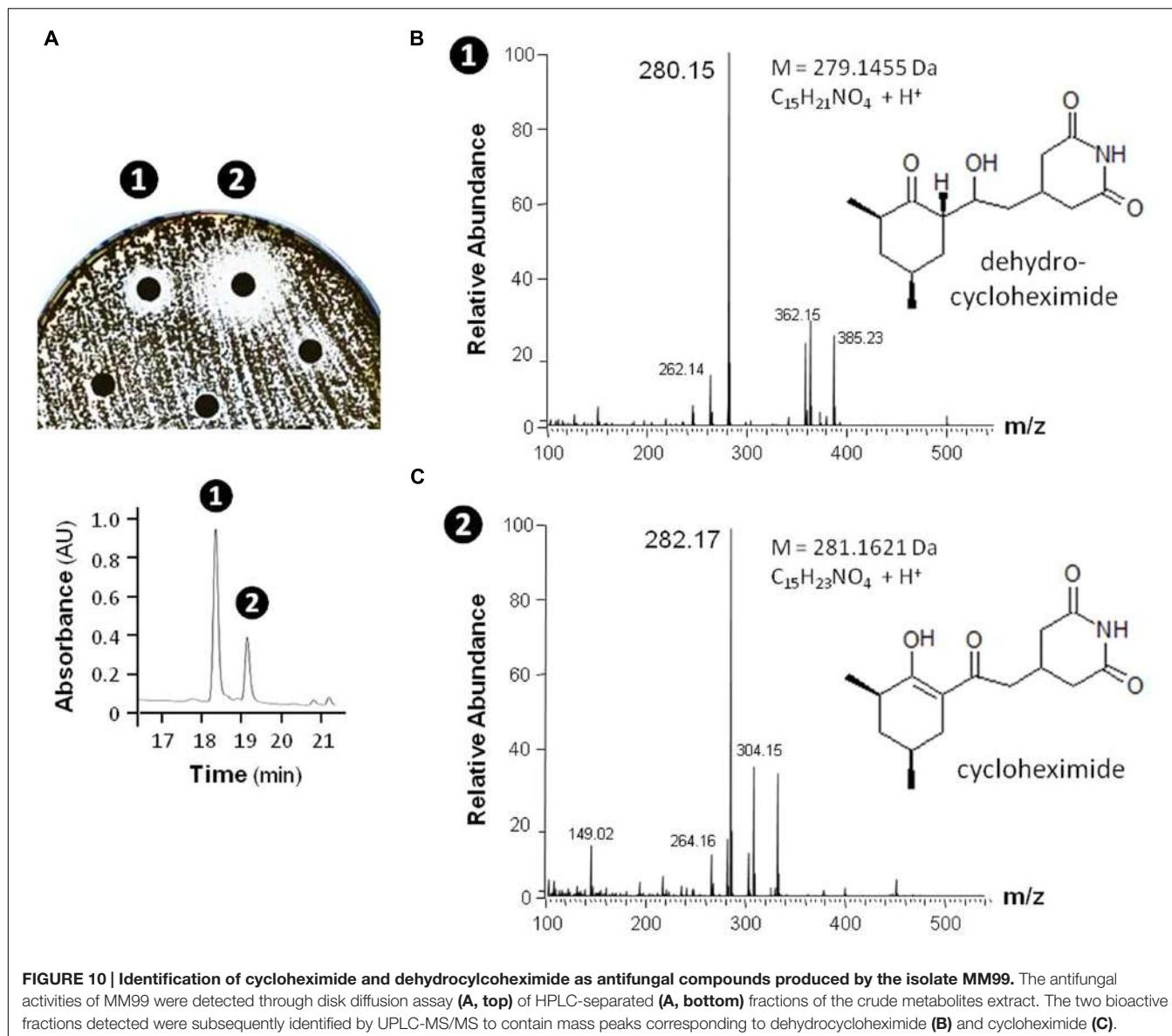


FIGURE 10 | Identification of cycloheximide and dehydrocycloheximide as antifungal compounds produced by the isolate MM99. The antifungal activities of MM99 were detected through disk diffusion assay (**A, top**) of HPLC-separated (**A, bottom**) fractions of the crude metabolites extract. The two bioactive fractions detected were subsequently identified by UPLC-MS/MS to contain mass peaks corresponding to dehydrocycloheximide (**B**) and cycloheximide (**C**).

drug-resistant strain, *C. albicans* 'R' (**Figures 6 and 8B**). The most bioactive isolate in terms of the strength and the number of inhibited fungal strains was found to be MM24, while MM10 and MM107 did not show any suppression under conditions tested (**Figure 6**). The latter two isolates, together with MM68, generally represented the least prolific antimicrobial producers (**Figure 9**). The correlation between phylogenetic inference and detected activities was significant for moonmilk phylotypes MM6 and MM17, as well as MM5 and MM59 (**Figure 6**). Remarkably, most of our isolates displayed very strong growth inhibitory properties against the clinically relevant filamentous fungi – *R. argillacea*. This mold, belonging to the so-called *R. argillacea* species complex, is a causative agent of chronic infections of cystic fibrosis (CF) and chronic granulomatous disease (CGD) patients, displaying tolerance to various antifungals (Giraud et al., 2013). That almost all our isolates display high GI activity against

R. argillacea suggests the involvement of one or more molecules commonly produced by cultivable moonmilk streptomycetes. The identification of active compound(s) is currently under investigation.

In order to provide a first evidence that the observed antimicrobial activities are indeed caused by the production of small inhibitory molecules, and do not result from other factors, such as enzymatic degradation, a single isolate – MM99 – was selected to characterize the compound(s) responsible for its antifungal response. For this purpose, MM99 was cultivated on GYM media, which triggered its antifungal activity, with the media collected for metabolite extraction and separation by HPLC. The two most active HPLC fractions were collected (**Figure 10A**), and subjected to UPLC-ESI-MS/MS analysis, which identified two masses (281.1626 and 279.1470 Da). These two peaks corresponded to cycloheximide and its

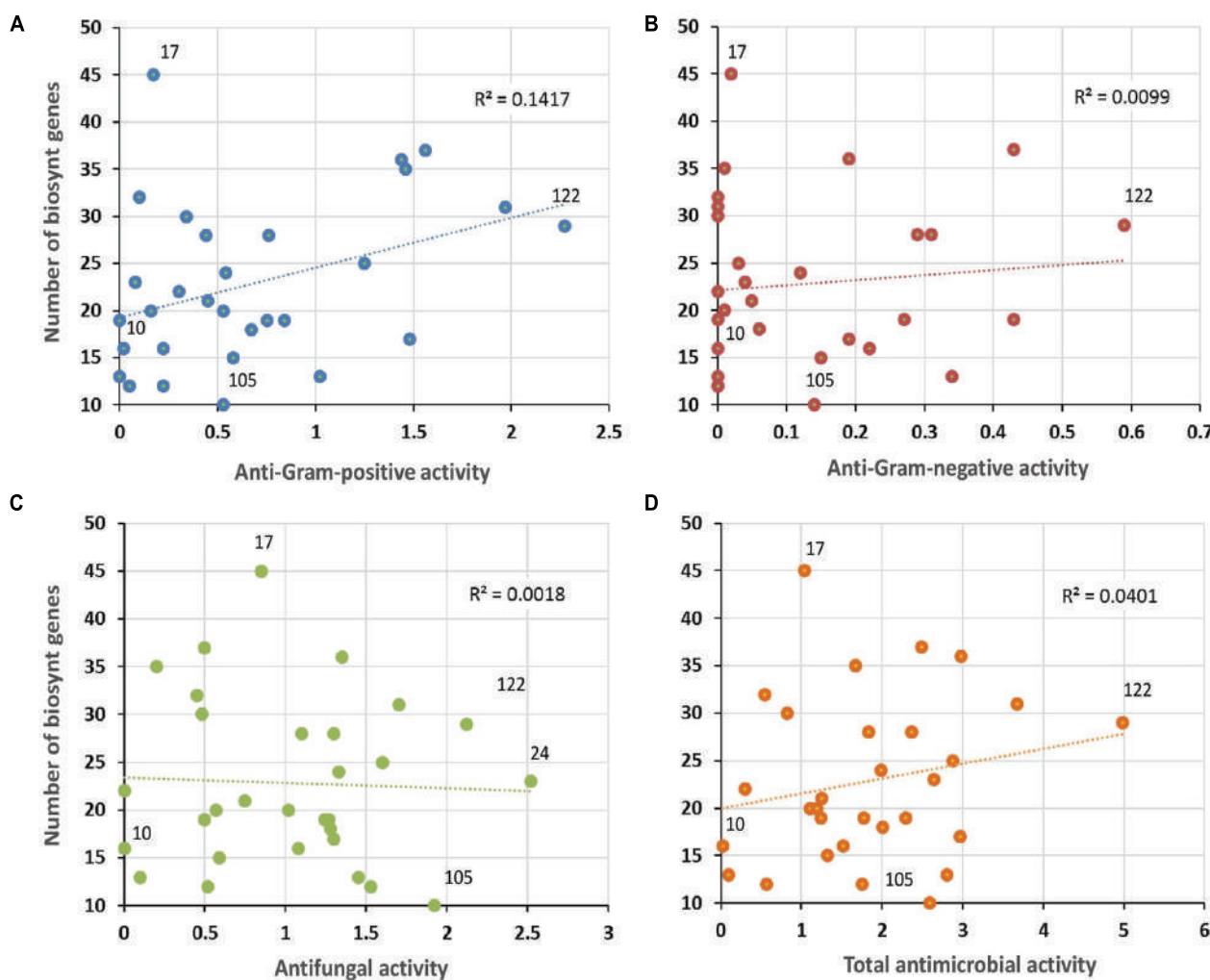


FIGURE 11 | Correlation between antimicrobial activities and predicted secondary metabolite biosynthetic genes. The vertical axis refers to the sum of PKS-I, PKS-II, PKS-III, and NRPS biosynthetic genes identified in each phylotype representative by the antiSMASH software. Horizontal axes represent the mean of the antibacterial activity (against Gram-positive bacteria **(A)**, or Gram-negative bacteria **(B)**, the antifungal activity **(C)**, and the total antimicrobial activity **(D)** of each phylotype representative. Note the non-significant R-squared (R^2) values for each plot, which reflect weak correlation between antimicrobial activities and the genetic potential of each isolate to produce secondary metabolites. The isolates that displayed the lowest (MM10) and the highest antibacterial (MM122) and antifungal (MM24) activities, and the smallest (MM105) and the largest (MM17) numbers of secondary metabolite biosynthetic genes, are highlighted in the plots.

precursor, dehydrocycloheximide, respectively (Figures 10B,C). Cycloheximide is a known inhibitor of eukaryotic protein synthesis, which was first isolated from *Streptomyces griseus* and previously known as actidione (Whiffen et al., 1946; Leach et al., 1947). To additionally confirm that our newly isolated antifungal compound was indeed cycloheximide, we compared the MS/MS fragments profile between the compound isolated from MM99 (Figure 10C) and a cycloheximide standard. Identical fragmentation patterns of both samples (Supplementary Figure S2), ultimately confirmed that cycloheximide was responsible – at least in part – for the antifungal activity of the isolate MM99. The identification of this single molecule is representative of ongoing efforts that aim to exhaustively identify the bioactive molecules of cultivable *Streptomyces* from moonmilk deposits.

Genome Mining

Complementary to *in vitro* antimicrobial screenings, we have carried out in parallel a large scale genome mining investigation. For this purpose, the draft genomic assemblies were examined for the presence of biosynthetic genes encoding polyketide synthases – PKSs (including PKS-I, PKS-II, and PKS-III), as well as NRPSs, which are collectively responsible for production of bioactive molecules in Actinobacteria. Notably, 100% of evaluated strains encoded at least two NRPS genes, 97% were found to possess PKS-I, 94% PKS-II, and 48% PKS-III genes (Figure 9). This clearly indicates a significant predisposition of moonmilk isolates to secrete bioactive secondary metabolites. The isolate MM17 was found to carry the highest total number of biosynthetic genes (45), while MM105 harbored only 10 of them, being classified as the weakest potential antimicrobial

producer (**Figure 9**). Comparison of the biosynthetic genes content and the antimicrobial activities of each individual phylotype did not show significant correlation, with only small relationship observed for anti-Gram positive test (**Figure 11**). Remarkably, MM17 although encoding the highest amount of secondary metabolites genes displayed one of the weakest antimicrobial activities (**Figure 11**). This observation clearly highlights the urgency for finding appropriate culture conditions and triggers which would awaken silent metabolite clusters.

CONCLUSION

In this work we have isolated 78 cultivable *Streptomyces* strains from three moonmilk deposits collected in the Grotte des Collemboles, Comblain-au-Pont (Belgium), and assessed their capacity to secrete compounds with antibacterial and antifungal activities. Surprisingly, the isolated strains were found to exclusively belong to the single *Streptomyces* genus, despite other cultivable-dependent and -independent approaches revealed the presence of many other Actinobacteria in moonmilk speleothems (Cañaverales et al., 1999; Rooney et al., 2010; Portillo and Gonzalez, 2011; Axenov-Gribanov et al., 2016). Hence, a more specific protocol for the isolation of rare Actinobacteria is currently tested in order to assess their potential in producing bioactive natural compounds. Phylogenetic analysis revealed the novelty of the selected isolates, and suggests that they might indeed represent indigenous moonmilk populations adapted to life in an inorganic and oligotrophic environment. Overall, antimicrobial screening showed that moonmilk strains were much more active against fungi, than against the bacterial reference strains, which was previously observe for plant-associated actinobacterial isolates (Bascom-Slack et al., 2009; Verma et al., 2009; Zhao et al., 2011). Identification of bioactive compounds is under investigation, particularly molecules active against *R. argillacea*. A complementary, *in silico* genome mining approach additionally revealed a high richness and diversity of secondary metabolite gene clusters, as evidenced by the presence of numerous NRPSs, and PKSs genes. Consequently, an effort has to be made in order to find cues and triggers that would activate the expression of these biosynthetic clusters. Our findings extend the previous data related to broad spectrum

antimicrobial activities reported for cave microbiomes of volcanic caves (Cheeptham et al., 2013; Rule and Cheeptham, 2013), and karstic caves (Yücel and Yamaç, 2010), including moonmilk speleothems (Axenov-Gribanov et al., 2016). They provide additional evidence for subterranean systems to be a promising target for bioprospecting for novel bioactive molecules.

AUTHOR CONTRIBUTIONS

MM, DA, LM, HB, and MoC collected and processed the samples. MM, DA, LM, AN, MaC, NS, M-PH, MH, DB, and SR performed experiments and analyzed the data. MM, AN, MaC, PD, MH, DB, and SR did bioinformatics, phylogeny analyses, and genome mining. All authors participated to the writing and revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01455>

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Broad Spectrum Antimicrobial Activity of Forest-Derived Soil Actinomycete, *Nocardia* sp. PB-52

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A mesophilic actinomycete strain designated as PB-52 was isolated from soil samples of Pobitora Wildlife Sanctuary of Assam, India. Based on phenotypic and molecular characteristics, the strain was identified as *Nocardia* sp. which shares 99.7% sequence similarity with *Nocardia niigatensis* IFM 0330 (NR_112195). The strain is a Gram-positive filamentous bacterium with rugose spore surface which exhibited a wide range of antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), Gram-negative bacteria, and yeasts. Optimization for the growth and antimicrobial activity of the strain PB-52 was carried out in batch culture under shaking condition. The optimum growth and antimicrobial potential of the strain were recorded in GLM medium at 28°C, initial pH 7.4 of the medium and incubation period of 8 days. Based on polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) gene-targeted PCR amplification, the occurrence of both of these biosynthetic pathways was detected which might be involved in the production of antimicrobial compounds in PB-52. Extract of the fermented broth culture of PB-52 was prepared with organic solvent extraction method using ethyl acetate. The ethyl acetate extract of PB-52 (EA-PB-52) showed lowest minimum inhibitory concentration (MIC) against *S. aureus* MTCC 96 (0.975 µg/mL) whereas highest was recorded against *Klebsiella pneumoniae* ATCC 13883 (62.5 µg/mL). Scanning electron microscopy (SEM) revealed that treatment of the test microorganisms with EA-PB-52 destroyed the targeted cells with prominent loss of cell shape and integrity. In order to determine the constituents responsible for its antimicrobial activity, EA-PB-52 was subjected to chemical analysis using gas chromatography-mass spectrometry (GC-MS). GC-MS analysis showed the presence of twelve different chemical constituents in the extract, some of which are reported to possess diverse biological activity. These results confirmed that the presence of bioactive constituents in EA-PB-52 could be a promising source for the development of potent antimicrobial agents effective against wide range of microbial pathogens including MRSA.

Keywords: *Nocardia* sp., antimicrobial activity, microbial pathogens, biosynthetic genes, culturing conditions, SEM, GC-MS

INTRODUCTION

The discovery of antibiotics like penicillin and other antimicrobial agents to treat infectious diseases has revolutionized the field of medicine in the mid-twentieth century. These discoveries have led to the development of improved antibiotics with a hope to serve humanity well (Walsh, 2003). However, due to overuse or misuse of antibiotics over a prolonged period, most of the pathogens have become resistant to the antibiotic therapy. These pathogens have accumulated a large number of resistance elements encoded by genes found both within the genome and plasmids, greatly limiting the therapeutic options (Wright, 2012). Thus, there is an immense need for the discovery and development of new antibiotics to effectively target these deadly pathogens that cause life-threatening infections. Actinomycetes are one of the most efficient groups of natural bioactive metabolite producers such as antibiotics, enzyme inhibitors, immunomodifiers, plant growth promoting substances, and many other compounds useful to mankind (Fiedler et al., 2004; Shivalata and Satyanarayana, 2015). Among actinomycetes, around 75% of commercially useful antibiotics such as ivermectin, tetracycline, streptomycin, nystatin etc. are produced by the dominant genus *Streptomyces* (Miao and Davies, 2010). But, in the past two decades, there has been a decline in the discovery of novel metabolites from *Streptomyces* as culture extracts usually yield disappointingly high number of previously described molecules (Qin et al., 2009; Aouiche et al., 2011). As such, new sources of bioactive metabolites from another group of actinomycetes, known as rare actinomycetes from different ecological niches have promoted recent advances in the discovery of new antibiotic molecules (Lazzarini et al., 2000; Lee et al., 2014; Azman et al., 2015; Nimaichand et al., 2015). The genus *Nocardia*, *Saccharopolyspora*, *Micromonospora*, *Streptosporangium*, *Streptoverticillium*, *Actinoplanes*, and *Actinomadura* are considered as the rare group of actinomycetes. It is because these microbes are difficult to isolate and maintain under conventional conditions (Berdy, 2005). Amongst the rare actinomycetes, numerous interesting biologically active compounds have been reported from the genus *Nocardia* such as nargenicin (Celmer et al., 1980), transvalencin (Hoshino et al., 2004), nocardithiocin (Mukai et al., 2009) etc.

Nocardia is a genus under the family Nocardiaceae of order Corynebacteriales within the class Actinomycetes (Goodfellow et al., 2012). The genus was first proposed by Trevisan (1889) and was named in honor of Edmond Nocard, who in 1888 described the first species (Kageyama et al., 2004a). *Nocardia* is a Gram-positive, aerobic, filamentous branching bacillus that is partially acid fast and ~86 species have been reported in the genus *Nocardia* (Brown-Elliott et al., 2015). It is represented by a list of chemical markers, including the presence of mycolic acids, meso-DAP, galactose, and arabinose and DNA G + C content of 63–72% (Goodfellow, 1992). However, little attention has been paid to *Nocardia* from where we continue our interest to extract biologically active compounds from this group of bacteria. They are prominent for their ability to produce a wide variety of biologically active compounds; however, some are also known to

be opportunistically pathogenic to humans and animals (Chun and Goodfellow, 1995).

The plausibility of finding new bioactive molecules from *Nocardia* could be increased by shifting the search from routinely explored ecological niches to unexplored ones (Manikkam et al., 2014). The poorly explored environments contain highest populations of actinomycetes with valuable antimicrobial secondary metabolites as reported by Ara et al. (2007). Isolation of microorganisms from poorly explored areas of the world like Jordan (Saadoun and Gharaibeh, 2003), Antarctica (Lee et al., 2012), and certain ecological niches of Northeast India (Bordoloi et al., 2001; Debnath et al., 2013) reflects that these habitats should be carefully explored for novel microorganisms and their valuable bioactive products. Northeast India is a part of the Indo-Burma mega-biodiversity hotspot (Myers et al., 2000). The influence of the local environment in the biodiversity hotspots might result in the evolution of novel secondary metabolic pathways in organisms (Glover, 1995). Pobitora Wildlife Sanctuary of Assam is largely an unscreened forest ecosystem and thus an unexplored source of actinomycetes and biologically active secondary metabolites. Many actinomycetes from forest ecosystems are known to produce polyketides and non-ribosomal peptides by type-I and type-II polyketide pathways and non-ribosomal peptide synthase pathways which are the hallmark of secondary metabolites production in this group of bacteria (Passari et al., 2015).

Our continuous screening for new bioactive metabolites from rare actinomycetes resulted in the isolation of a promising *Nocardia* strain designated as PB-52 from the soil samples of Pobitora Wildlife Sanctuary of Assam, India. This sanctuary (38.8 sq km) lies in the sub-tropical zone and is situated in the flood plains of river Brahmaputra in Assam, India. In this work, we aimed to investigate the antimicrobial biosynthetic potential of PB-52 strain against a wide range of microbial pathogens. Optimization of different culture conditions like fermentation media, temperature, pH and period of incubation was performed to facilitate improved growth and antimicrobial activity of the strain. To evaluate the antimicrobial potency of ethyl acetate extract of PB-52 (EA-PB-52), various techniques like MIC determination against microbial pathogens, rate kill assay, interaction with the test microbial pathogens by SEM analysis was performed. The chemical constituents present in EA-PB-52 were further identified using GC-MS. The outcome of this research lays the foundation for performing in-depth studies focusing on the development of potent antimicrobial agents effective against a broad range of disease-causing microbial pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans*.

MATERIALS AND METHODS

Sampling Site and Sample Collection

Soil samples were collected in the month of April, 2013 from varied locations of Pobitora Wildlife Sanctuary ($26^{\circ}12'$ to $26^{\circ}16'$ N and $91^{\circ}58'$ to $92^{\circ}05'$ E) of Assam, India. The Sanctuary experiences semi-dry hot climate in summer (37 – 39°C) and cold in winter (6 – 7°C) with an average humidity of 75% and annual

rainfall of 1500–2600 mm. The habitat comprises of alluvial grassland with hilly forests. Soil samples each weighing ~50 g was collected randomly from 5 to 20 cm depth after removing the upper surface layer of the top soil. The samples were transferred aseptically in sterile zip-lock bags and transported to the laboratory on the same day.

Isolation of the Actinomycete Strain

During the screening of actinomycetes from soil samples, PB-52 strain was isolated by serial dilution technique in actinomycetes isolation agar medium (Himedia, India) amended with rifampicin (2.5 µg/mL) and amphotericin B (75 µg/mL) after incubation at 28°C for 7–10 days (Thakur et al., 2007). The pure culture of the isolate was sub-cultured on GLM agar medium (Yeast extract, 3 g; malt extract, 3 g; peptone Type I, 5 g; starch, 10 g; agar, 20 g; distilled water, 1000 mL; pH 7.4) and preserved in 15% glycerol at –80°C for future use.

Identification and Characterization of the Actinomycete Strain

Cultural Characteristics

The cultural characteristic of PB-52 strain was examined by growing the strain in different culture media. Micromorphology of the strain was examined by using cover slip insertion method (Williams et al., 1989) on GLM medium. The morphology and ornamentation of the spore chain was observed by scanning electron microscopy (Kumar et al., 2011). Utilization of carbon and nitrogen was determined by growth of the strain on ISP Medium No. 9 supplemented with 1% carbon and 1% nitrogen sources respectively at 28°C. Temperature range (15–45°C), pH range (4–11) and NaCl tolerance for growth (1–5% NaCl, w/v) was determined on ISP Medium No. 4 (Shirling and Gottlieb, 1966). Hydrolysis of starch, cellulose, casein, tween 20, tween 80, liquefaction of gelatin, nitrate reduction and other biochemical tests were assessed by following the methods of Gordon et al. (1974). Whole-cell hydrolysates were determined according to Lechevalier and Lechevalier (1970). Sensitivity and resistance of PB-52 strain to nineteen standard antibiotics were detected by disc diffusion method (Kumar V. et al., 2014a).

Molecular Identification

DNA Extraction

Genomic DNA was isolated according to Sambrook and Russell (2001) with slight modifications. For isolation of genomic DNA, PB-52 strain was grown in GLM broth on a rotary shaker (150 rpm) at 28°C, pH 7.4 for 4 days. The cells were harvested by centrifugation (8000 rpm, 5 min), washed two times with sterile water and suspended in 800 µL lysis buffer (100 mM Tris-HCl, 20 mM EDTA, 250 mM NaCl, 2% SDS and 1 mg/mL lysozyme). 2 µL RNase was added and the sample was incubated at 37°C for 3 h. 2 µL proteinase K was added to the sample and incubated at 65°C for 30 min. 800 µL phenol, chloroform (1:1) was added and centrifuged (12,000 rpm, 5 min). The upper aqueous layer was collected, mixed with equal volume of chloroform, isoamyl alcohol (24:1) and centrifuged (12,000 rpm, 5 min). Again, the upper layer was collected, mixed with 0.1 volume sodium acetate along with 2 volume 96% ethanol and incubated at –20°C

for 1 h and centrifuged (12,000 rpm, 15 min). The pellet was washed first with 300 µL 70% ethanol and then with 90% ethanol (8000 rpm, 10 min each). The pellet was air dried and suspended in 30 µL TE buffer (pH 7.7). DNA was analyzed by 1% agarose gel electrophoresis.

PCR Amplification

16S rRNA gene was amplified using universal eubacterial primer set, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3'), and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). PCR reactions were performed in Proflex PCR System (Applied Biosystems, USA) in a total volume of 50 µL reaction mixture containing 50 ng template DNA, 1X Taq DNA polymerase buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 U Taq DNA polymerase enzyme and 0.2 µM of each primer. The thermal cycling conditions were programmed as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min and final extension at 72°C for 10 min. The amplified products were determined by 1.8% (w/v) agarose gel electrophoresis. The amplified products were further purified using GenElute PCR Clean-Up Kit (Sigma Aldrich, USA) and the purified PCR products were sequenced by automated DNA sequencer with specific primers using the facility at Xcelris Genomics Lab Ltd. (Ahmedabad, India).

Phylogenetic Analysis

Identification of phylogenetic neighbors and calculation of pairwise sequence similarities of 16S rRNA gene of PB-52 strain were carried out using BLASTN (Altschul et al., 1990) and EzTaxon server (<http://www.eztaxon.org/>; Kim et al., 2012). Top 14 reference sequences with highest scores were selected for multiple sequence alignment which was performed by CLUSTAL W program (Thompson et al., 1997). The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987) using MEGA version 4.0 (Tamura et al., 2007). The support of each clade was determined by bootstrap analysis performed with 1000 replications (Felsenstein, 1985). The GenBank accession number for the partial 16S rRNA gene sequence of PB-52 strain is KM406386.

Evaluation of Antimicrobial Activity of PB-52 Strain

Test Microorganisms

The following test microorganisms were used for the experiment. Gram-positive bacteria: *S. aureus* MTCC 96, *S. aureus* MTCC 3160, MRSA ATCC 43300, *S. epidermidis* MTCC 435, *Bacillus subtilis* MTCC 441, *B. cereus* MTCC 1272, *B. megaterium* MTCC 8075, *Micrococcus luteus* MTCC 1538; Gram-negative bacteria: *Escherichia coli* MTCC 40, *E. coli* MTCC 739, *Serratia marcescens* MTCC 97, *Klebsiella pneumoniae* MTCC 3384, *K. pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* MTCC 741, *P. aeruginosa* MTCC 424, *P. aeruginosa* MTCC 2582, *Proteus vulgaris* MTCC 426; Yeast: *C. albicans* MTCC 227, *C. tropicalis* MTCC 2208 and *C. albicans* ATCC 10231. All the MTCC strains were purchased from Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India and ATCC cultures were purchased from HiMedia, Mumbai. The bacterial

strains were cultured in nutrient agar medium at 37°C and yeasts strains were cultured on Sabouraud dextrose medium at 25°C. The test organisms were preserved at -70°C in glycerol stock vials for further study.

Antimicrobial Activity Assessment and Media Optimization

Strain PB-52 was screened for *in vitro* antimicrobial activity against test microorganisms by conventional spot inoculation method (Shomurat et al., 1979) on GLM agar medium after 8 days of incubation at 28°C. The inhibition zone was observed after 24–48 h incubation at 37°C for bacteria and at 25°C for yeasts. Each experiment was conducted in three replicates and the average size of PB-52 colony diameter and mean value of inhibition zone diameter (mm ± SD) of test microorganisms was calculated.

The selection of best culture medium for growth and antimicrobial activity of PB-52 was performed by growing the strain in different growth media such as GLM broth, Thronton's broth (K_2HPO_4 , 1 g; KNO_3 , 0.5 g; $MgSO_4 \cdot 2H_2O$, 0.2 g; $CaCl_2 \cdot H_2O$, 0.1 g; $NaCl$, 0.1 g; $FeCl_3$, 0.01 g; asparagine, 0.5 g; distilled water, 1000 mL), CSPY-ME broth, Actinomycetes broth and nutrient broth. The strain PB-52 was grown with continuous shaking on a rotary shaker (150 rpm) at 28°C, pH 7.4 for 8 days. Antimicrobial potential was evaluated against *S. aureus* MTCC 96 by disc diffusion method (Bauer et al., 1966) since this test organism was found to be more sensitive during antimicrobial evaluation by spot inoculation method. The experiment was repeated three times and its mean value was recorded.

Crude extract of PB-52 strain was recovered from the culture filtrate by solvent extraction using ethyl acetate in 1:1 ratio (v/v). The ethyl acetate extract of PB-52 (EA-PB-52) obtained by evaporation of ethyl acetate was prepared by dissolving it in 10% dimethyl sulphoxide (DMSO) at a concentration of 1 mg/mL prior to antimicrobial bioassay. 20 µL EA-PB-52 was loaded on to sterile discs (6 mm diameter) placed on nutrient agar plates spread with bacterial test organisms (0.5 McFarland turbidity standards). Similarly, the experiment was conducted with *Candida* species on sabouraud dextrose agar plates. 10% DMSO loaded disc was used as a negative control while rifampicin (20 µg/disc) for bacteria and amphotericin B (30 µg/disc) for *Candida* species served as positive controls. Antimicrobial activity was observed after 24–48 h incubation at 37°C for bacteria and at 25°C for yeasts. Each experiment was conducted in three replicates and the mean value of inhibition zone diameter was calculated. Among all the different liquid media evaluated for antimicrobial activity, GLM was found to be the best medium for PB-52 strain; hence, it was selected as the production medium for further studies.

Effect of Temperature, pH and Incubation Period on Growth and Antimicrobial Activity

GLM medium was inoculated with PB-52 strain to study the growth response and antimicrobial activity at different range of temperatures from 20 to 40°C at 150 rpm for 8 days at pH 7.4. To

study the effect of pH, different ranges of acidic to alkaline pH-values (5–10) were used for the growth and antibiotic production in GLM broth at 28°C for 8 days. Similarly, incubation periods were observed up to 12 days to determine the optimum growth and antibiotic production of PB-52 in GLM broth at 28°C, pH 7.4. The antimicrobial activity of PB-52 was assessed against *S. aureus* MTCC 96 by disc diffusion method (El-Gendy et al., 2008; Thakur et al., 2009) in terms of diameter of inhibition zone.

PCR Amplification and Sequencing of Biosynthetic Genes of PB-52 Strain (PKS-I and NRPS)

Degenerate primers K1F (5'-TSA AGT CSA ACA TCG GBC A-3') and M6R (5'- CGC AGG TTS CSG TAC CAG TA-3') were used for amplification of PKS-I ketosynthase and methylmalonyltransferase domain sequences and primers A3F (5'-GCS TAC SYS ATS TAC ACS TCS GG-3') and A7R (5'-SAS GTC VCC SGT SCG GTA S-3') were used for amplifications specific for NRPS adenylation domain sequences (Ayuso-Sacido and Genilloud, 2005). PCR reactions were performed in Proflex PCR System (Applied Biosystems, USA) in a final volume of 50 µL consisting of template DNA (50 ng), 1X Taq DNA polymerase buffer, $MgCl_2$ (1.5 mM), each dNTP (0.2 mM), 1 U Taq DNA polymerase enzyme and each primer (0.2 µM). For increasing the specificity, touchdown PCR was performed for the amplification of PKS-I genes. In touchdown PCR, annealing temperature was set 10°C above the expected annealing temperature (56.8°C) and decreased by 1°C every second cycle until a touchdown of 46.8°C, at which temperature 25 additional cycles were carried out. Denaturation was carried out at 94°C for 1 min, primer annealing was performed at the appropriate temperature (46.8°C) for 1 min, and primer extension at 72°C for 2 min followed by the final extension at 72°C for 10 min. The thermal cycling conditions for the amplification of NRPS genes were programmed as: initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 1 min, 63°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min.

The amplified products were purified by GenElute PCR Clean-Up Kit (Sigma Aldrich, USA). The purified PCR products were sequenced by automated DNA sequencer with the specific PKS-I and NRPS primers using the sequencing facility at First BASE Laboratories, Malaysia. The resultant sequence was analyzed using BLAST N. The PKS-I (1040 bp fragment) and NRPS (647 bp fragment) gene sequences were deposited to GenBank under the accession number KU721843 and KU721842, respectively.

Determination of Minimum Inhibitory Concentration (MIC) of EA-PB-52

MIC was determined as illustrated by the Clinical Laboratory Standards Institute (CLSI; 2012) and Andrews (2001) with slight modifications using broth dilution method. An inoculum of 1×10^5 CFU/mL of test microorganisms (log phase culture) was added to 5 mL of Mueller Hinton broth (for bacterial test organisms) and Sabouraud dextrose broth (for yeasts) in different test tubes and incubated at room temperature for 24–48 h. EA-PB-52 was dissolved in 10% DMSO (1000 µg/mL) and two-fold

serial dilutions of the extract was prepared for MIC tests (62.5–0.975 µg/mL). MIC was determined after 48 h of incubation of the test microorganisms in the presence of EA-PB-52. 10 µl of the test microorganisms were removed from each tube and spread on Mueller Hinton agar plates/sabouraud dextrose agar plates. The growth of test organisms was observed after 24 h of incubation at 37°C for bacteria and 25°C for yeasts. MIC was recorded as the lowest concentration of the antimicrobial compound which inhibits the visible growth of the inoculated test microorganisms completely after 24–48 h. Control was prepared using 10% DMSO without antimicrobial agent which should be turbid (negative control) while control with standard antibiotic such as rifampicin, streptomycin, and amphotericin B should be clear (positive control).

Rate of Kill Assay of EA-PB-52

Antimicrobial assay for the rate of killing of test microorganisms by EA-PB-52 was done in accordance with the description of Eliopoulos and Moellering (1996) using modified plating technique. EA-PB-52 was inoculated into 10 mL Mueller Hinton broth for bacterial test microorganisms and Sabouraud dextrose broth for yeasts at $\frac{1}{2} \times$ MIC, 1 \times MIC and 2 \times MIC. Broth with EA-PB-52 at the test concentrations without the test microorganisms and broth without EA-PB-52 with test organisms served as controls. 1 \times 10⁵ CFU/mL inoculum was used to inoculate 10 mL of both control and test bottles. The bottles were incubated at 37°C for bacteria and 25°C for yeasts on an orbital shaker at 150 rpm. 100 µl aliquot was removed from the culture medium after 0, 4, and 8 h incubation to determine CFU/mL by the plate count technique (Cruickshank et al., 1975) by plating 25 µl of each of the dilutions. After incubation, emergent colonies were counted, CFU/mL was determined and compared with the count of the control culture without EA-PB-52. 10% DMSO was used as control. The experiments were conducted in replicates and the mean value was obtained. The results were expressed as positive or negative log₁₀ values (Baltch et al., 1998).

Interaction of EA-PB-52 with Test Microorganisms by SEM Analysis

EA-PB-52 with strong antimicrobial activity was further studied for its possible effects on targeted cells of *P. aeruginosa* MTCC 741 and *C. albicans* MTCC 227 by SEM according to Supaphon et al. (2013) with slight modifications. Test microorganisms were grown overnight and then treated with 1 \times MIC EA-PB-52. Cells were harvested after 24 h of incubation and washed with phosphate buffered saline (PBS), pH 7.4. The cells were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h and dehydrated in series of increasing concentrations of acetone (30–100%, v/v) for 10 min. The cells were dried for 30–45 min and mounted onto steel stub with double-sided carbon tape. Samples were coated with a film of gold-palladium alloy under vacuum and scanned under scanning electron microscope (Zeiss Sigma VP, Germany).

GC-MS Analysis of EA-PB-52

Identification of the chemical compounds present in EA-PB-52 was analyzed using GC-MS as previously described (Ser et al.,

2015b; Sun et al., 2015) with slight modifications. The sample was dissolved in spectroscopy-grade methanol and filtered through 0.2 µm filter. GC-MS analysis was performed on Shimadzu GC 2010 plus with triple quadrupole MS (TP-8030) fitted with EB-5 MS column (length- 30 m, thickness-0.25 µm, internal diameter-25 mm). The oven programme started at 40°C, held for 5 min and then ramped at 10°C/min to 280°C, held for 10 min and then again raised to 285°C at 5°C/min and finally held for 10 min. Sample was injected at 300°C using helium as carrier gas (1 mL/min), split at the ratio of 1:20. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV with a continuous scan from 45 to 600 m/z. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST, USA) library.

Detection of Polyenic or Non-polyenic Antimicrobial Activity of EA-PB-52

The polyene like activity of EA-PB-52 was determined by ergosterol agar plate method by using ergosterol as the reversal agent to test for its ability to reverse the inhibition of *C. albicans* MTCC 227 caused by the metabolite (Thakur et al., 2007). EA-PB-52 was dissolved in methanol and its absorption spectrum was scanned in the UV-vis region (200–800 nm) by using a Nanodrop 2000c UV-vis spectrophotometer (Thermo Fisher Scientific, USA).

Statistical Analysis

All experiments were performed in biological triplicate and repeated for three times. The data was expressed as the mean ± standard deviation of mean of the three replicates. Duncan's multiple range test was performed to compare that the sample means differ significantly from each other at a significant level of $P < 0.05$ (Gomez and Gomez, 1984).

RESULTS

Characterization of PB-52 Strain

Actinomycete strain PB-52 isolated from soil samples of Pobitora Wildlife Sanctuary of Assam, India, was aerobic, Gram-positive, and filamentous in nature (Figure 1A). The branched vegetative hyphae were light brown in color while aerial mycelium with orange color was sparse with a patchy distribution. The strain produced faint brown soluble pigment after incubation for more than 20 days at 28°C. SEM analysis revealed that the aerial mycelia formed long, straight to rectiflexibilis spore chains with rugose spore surface (Figure 1B). The cultural characteristics of PB-52 strain in different media are shown in Table 1, where the strain showed good growth on all the media except Mueller Hinton agar and Omeliansky's agar medium. The strain was found to grow between the temperature range of 20–42°C, pH 5–11 with optimal growth temperature at 28°C, pH 7.4, and in the presence of upto 5% NaCl (w/v) with optimum at 1–3% NaCl. The morphological, physiological, and biochemical characteristics of PB-52 and its antibiotic sensitivity are shown in Table 2. The whole-cell hydrolysates of PB-52 were rich in the meso-diaminopimelic acid along with galactose and arabinose as characteristic whole cell sugars.

Partial 16S rRNA gene sequence (1259 nucleotides) of PB-52 strain was determined and submitted to NCBI GenBank database under the accession number KM406386. The strain showed highest 16S rRNA gene sequence similarities with *Nocardia niigatensis* IFM 0330 (NR_112195; 99.7%). The phylogenetic tree also indicated its closest similarity to *N. niigatensis* based on Neighbor-joining method (Figure 2). The phenotypic and genomic data indicated that PB-52 strain represented a strain of the genus *Nocardia* for which the strain was referred to as *Nocardia* sp. strain PB-52.

Antimicrobial Potential of PB-52 Strain

During the screening of PB-52 strain for drug discovery by spot inoculation method in GLM agar medium, it was observed that PB-52 exhibited excellent activity by inhibiting Gram-positive

bacteria, Gram-negative bacteria and yeasts. The average size of the colony of PB-52 in GLM medium was (7 ± 2) mm in diameter after 8 days of incubation at 28°C. The maximum mean value of inhibition zones (mm \pm SD) was found against *S. aureus* MTCC 96 (inhibition zone diameter of 36 ± 0.8), followed by

TABLE 1 | Cultural characteristics of *Nocardia* sp. PB-52 on different media.

Medium	Aerial mycelium color	Substrate mycelium color	Diffusible pigment	Growth
Actinomycetes isolation agar	Brown	Dark brown	–	+++
Streptomyces agar	Light orange	Orange	–	++
GLM medium	Orange	Light brown	Faint Brown	+++
CSPY-ME medium	Pink	Brown	–	+++
Mueller Hinton Agar	Light brown	Brown	–	+
Nutrient agar	White	Cream	–	++
Sabouraud Dextrose Agar	Orange	Cream	–	++
Thronton's medium	Orange	Brown	–	++
Omeliansky's agar	White	Brown	–	+
ISP 2	Pink	Orange	–	+++
ISP 3	White	Brown	–	++
ISP 4	White	Cream	–	+++
ISP 7	Orange	Cream	–	++

++, Good growth; ++, Moderate growth; +, Poor growth; –, No growth.

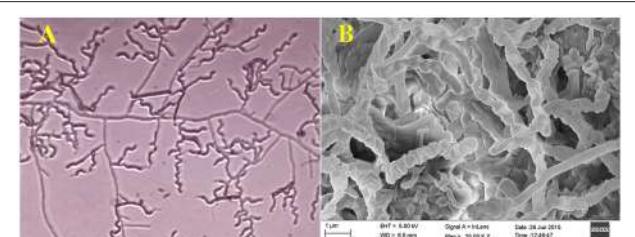


FIGURE 1 | Microscopic view showing spore chain of *Nocardia* sp. PB-52 on GLM agar (A) Micro morphology using cover slip insertion method; (B) Scanning electron micrograph view.

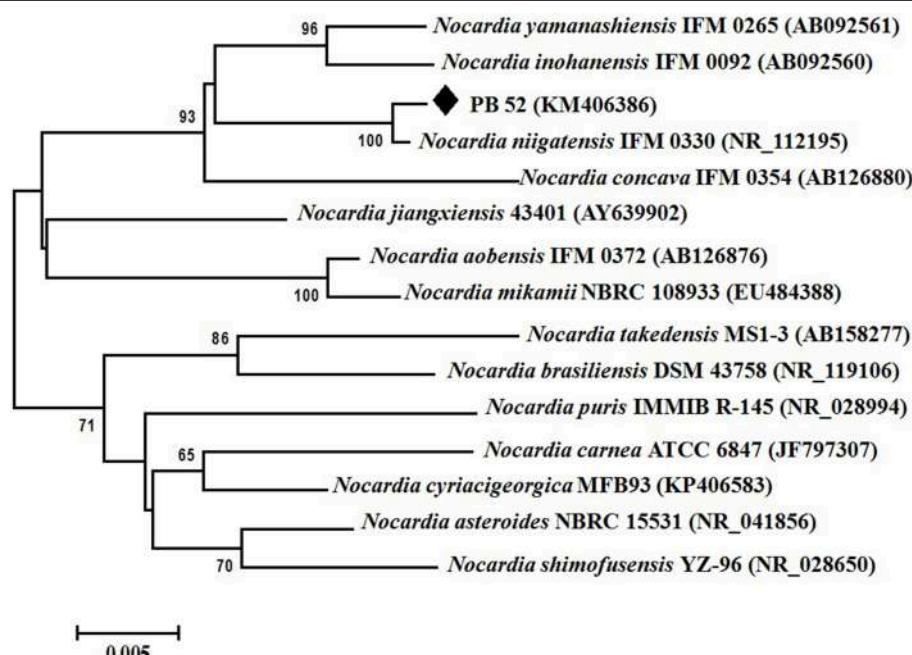


FIGURE 2 | Phylogenetic tree of *Nocardia* sp. PB-52 and the closest *Nocardia* species showing phylogenetic relationships based on the 16S rRNA gene sequences using neighbor-joining method. Bootstrap percentages based on 1000 resamplings are listed at nodes, only values above 50% are given. Bar, 0.005 substitutions per nucleotide position.

TABLE 2 | Morphological, physiological, and biochemical characteristics of *Nocardia* sp. PB-52.

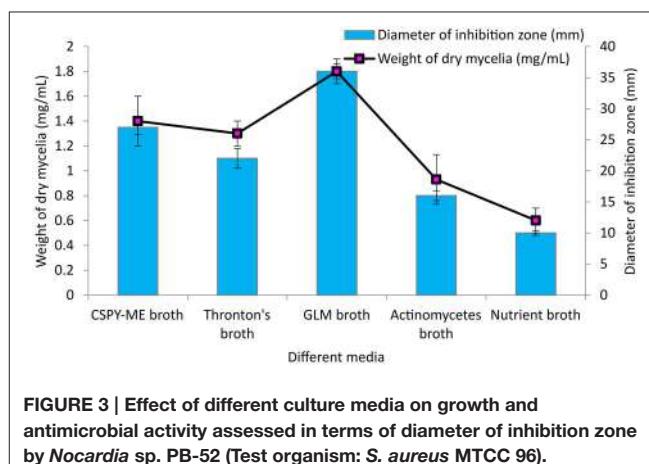
Characteristics	Result
MORPHOLOGICAL	
Aerial mycelium color	Orange
Substrate mycelium color	Brown
Diffusible pigment	Faint brown
Melanin pigment	–
Spore chain morphology	Straight to rectiflexibiles
Spore surface	Rugose
PHYSIOLOGICAL	
Temperature range for growth	20–42°C
Optimum temperature for growth	28°C
pH range for growth	5–11
Optimum pH for growth	7.4
NaCl tolerance	Up to 5%
BIOCHEMICAL	
Gram reaction	Positive
Utilization of	
Glucose	+
Fructose	+
Arabinose	–
Mannitol	+
Inositol	+
Adonitol	+
Galactose	–
Sucrose	+
Xylose	+
Lactose	+
Maltose	+
Starch	+
Glycerol	+
Erythritol	–
Sorbitol	–
Rhamnose	–
Gluconate	–
Carboxy methyl cellulose	+
Citrate	–
Urea	+
Ammonium chloride	+
Ammonium sulfate	+
Sodium nitrate	+
Glycine	+
Asparagine	+
Casein	–
Tween 20	–
Tween 80	+
Nitrate reduction	+
Gelatin liquefaction	–
Cell wall amino acids	Meso-diaminopimelic acid
Cell wall sugars	Arabinose, galactose

(Continued)

TABLE 2 | Continued**ANTIBIOTIC SENSITIVITY ($\mu\text{g/disc}$)**

Vancomycin (30)	S
Chloramphenicol (30)	S
Oxacillin (1)	R
Ciprofloxacin (5)	S
Co-trimoxazole (25)	R
Streptomycin (10)	S
Methicillin (5)	S
Ampicillin (10)	R
Penicillin-G (10)	S
Gentamicin (120)	R
Nalidixic acid (30)	S
Erythromycin (5)	S
Norfloxacin (10)	S
Amphotericin B (100)	R
Clotrimazole (10)	S
Fluconazole (25)	R
Itraconazole (10)	R
Ketoconazole (10)	S
Nystatin (100)	R

+, Positive for test; –, Negative for test; S for Sensitivity; R for Resistant.

**FIGURE 3 | Effect of different culture media on growth and antimicrobial activity assessed in terms of diameter of inhibition zone by *Nocardia* sp. PB-52 (Test organism: *S. aureus* MTCC 96).**

E. coli MTCC 40 (31 ± 1.2) and MRSA ATCC 43300 (30 ± 0.8). Maximum growth inhibition among yeast test pathogens was observed in *C. albicans* MTCC 227 (27 ± 0.4). The detailed antimicrobial profiles in GLM agar medium are shown in Table 3.

Among the different liquid culture media evaluated for the production of the bioactive compounds in shake flask condition, GLM was found to be most appropriate for growth and antimicrobial activity assessed in terms of diameter of inhibition zone by PB-52 strain (Figure 3). The culture filtrate of PB-52 grown on GLM medium exhibited maximum zone of inhibition against *S. aureus* MTCC 96 followed by the culture filtrate grown on CSPY-ME broth while lowest activity was observed with the one grown on nutrient broth. The antimicrobial activity of EA-PB-52 along with the controls (10% DMSO as negative control and standard antibiotics as positive control) against *S. aureus* MTCC 96 and *C. albicans* MTCC 227 is shown in Figure 4.

TABLE 3 | Antimicrobial activity and MIC ($\mu\text{g/mL}$) of *Nocardia* sp. PB-52 by broth dilution method.

Test microorganisms	*Zone of inhibition (mm)	MIC of EA-PB-52 ($\mu\text{g/mL}$)	MIC of Rif ($\mu\text{g/mL}$)	MIC of Strep ($\mu\text{g/mL}$)	MIC of Amp B ($\mu\text{g/mL}$)
GRAM-POSITIVE BACTERIA					
<i>S. aureus</i> MTCC 96	36 ^k \pm 0.8	>0.975	>1.95	>6.25	NA
<i>S. aureus</i> MTCC 3160	29 ^{hi} \pm 0.8	>1.95	>1.95	>12.5	NA
<i>S. epidermidis</i> MTCC 435	23 ^{de} \pm 1.6	>7.81	>3.125	>6.25	NA
<i>B. subtilis</i> MTCC 441	28 ^{ghi} \pm 0.8	>1.95	>1.95	>6.25	NA
<i>B. cereus</i> MTCC 1272	22 ^d \pm 0.4	>7.81	>6.25	>12.5	NA
<i>B. megaterium</i> MTCC 8075	26 ^{fgh} \pm 0.4	>3.9	>3.12	>3.12	NA
<i>M. luteus</i> MTCC 1538	22 ^d \pm 1.6	>7.81	>0.97	>6.25	NA
MRSA ATCC 43300	30 ^{ij} \pm 0.8	>1.95	>25	>50	NA
GRAM-NEGATIVE BACTERIA					
<i>E. coli</i> MTCC 40	31 ^j \pm 1.2	>3.9	>6.25	>3.12	NA
<i>E. coli</i> MTCC 739	25 ^{ef} \pm 0.4	>3.9	>50	—	NA
<i>S. marcescens</i> MTCC 97	18 ^b \pm 1.2	>31.2	>12.5	>3.12	NA
<i>K. pneumoniae</i> MTCC 3384	27 ^{fgh} \pm 1.6	>1.95	>25	—	NA
<i>K. pneumoniae</i> ATCC 13883	15 ^a \pm 1.6	>62.5	>50	—	NA
<i>P. aeruginosa</i> MTCC 741	29 ^{hij} \pm 0.8	>1.95	>25	>25	NA
<i>P. aeruginosa</i> MTCC 424	19 ^{bc} \pm 1.2	>15.6	—	>12.5	NA
<i>P. aeruginosa</i> MTCC 2582	26 ^{fgh} \pm 0.8	>3.9	>50	>25	NA
<i>P. vulgaris</i> MTCC 426	17 ^a \pm 0.8	>31.25	>25	>6.25	NA
YEAST					
<i>C. albicans</i> MTCC 227	27 ^{fgh} \pm 0.4	>1.95	NA	NA	>0.97
<i>C. tropicalis</i> MTCC 2208	23 ^{de} \pm 0.2	>7.81	NA	NA	>0.48
<i>C. albicans</i> ATCC 10231	21 ^{cd} \pm 1.6	>7.81	NA	NA	>1.95

*Zone of inhibition by spot inoculation method on GLM agar medium. Average size of colony of PB-52 in GLM agar was (7 ± 2) mm in diameter after 8 days of incubation at 28°C.

Zone of inhibition values are given as mean \pm SD ($n = 3$). Values having different superscripts (a-k) differ significantly ($P < 0.05$).

Rif, Rifampicin (antibacterial agent); Strep, Streptomycin (antibacterial agent); Amp B, Amphotericin B (antifungal agent); NA, not applicable; —, No activity.

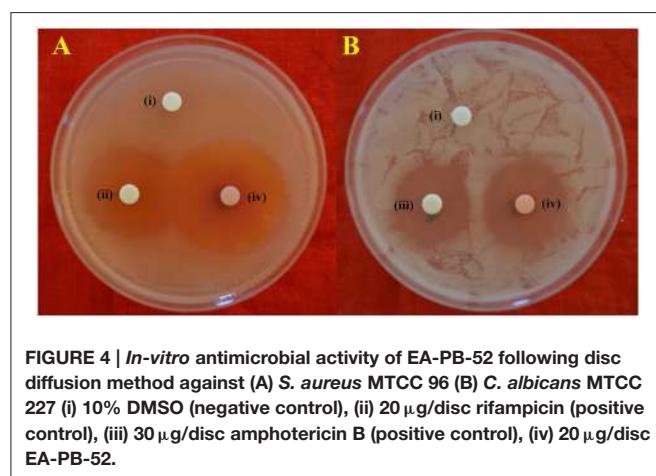


FIGURE 4 | *In-vitro* antimicrobial activity of EA-PB-52 following disc diffusion method against (A) *S. aureus* MTCC 96 (B) *C. albicans* MTCC 227 (i) 10% DMSO (negative control), (ii) 20 $\mu\text{g}/\text{disc}$ rifampicin (positive control), (iii) 30 $\mu\text{g}/\text{disc}$ amphotericin B (positive control), (iv) 20 $\mu\text{g}/\text{disc}$ EA-PB-52.

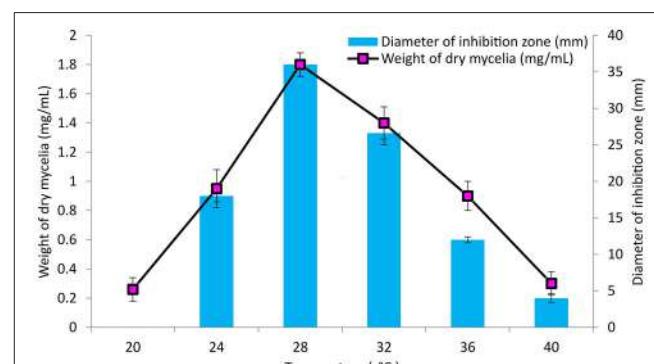


FIGURE 5 | Effect of temperature on growth and antimicrobial activity assessed in terms of diameter of inhibition zone by *Nocardia* sp. PB-52 (Test organism: *S. aureus* MTCC 96).

Effect of Cultural Parameters on Growth and Antimicrobial Activity

The culture conditions for growth and antimicrobial evaluation by PB-52 strain was studied on GLM medium. PB-52 displayed a narrow range of incubation temperature for its good growth and production of antibiotics. 28°C was found to be the optimum temperature for highest growth and maximum antimicrobial activity by the strain (Figure 5). The organism appeared to be

mesophilic in terms of its growth at optimum temperature. However, poor growth and decreased antibiotic production were evident at higher incubation temperature. The maximum growth as well as highest antibiotic production was obtained at pH-value of 7.4 (Figure 6). However, adverse growth and antibiotic production were observed at pH-values above and below neutral. Maximum incubation period of 8 days is required for best growth and antibiotic production under shake flask conditions

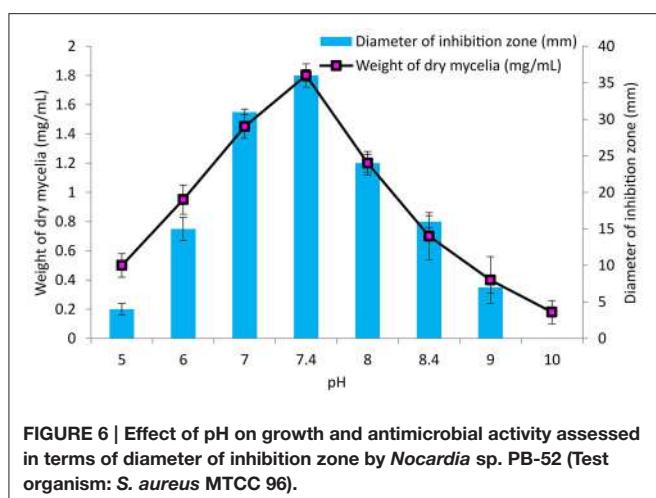


FIGURE 6 | Effect of pH on growth and antimicrobial activity assessed in terms of diameter of inhibition zone by *Nocardia* sp. PB-52 (Test organism: *S. aureus* MTCC 96).

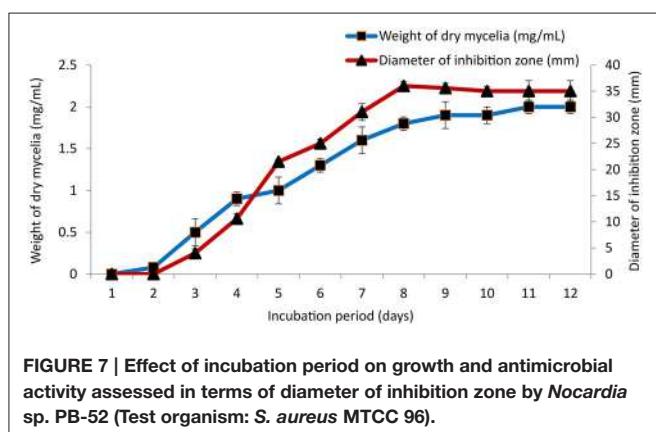


FIGURE 7 | Effect of incubation period on growth and antimicrobial activity assessed in terms of diameter of inhibition zone by *Nocardia* sp. PB-52 (Test organism: *S. aureus* MTCC 96).

(Figure 7). Antimicrobial activity by PB-52 strain was assessed in terms of diameter of inhibition zone.

Analysis of PKS-I and NRPS Genes

Polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) are biosynthetic enzymes responsible for the production of different classes of bioactive molecules in Actinomycetes (Hodges et al., 2012). PCR amplification of PKS-I gene of the strain PB-52 resulted in a band size of 1200–1400 bp fragments (Figure 8A) using K1F/M6R primers corresponding to PKS-I ketosynthase and methyl-malonyl-CoA transferase modules of the polyketide synthase gene. Sequencing of the PKS-I gene fragment yielded a sequence length of 1040 bp (NCBI accession no. KU721843). PB-52 strain showed highest PKS-I gene sequence similarities (89%) with *Streptomyces olivoviridis* strain O855 clone 22 modular polyketide synthase gene (NCBI accession no. FJ405974). The NRPS amplicon was found to be 700 bp size (Figure 8B) using A3F/A7R specific primers for NRPS genes targeting NRPS adenylation domain sequences. Sequencing of the NRPS gene fragment yielded 647 bp sequence length (NCBI accession no. KU721842). NRPS sequence of PB-52 showed highest similarities (76%) to *Streptomyces* sp. Sp080513GE-23 gene for non-ribosomal peptide synthetase

(NCBI accession no. AB492018). The closest similarity of PKS-I and NRPS sequences of PB-52 to the closest relatives in GenBank is shown in Table S1.

MIC of EA-PB-52

MIC values of EA-PB-52 ranging from 62.5 to 0.975 µg/mL were performed against all the test microorganisms by broth dilution method. The quantitative efficiency of EA-PB-52 against these organisms was estimated as MIC and it has got lowest MIC against *S. aureus* MTCC 96 (0.975 µg/mL) whereas highest was recorded against *K. pneumoniae* ATCC 13883 (62.5 µg/mL; Table 3). According to CLSI recommendations for MIC, *K. pneumoniae* ATCC 13883 was found to be resistant to EA-PB-52 (MIC: 62.5 µg/mL) since ≤ 8 µg/mL was taken as susceptible, ≤ 16 µg/mL as intermediate and ≥ 32 µg/mL as resistant (CLSI, 2012). It was observed that 10% DMSO (control) had no inhibitory effect on the test microorganisms.

Time-Kill Assay

The time-kill assay was conducted for EA-PB-52 against all the test microorganisms. Table 4 shows the changes in the \log_{10} CFU/mL of viable colonies of the test microorganisms following exposure to different concentration of EA-PB-52 which is expressed in terms of \log_{10} CFU/mL change. It is based on the conventional bactericidal activity standard, i.e., 3 \log_{10} CFU/mL or greater reduction in the viable colony count (Pankey and Sabath, 2004). After incubating the test microorganisms for 4 h with 1 \times MIC and 2 \times MIC of EA-PB-52, the average log reduction in the viable cell count ranged between -0.157 \log_{10} and 1.897 \log_{10} CFU/mL. While after 8 h of incubation, the average log reduction in the viable cell count ranged between -2.092 \log_{10} and 0.864 \log_{10} CFU/mL. After 4 h of incubation of the test microorganisms with 1 \times MIC, the average log reduction in the viable cell count ranged between 0.947 \log_{10} and 1.897 \log_{10} while incubating the test microorganisms with 2 \times MIC resulted in the average log reduction in the viable cell count ranging between -0.157 \log_{10} and 0.891 \log_{10} . After 8 h of incubation with 1 \times MIC resulted in average log reduction in the viable cell count between 0.342 \log_{10} and 0.864 \log_{10} while 2 \times MIC resulted in the average log reduction in the viable cell count ranging between -2.092 \log_{10} and -1.213 \log_{10} . The highest log reduction in cell density with EA-PB-52 was observed in *S. aureus* MTCC 96 (-2.092 \log_{10} CFU/mL) followed by *E. coli* MTCC 40 (-1.917 \log_{10} CFU/mL) and MRSA ATCC 43300 (-1.908 \log_{10} CFU/mL), while least log reduction in cell density was observed in *K. pneumoniae* ATCC 13883 (-1.213 \log_{10} CFU/mL) followed by *P. vulgaris* MTCC 426 (-1.325 \log_{10} CFU/mL). No inhibitory effect was observed with 10% DMSO (control) on the test microorganisms.

SEM Analysis

Antibacterial and anti-candidal activity of the EA-PB-52 was evaluated against *P. aeruginosa* MTCC 741 and *C. albicans* MTCC 227 by SEM analysis. SEM study revealed that treatment of the test microorganisms with 1 \times MIC EA-PB-52 showed considerable morphological alterations in the cells

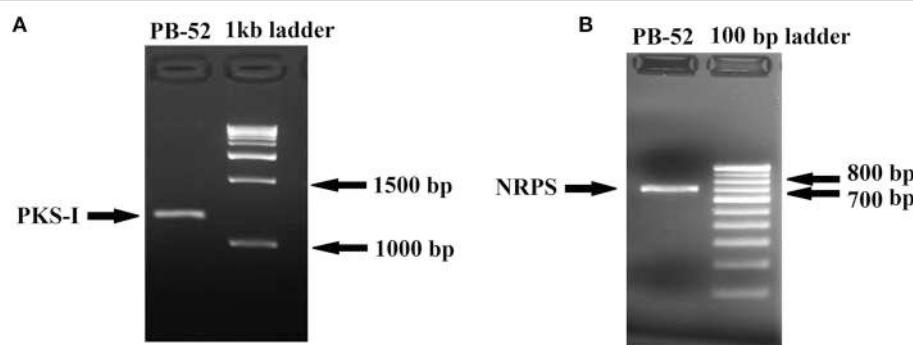


FIGURE 8 | Agarose gel electrophoresis of PCR amplified products of *Nocardia* sp. PB-52. (A) Selective amplification of PKS-I using K1F/M6R specific primers; (B) Selective amplification of NRPS using A3F/A7R specific primers.

TABLE 4 | *In vitro* time-kill assessment of EA-PB-52 against the test microorganisms.

Test microorganisms	\log_{10} kill (1/2) \times MIC			\log_{10} kill 1 \times MIC			\log_{10} kill 2 \times MIC		
	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
GRAM-POSITIVE BACTERIA									
<i>S. aureus</i> MTCC 96	2.452	2.772	4.330	2.456	0.947	0.342	2.468	-0.157	-2.092
<i>S. aureus</i> MTCC 3160	2.510	3.245	3.998	2.487	1.263	0.582	2.433	0.422	-1.775
<i>S. epidermidis</i> MTCC 435	2.853	3.421	4.227	2.610	1.512	0.539	2.717	0.531	-1.717
<i>B. subtilis</i> MTCC 441	2.329	3.022	4.172	2.311	1.235	0.492	2.352	0.544	-1.842
<i>B. cereus</i> MTCC 1272	2.115	2.914	4.013	2.302	1.429	0.483	2.196	0.436	-1.741
<i>B. megaterium</i> MTCC 8075	2.277	3.510	4.734	2.256	1.197	0.548	2.290	0.282	-1.869
<i>M. luteus</i> MTCC 1538	2.314	3.219	3.816	2.334	1.315	0.513	2.105	0.587	-1.512
MRSA ATCC 43300	2.412	2.868	3.797	2.249	1.206	0.429	2.270	0.124	-1.908
GRAM-NEGATIVE BACTERIA									
<i>E. coli</i> MTCC 40	2.212	2.842	3.714	2.368	1.191	0.404	2.292	-0.112	-1.917
<i>E. coli</i> MTCC 739	2.401	3.124	3.874	2.427	1.374	0.473	2.218	0.677	-1.880
<i>S. marcescens</i> MTCC 97	2.316	2.997	4.512	2.212	1.529	0.597	2.307	0.528	-1.563
<i>K. pneumoniae</i> MTCC 3384	2.312	3.046	4.147	2.299	1.434	0.501	2.333	0.648	-1.693
<i>K. pneumoniae</i> ATCC 13883	2.216	3.934	4.729	2.137	1.897	0.864	2.296	0.891	-1.213
<i>P. aeruginosa</i> MTCC 741	2.142	3.013	3.976	2.312	1.463	0.413	2.133	0.432	-1.436
<i>P. aeruginosa</i> MTCC 424	2.314	3.814	5.214	2.367	1.545	0.456	2.121	0.612	-1.517
<i>P. aeruginosa</i> MTCC 2582	2.202	2.889	4.318	2.311	1.385	0.567	2.273	0.418	-1.493
<i>P. vulgaris</i> MTCC 426	2.013	3.214	4.813	2.213	1.662	0.727	2.115	0.723	-1.325
YEAST									
<i>C. albicans</i> MTCC 227	2.104	3.116	3.937	2.204	1.229	0.435	2.316	0.499	-1.865
<i>C. tropicalis</i> MTCC 2208	2.049	3.313	4.712	2.014	1.575	0.541	2.179	0.583	-1.630
<i>C. albicans</i> ATCC 10231	2.098	3.236	4.213	2.212	1.305	0.512	2.513	0.513	-1.518

including shrinkage and deformity leading to prominent cell shape loss and integrity. The control cells treated with 10% DMSO appeared smooth with intact cell surface (Figure 9).

GC-MS Analysis

Chemical composition of EA-PB-52 was done using GC-MS. Twelve chemical compounds were identified by comparison of their mass spectra with the NIST library based on their retention time, molecular weight, and molecular formula shown in Table 5 and the chemical structures were illustrated in Figure 10. The

peak area of the compound is directly proportional to its quantity in EA-PB-52 (Figure S1).

Detection of Polyenic or Non-polyenic Antimicrobial Activity of EA-PB-52

The ergosterol test of EA-PB-52 showing antimicrobial activity indicated the absence of polyene class of antibiotics. The nature of the metabolite was thus found to be non-polyene. The UV-vis spectrum of the EA-PB-52 in methanol showed the presence of three distinct peaks at 247, 260, and 296 nm where maximum absorption peak was observed at 296 nm (Figure S2).

TABLE 5 | Chemical compounds detected in EA-PB-52 by GC-MS analysis.

Compound name	Similarity (%)	RT	MW	Area (%)	Nature of compound	Activity	References
(Z)-3-tridecene	95	18.27	182	5.14	Hydrocarbon	No activity reported	
3,5-bis(1,1-dimethylethyl)-phenol	91	19.93	206	34.43	Phenolic compound	No activity reported	
(Z)-3-tetradecene	95	20.94	196	7.90	Hydrocarbon	Antimicrobial	Natarajan and Dhas, 2014
Dodecyl acrylate	96	22.13	240	6.01	Ester	Antibacterial	Manilal et al., 2009
2,4-di-t-butyl-6-nitrophenol	63	22.22	251	4.32	Phenolic compound	Antimicrobial	Gutierrez et al., 2009
Hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione	90	23.11	154	5.03	Pyrrolizidine	Antimicrobial Antioxidant	Narasaiah et al., 2014; Ser et al., 2015a
(E)-5-Eicosene	95	23.32	280	5.07	Hydrocarbon	Antimicrobial	Elavarasi et al., 2014
3,5-dihydroxy-4,4-dimethyl-2,5-cyclohexadien-1-one	81	24.08	154	3.60	Quinone	No activity reported	
Hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione	89	25.11, 25.17	210	9.04	Pyrrolizidine	Antimicrobial	Manimaran et al., 2015
(E)-9-Octadecene	94	25.47	252	2.21	Hydrocarbon	Antimicrobial	Cao et al., 2014; Okwu and Ighodaro, 2010
Trichloroacetic acid, hexadecyl ester	87	27.42	387	1.03	Acid	Cytotoxic, Antioxidant	Luo et al., 2014
Hexahydro-3-(phenylmethyl)-pyrrolo[1,2-a]pyrazine-1,4-dione	82	29.08	244	16.22	Pyrrolizidine	Antimicrobial, Nematicidal	Dashti et al., 2014; Wang et al., 2014

RT is retention time; MW is molecular weight of compounds.

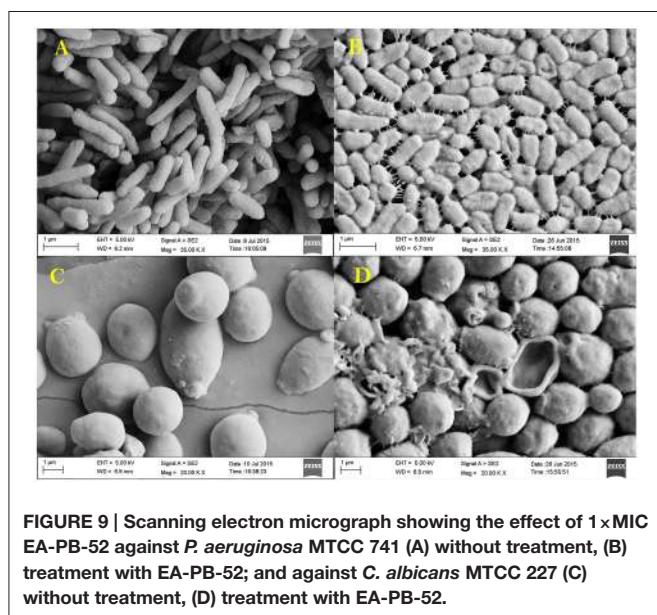


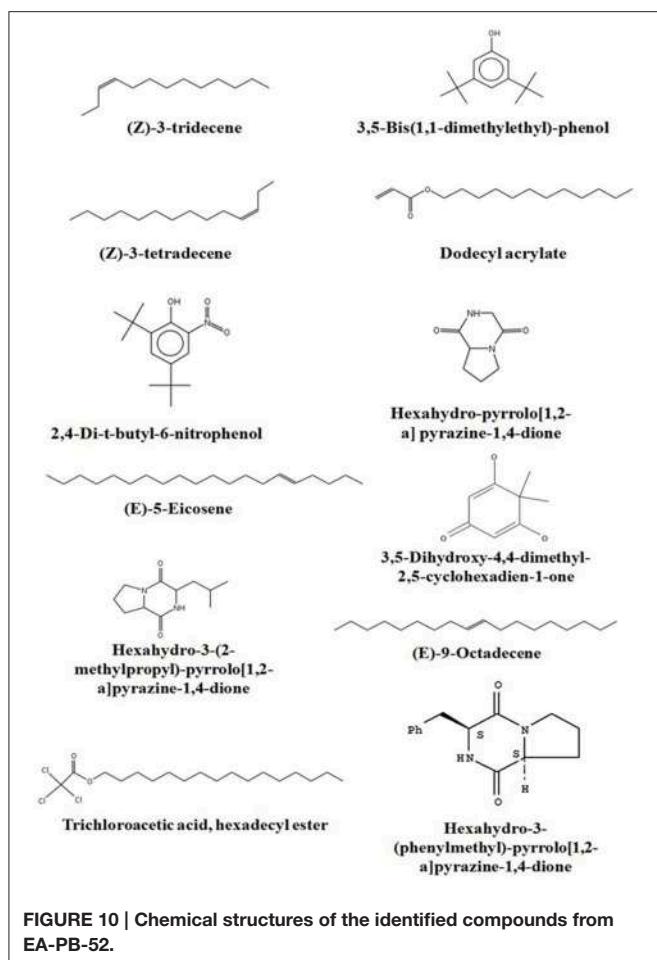
FIGURE 9 | Scanning electron micrograph showing the effect of 1×MIC EA-PB-52 against *P. aeruginosa* MTCC 741 (A) without treatment, (B) treatment with EA-PB-52; and against *C. albicans* MTCC 227 (C) without treatment, (D) treatment with EA-PB-52.

DISCUSSION

Actinomycetes remain as the most proliferant producers of small molecule diversity including antimicrobials, diverse group of enzymes and useful secondary metabolites with an incredible variety of biological activities (Berdy, 2005). Depending on the abundance of actinomycetes in the soil, 99% of the diverse species has still been unexplored (Davies, 1999). Isolation of these novel species from unexplored habitats increases the possibility of discovery of new types of microbial products with

new types of activities. Many ecosystems have still been poorly investigated and thus, there is a remarkable likelihood to exploit novel microorganisms with diverse biological activities from such ecosystems. There are several reports available regarding the study of metabolic pathways leading to the production of novel secondary metabolites from different microorganisms exploited from untapped ecosystems (Glover, 1995; Tiwari et al., 2014). Actinomycetes from diverse environments have been explored in the last few decades for their capability to produce a wide variety of natural bioactive compounds (Mitra et al., 2008). Isolation of actinomycetes for the production of antimicrobial secondary metabolites from the forest ecosystems of Northeast India is reported by several researchers (Thakur et al., 2007; Talukdar et al., 2012; Sharma et al., 2014; Passari et al., 2015). After literature review, it has been found that Pobitora Wildlife Sanctuary of Assam, India is an unexplored ecosystem and no considerable data could be found regarding bioactivity prospective of its microflora. Thus, we have an additional advantage by isolating a *Nocardia* strain selectively designated as PB-52 having broad spectrum bioactivity against a wide range of pathogens including MRSA from this unexplored habitat. Pobitora Wildlife Sanctuary is a part of the mega biodiversity hotspot which indicates that it is a very dynamic ecosystem where the occurrence of novel microflora may be very likely (Myers et al., 2000). This study signifies the first report on genus *Nocardia* isolated from this untouched habitat.

Based on the comparative study of 16S rRNA gene sequence and phylogenetic relationship, PB-52 strain lies in clade with *N. niigatensis* IFM 0330 (NR_112195). PB-52 shared 99.7% sequence similarity with type strain *N. niigatensis* IFM 0330 with 77 nucleotide differences, where PB-52 has a stretch of unique 64



nucleotides at 881 to 944 sites in the middle of the sequence which is absent in the type *Nocardia* strains included in the phylogenetic tree. PB-52 differs from *N. niigatensis* IFM 0330 phenotypically where PB-52 possess orange aerial mycelium with light brown vegetative mycelium while *N. niigatensis* IFM 0330 (Kageyama et al., 2004b) produced white aerial mycelium with orange-tan vegetative mycelium. The spore surface of PB-52 was rugose while *N. niigatensis* had smooth spore surface, PB-52 utilized adonitol and maltose while *N. niigatensis* could not. *N. niigatensis* could grow up to a maximum temperature of 37°C while PB-52 grew up to 42°C.

Nocardia strain PB-52 showed promising broad spectrum antagonistic activity both in agar medium as well as in culture broth against different bacteria and yeasts. Similar findings were reported by a subset of the researchers (Kavitha et al., 2009, 2010; Mukai et al., 2009). The results signified that the bioactive metabolites having antimicrobial activity were extracellular in nature. Most of the bioactive secondary metabolites including antibiotics produced by actinomycetes are previously reported to be extracellular products (Augustine et al., 2005; Kumar P. S. et al., 2014b). The results of antimicrobial activity of PB-52 strain showed that it secretes broad spectrum antagonistic metabolites which showed the capacity to inhibit the growth of Gram-positive bacteria, Gram-negative bacteria, and *Candida*

species. These results are consistent with previous reports of Sun et al. (2007) and Kavitha et al. (2009). The study for evaluation of antimicrobial activity generally involves the search of suitable culture medium. GLM was found to be the best suitable medium for the growth and evaluation of antimicrobial activity of *Nocardia* sp. PB-52 among the tested media. It was obvious from the findings that the antimicrobial activity of PB-52 was positively influenced by the presence of starch as carbon and peptone as the nitrogen source in the medium. This result is quite similar to the previously reported study of El-Gendy et al. (2008) where it was shown that starch was the most suitable carbon source for antibiotic production by *Nocardia* sp. ALAA 2000. It has also been shown that the production of antibiotics in different organisms is strongly influenced by the nature of carbon and nitrogen sources (Vilches et al., 1990). Extraction of the culture broth of PB-52 strain using ethyl acetate led to the recovery of EA-PB-52 consisting of a mixture of metabolites which showed a wide range of activity against the selected test microorganisms. El-Gendy et al. (2008) and Kavitha et al. (2009) have also reported antimicrobial activity of ethyl acetate extracted compound of *Nocardia levis* MK-VL_113 and *Nocardia* sp. ALAA 2000 respectively showing broad spectrum bioactivity.

It is essential to determine the efficiency of the organism during its growth with different culture conditions for determination of the factors that intensify the production of antimicrobial compounds in culture media. PB-52 strain is a mesophilic organism and its capacity of producing antibiotic was consecutively increased with the increase in temperature up to 28°C and pH of 7.4 as well. Complete biosynthesis of antimicrobial compounds production of PB-52 was established on the 8th day of growth. Our result is consistent with the findings of El-Gendy et al. (2008) who reported that *Nocardia* sp. ALAA 2000 showed maximum growth and antibiotic production at 28°C, pH up to 7.4. As reported by Griffiths and Saker (2003), *Cylindrospermopsis raciborskii* produced highest bioactive metabolites when the bacterium moved into the post-exponential phase of growth, while Egorov (1985) suggested that highest antagonistic potential was directly proportional to the value of the biomass. These results signify that cultural environmental aspects like temperature, pH, and incubation period have profound influence on growth and antimicrobial potential of *Nocardia* sp. PB-52 as reported in genus *Nocardia* by El-Gendy et al. (2008).

Non-ribosomal peptides and polyketides are structurally varied group of compounds and have significant biological roles to play. Myriad of bioactive natural products belonging to these two diverse groups have a wide range of applications in the field of medicine, agriculture, and veterinary science (Cane and Walsh, 1999). PCR detection of genes encoding PKS-I and NRPS might be responsible for both of their involvement in the regulation of antimicrobial activity in PB-52. The result is comparable with the studies of Ding et al. (2013) and Passari et al. (2015) where it was clearly shown that few actinomycetes possessing antimicrobial activity were positive for the presence of both of these two biosynthetic pathway genes in their genome. Zhang et al. (2014) also reported that antibacterial activity in *Lysobacter enzymogenes* is regulated by the involvement of both a PKS and

NRPS biosynthetic gene. This serves as useful information for discovery of novel bioactive secondary metabolites from PB-52 strain.

MIC of EA-PB-52 at very low concentrations (as low as 0.975 µg/mL) against all the test microorganisms supports the popular notion that *Nocardia* can be one of the best source of potent antimicrobial agents that can be of significance in the treatment of infectious diseases especially those caused by clinically resistant pathogens, such as MRSA, *P. aeruginosa*, *C. albicans* etc. (Oskay et al., 2004). Our result is comparable with the report of Kumar P. S. et al. (2014b) where the crude ethyl acetate extracted product of *Streptomyces lavendulae* strain SCA5 showed good antimicrobial activity against Gram-positive and Gram-negative bacteria with the MIC-value of 125 µg/mL and the MIC-value against fungi was reported to be 31.25 µg/mL. In addition, Teng_hern et al. (2015) and Ser et al. (2015b) showed that the crude extract of *Streptomyces* sp. MUM256 and *S. pluripotens* MUSC 137 respectively exhibited good antioxidant and anticancer property. The effect of incubating the test microorganisms at 2 × MIC EA-PB-52 lead to rapid decrease in the average log of the viable cells counts whose value was observed to be higher than that treated with 1 × MICs. The considerable decrease in cell counts between 4 and 8 h of incubation period signifies that EA-PB-52 is highly bactericidal/fungicidal seeing that the population of the test microorganisms were almost totally wiped out after 8 h of incubation. Furthermore, the net growth of all the test microorganisms was observed when administered with ½ × MICs of EA-PB-52. Growth inhibition and efficiency of EA-PB-52 was ascertained to be dosage and time-dependent which produces discrete time-kill profiles for the tested microorganisms. Similar antimicrobial activity had also been reported by Olajuyigbe and Afolayan (2012) and Singh et al. (2014). The strong antimicrobial activity of EA-PB-52 was further confirmed by SEM studies which lead to morphological changes in the selected test microorganisms leading to shrinkage and cytosolic loss of the cells. These results are in conformity with the studies of Supaphon et al. (2013) and Nurkanto and Julistiono (2014).

Actinomycetes are known to produce a wide variety of bioactive secondary metabolites possessing diverse biological activity. There are plentiful reports available incorporating the study of GC-MS for chemical analysis of these microbial metabolites (Jog et al., 2014; Teng_hern et al., 2015; Ser et al., 2015a,b). For instance, study by Selvin et al. (2009) illustrated several antimicrobial agents isolated from *Nocardiopsis dassonvillei* MAD08 by using GC-MS. Also, Kim et al. (2008) and Ser et al. (2015a) reported the detection of bioactive compound protocatechualdehyde and an antioxidative agent hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione in the extract of *S. lincolnensis* M-20 and *S. mangrovisoli* sp. nov., respectively with the help of GC-MS. As such, in this study EA-PB-52 was subjected to GC-MS analysis and twelve chemical compounds were detected with different retention time and abundance. The identified compounds comprised of phenolic compounds, pyrrolizidines, quinones, hydrocarbons, esters, and acids. Phenolic compounds are commonly known as potent antimicrobial agents as well

as antioxidant agents as they possess hydrogen-donating capability to reduce free radicals (Yogeswari et al., 2012). Recently, the study conducted by Kumar P. S. et al. (2014c) showed highest antimicrobial activity in the GC-MS fractions containing the highest amount of phenolic compounds. 3,5-bis (1,1-dimethylethyl)-phenol and 2,4-di-t-butyl-6-nitrophenol were the two phenolic compounds detected in EA-PB-52. 3,5-bis (1,1-dimethylethyl)-phenol constituted 34.43% of the total constituents present in EA-PB-52. Antimicrobial activity of 2,4-di-t-butyl-6-nitrophenol is already reported (Kumar P. S. et al., 2014c) but there is no report of 3,5-bis (1,1-dimethylethyl)-phenol as an antimicrobial agent. Roy et al. (2015) reported surfactant activity of 3,5-bis (1,1-dimethylethyl)-phenol in *Nocardiopsis* VITSISB isolated from Marina beach, India. The pyrrolizidine compounds present in EA-PB-52 include hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione, and hexahydro-3-(phenylmethyl)-pyrrolo[1,2-a]pyrazine-1,4-dione. These compounds had been reported to possess promising antimicrobial activity (Dashti et al., 2014; Manimaran et al., 2015), nematicidal activity (Wang et al., 2014), and antioxidant activity (Ser et al., 2015a). Another study conducted by Devi and Wahab (2012) illustrated that hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione in endophytic fungi isolated from *Camellia sinensis* possess strong antimicrobial activity. Furthermore, a quinone compound, 3,5-dihydroxy-4,4-dimethyl-2,5-cyclohexadien-1-one was detected in the PB-52 crude extract for which no antimicrobial activity has been reported till now. Previous study by Sher (2009) demonstrated that the antimicrobial effects of quinines are due to the fact that they are known to complex with nucleophilic amino acids in protein irreversibly. This often leads to loss of function and inactivation of the protein. Asolkar et al. (2010) reported a quinone antibiotic from *Salinispora arenicola* effective against MRSA and other drug-resistant pathogens. Hydrocarbon compounds such as (Z)-3-tetradecene, (E)-5-eicosene, and (E)-9-octadecene are reported to possess antagonistic potential against a wide range of pathogens by a subset of researchers (Cao et al., 2014; Elavarasi et al., 2014; Natarajan and Dhas, 2014). Manilal et al. (2009) reported antibacterial activity of dodecyl acrylate produced by red algae, *Falkenbergia hillebrandii* against multidrug resistant human pathogens. Antimicrobial activity of (Z)-3-tridecene is not available till now. According to the recent reports by Luo et al. (2014), trichloroacetic acid, hexadecyl ester is reported to possess both antioxidant and anticancer activity along with its antimicrobial nature. These compounds are well recognized for their antimicrobial activity and together they may be responsible for the broad spectrum antimicrobial activity of EA-PB-52 against the wide range of test microorganisms. Previous reports by Narayana et al. (2008), Selvin et al. (2009), Ser et al. (2015a,b), and Teng_hern et al. (2015) demonstrated the combinatorial effect of bioactive compounds from GC-MS analysis. Thus, we propose that these compounds could be the key contributing factor for the antimicrobial activities of EA-PB-52. Further, the study of other biological activities of the metabolites produced by the strain PB-52 is the subject of future investigation.

CONCLUSION

During the exploration of rare actinomycetes prevailing in forest-derived soil samples of Pobitora Wildlife Sanctuary of Assam, India, *Nocardia* sp. PB-52 was isolated by serial dilution technique. Based on phenotypic and molecular characteristics, the strain was identified as *Nocardia* sp. which shares 99.7% sequence similarity with *N. niigatensis* IFM 0330 (NR_112195). However, the differential phenotypic characteristics on agar media and utilization of carbon sources mainly adonitol and maltose reveal that the strain PB-52 may be classified within the genus *Nocardia* as a different or novel species. Thus to confirm it, further experiments are required such as cellular fatty acid composition, DNA-DNA relatedness value, whole cell sugar analysis etc. of strain PB-52 along with the closest species *N. niigatensis* IFM 0330 and additional type strains.

Extracellular metabolite produced by PB-52 strain exhibited a wide range of antimicrobial activity against Gram-positive bacteria including MRSA, Gram-negative bacteria and yeasts. The antimicrobial potential of *Nocardia* sp. PB-52 was positively influenced by appropriate carbon and nitrogen supplements in the GLM culture media along with the optimum cultural conditions. GLM media constitute of starch and peptone as the principal carbon and nitrogen sources respectively. The maximum production of the antimicrobial compounds was attained on the 8th day of growth at a temperature of 28°C with pH 7.4.

The antimicrobial activity of EA-PB-52 was further confirmed by SEM studies where considerable morphological alterations were observed in the test microbial pathogens. GC-MS analysis revealed that the broad spectrum antimicrobial activity of EA-PB-52 was due to the presence of biologically active compounds. Twelve different compounds were detected in EA-PB-52 which comprised of phenolic compounds, pyrrolizidines, quinones, hydrocarbons, esters, and acids and some of them are already reported to possess antimicrobial or other biological activities. 3,5-bis (1,1-dimethylethyl)-phenol and 2,4-di-t-butyl-6-nitrophenol were the two phenolic compounds detected in EA-PB-52. Phenolic compounds are commonly known as potent antimicrobial agents as well as antioxidant agents. Antimicrobial activity of 2,4-di-t-butyl-6-nitrophenol is already documented but 3,5-bis (1,1-dimethylethyl)-phenol is not reported as an antimicrobial agent. As 3,5-bis (1,1-dimethylethyl)-phenol occupied 34.43% of the total constituents present in EA-PB-52, it might be involved in antimicrobial action.

The genus *Nocardia* was previously reported for the production of antimicrobial compounds against microbial pathogens. Mukai et al. (2009) reported nocardithiocin from *N. pseudobrasiliensis* IFM 0757 active against *Mycobacterium* and *Gordonia* species. Transvalencin A, an antifungal antibiotic is produced from *N. transvalensis* IFM 10065 (Hoshino et al., 2004). Kavitha et al. (2009) reported two bioactive compounds bis-(2-ethylhexyl) phthalate and bis-(5-ethylheptyl) phthalate from *N. levis* MK-VL_113 which showed antagonistic activity against gram-positive bacteria, gram-negative bacteria, yeast, and filamentous fungi. Celmer et al. (1980) reported that *N. argentinensis* produced nargenicin A1 which was found to be active against MRSA. In this work *Nocardia* sp. PB-52 isolated from the soil samples of Pobitora Wildlife Sanctuary, Assam, India exhibited a wide range of antimicrobial activity against Gram-positive bacteria including methicillin resistant *S. aureus* (MRSA), Gram-negative bacteria and yeasts. However, there is no report available regarding the antimicrobial activity of *N. niigatensis* IFM 0330 (NR_112195) which is the closest type strain of PB-52. From our results, it is evident that PB-52 strain could be a promising candidate for the development of potential antimicrobial drug active against a wide range of microbial pathogens including drug resistant microorganisms such as MRSA.

AUTHOR CONTRIBUTIONS

DT supervised the research work and guided the experimental design. MK provided the research work suggestion. PS conducted the experiments, analyzed the data. DT and PS prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00347>

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Biological Significance of Marine Actinobacteria of East Coast of Andhra Pradesh, India

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An attempt was made to identify actinobacterial strains present in the marine soil of East Coast regions viz., Chirala, Bapatla, and Peddaganjam, Andhra Pradesh; Kanyakumari, Tamil Nadu and Goa, Goa along with the study of their antimicrobial potential. Eight out of 73 actinobacterial strains isolated from these regions showed strong antimicrobial activity against Gram positive bacteria, Gram negative bacteria, and *Candida albicans*. Molecular identification (16S rRNA analysis) of the eight strains revealed that they belong to *Dietzia* sp., *Kocuria* sp., *Nocardiopsis* sp., and *Streptomyces* spp. ISP (International Streptomyces project) -1, ISP-2 and starch casein media supported high antimicrobial potential after 5–6 days of growth. Production of antimicrobials by the strains varied significantly with different carbon and nitrogen sources. Gas chromatography mass spectrometry (GCMS) analysis of volatile compounds produced by the strains illustrated an array of antimicrobial compounds such as 1, 2-benzene dicarboxylic acid, 2-piperidinone, pyrrolo[1,2-a]pyrazine-1,4-dion, phenyl ethyl alcohol, 3-phenyl propionic acid etc. Ours is the first report on the study and detection of above mentioned antimicrobial metabolites from *Dietzia* sp. (A3), *Kocuria* sp. (A5), and *Nocardiopsis* sp. (A7). By sequence based analysis for secondary metabolites, non-ribosomal peptide synthetase (NRPS) gene cluster was noticed in six strains (A2, A3, A4, A6, A7, and A8) and none of them had polyketide synthase (PKS) system. The present study intimates the biological potentiality of the actinobacterial strains isolated from East Coast of Andhra Pradesh, India which reveals further scope to investigate new bioactive compounds from them by employing both natural product chemistry and modern biotechnological aspects.

Keywords: marine actinobacteria, *Dietzia*, *Kocuria*, *Nocardiopsis*, *Streptomyces*

INTRODUCTION

Pathogens causing infectious diseases are rapidly developing resistance towards traditional antibiotics (Chambers and DeLeo, 2009; Morens and Fauci, 2013; Ventola, 2015); therefore, there is an urgent necessity to search for safer and more potent compounds with broader spectrum of activity (Devine et al., 2017; WHO, 2017). Natural compounds or their derived products persist as a lead hub for the discovery of novel medicines to treat most of the human diseases. It has also been estimated that about 60% of the drugs that are available now including penicillin, anthracycline, bleomycins A₂ and B₂, mitomycin C, doxorubicin, epothilones, camptothecin, lovastatin etc. are mostly obtained from natural products (Lam, 2007; Newman, 2008; Cragg and Newman, 2013; Newman and Cragg, 2016). By knowing their potent bioactivities, organic chemists have developed

new drugs using modern advancements in synthetic biology (Beghyn et al., 2008; Maier, 2015; Rodrigues et al., 2016).

Originally considered as an intermediate group between bacteria and fungi, Actinobacteria include Gram-positive bacteria with high G+C (>55%) content in their DNA which provide fifty percent of bioactive metabolites as recorded in the Dictionary of Natural Products (Barka et al., 2016). Initially, they were considered to be native to terrestrial habitats, but they are also common in marine ecosystems (Behie et al., 2017; Betancur et al., 2017) as evident by the isolation of various genera like *Agrococcus*, *Arthrobacter*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Pseudonocardia*, *Rhodococci*, *Streptomyces* etc. (Claverias et al., 2015). The rate of finding new bioactive metabolites from the species of soil habitats has reduced. Therefore, a few attempts have been made to understand the microbial diversity of marine sediments which are an inexhaustible resource for the search of new drugs (Hassan et al., 2017).

Adaptation of marine actinobacteria to extreme climatic conditions such as high salinity, high pressure, and high temperature have modified their physiological conditions to survive and elaborate novel bioactive metabolites (Blunt et al., 2015, 2016, 2017; Behie et al., 2017; Kamjam et al., 2017). Approximately, 41 species belonging to 8 genera have been recorded from four states (Maharashtra, Kerala, Tamil Nadu, and Andhra Pradesh) of Indian Peninsula (Sivakumar et al., 2007) and have been shown to yield new bioactive compounds (Ramesh and Mathivanan, 2009; Ramesh and William, 2012). East Coast regions of Andhra Pradesh, India have not been much explored for the presence of micro-organisms that could produce potent new drugs against several diseases. Therefore, an attempt was made to study the diversity of marine actinobacteria in East Coast as well as their antimicrobial potential.

MATERIALS AND METHODS

Collection of Marine Soil Samples

Marine soil samples were collected at a depth of 14 cm from the surface of different marine habitats viz., Suryalanka (Bapatla region), Chirala, Peddapalem (Peddaganjam region) located near East Coast of Andhra Pradesh, India along with Kanyakumari, Tamil Nadu and Goa, Goa. They were air-dried and pretreated with calcium carbonate (1:1 w/w) followed by drying in a hot air oven at 45°C for 1 h, in order to reduce the contamination with bacteria and molds (El-Nakeeb and Lechevalier, 1963; Kavitha et al., 2010).

Isolation of Actinobacterial Strains

International *Streptomyces* project (ISP-1, Tryptone glucose yeast extract) and ISP-2 (Yeast extract-malt extract-dextrose) agar media were prepared, sterilized at 121°C for 15 min and poured into Petri plates under aseptic conditions. Antibiotics such as streptomycin and amphotericin-B were added to the media just before pouring into Petri plates. Soil dilution plate technique was used for the isolation and enumeration of actinobacterial strains (Williams and Cross, 1971; Kavitha et al., 2010). Marine soil (1 g) sample pretreated with calcium carbonate was suspended in 100 ml of sterile distilled water followed by plating 0.1 ml of 10⁻³

serial dilution on different Petri dishes. After incubation of the plates at 30°C for 10 days, actinobacterial strains were isolated by observing the characteristics like tough and leathery colonies which are partially embedded into the agar (Jensen et al., 1991).

Screening of Actinobacterial Strains for Potent Antimicrobial Metabolites Using Agar Well Diffusion Method

The secondary metabolites produced by the actinobacterial strains were extracted by using standard protocol (Ellaiah et al., 2005; Kavitha et al., 2010). Under aseptic conditions, actinobacterial strains were inoculated individually into the seed medium (ISP-2 broth) and incubated for 24 h. After that, the seed culture (10% v/v) was transferred into the production medium (ISP-2) and allowed to ferment for 5 days at 28 ± 2°C, 180 rpm. Sterile Whatman No. 1 filter papers were used to separate biomass from their culture filtrates. The collected biomass of all the strains was dried in a hot air oven and the residual dry weight was measured (mg/100 ml). The secondary metabolites obtained from all the culture filtrates were extracted with ethyl acetate twice, pooled individually and condensed under vacuum to yield solid residues. The residues were then resuspended in ethyl acetate and checked for their antimicrobial activity against the overnight grown cultures of *Bacillus megaterium* (NCIM 2187), *B. subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424) whereas 24–48 h old culture of *Candida albicans* (MTCC 183) was tested for evaluating antifungal activity.

The secondary metabolites produced by the strains were tested for their antimicrobial potentiality using agar well diffusion assay (Cappuccino and Sherman, 2002). Luria agar and Potato dextrose agar media were employed for the growth of test bacteria and fungus, respectively. About 0.1 ml of test bacterial/fungal suspension was transferred into the corresponding media (100 ml) sterilized previously at 15 lbs pressure (121°C) for 15 min. The inoculated medium was thoroughly mixed, poured into Petri plates and allowed to solidify under aseptic conditions. After that, wells of around 5 mm diameter were drilled into the agar medium with the help of a sterilized cork borer. The solvent extracts (50 µl) prepared at a concentration of 5 mg/ml were added to each well and ethyl acetate alone served as control. The inoculated plates were incubated at 30°C for 24 h and the diameter of inhibition zone was measured for bacteria and fungus.

Antimicrobial potential of the metabolites produced by the actinobacterial strains was examined to select the potent ones among the 73 isolated actinobacterial strains. Eight potent strains were chosen to determine their taxonomic position through cultural and molecular 16S rRNA gene fragment analysis.

Taxonomic Studies of the Eight Actinobacterial Strains

Cultural and molecular (16S rRNA gene sequencing) analysis of the strains were studied. Different ISP media viz., ISP-1 (Tryptone-yeast extract agar), ISP-2 (Yeast extract malt extract dextrose agar), ISP-3 (Oat meal agar), ISP-4 (Inorganic

salts starch agar), ISP-5 (Glycerol-asparagine salts agar), ISP-6 (Peptone yeast extract iron agar medium), and ISP-7 (Tyrosine agar) as well as non ISP media like Czapek Dox, Gauze, Maltose tryptone, Nutrient, and Potato dextrose agar with initial pH 7.2 maintained at 30°C were used to monitor the characteristics of the organisms (Dietz and Theyer, 1980). Cultural characters including growth, color of the aerial mycelia and substrate mycelia with their pigmentation were recorded.

Molecular Identification of the Potent Actinobacterial Strains Through Genomic (16S rRNA Gene Fragment) Analysis

For the extraction of genomic DNA (gDNA), the cell mass of the strains grown individually in maltose tryptone broth at 30°C for 2–3 days was centrifuged at 10,000 rpm, 4°C for 20 min (Stach et al., 2003). The cells were resuspended immediately in 5 ml of TE25S buffer followed by rapid vortexing. The cells were exposed to heat shock treatment (kept in water bath at 90°C for 5–10 min with immediate cooling on ice) followed by the addition of 250 µl of lysozyme (100 mg/ml) for proper cell wall lysis. Two microliters of RNase A (20 mg/ml) was also added to this mixture and incubated at 37°C for 1–2 h with proper inversion at every 20 min. To this, 100 µl of pronase (20 mg/ml) and 300 µl of 10% SDS were added, mixed by inversion occasionally and incubated at 55°C for 2–3 h. Further, 1 ml of 5M NaCl and 325 µl of 10% CTAB were added, mixed thoroughly and incubated for 10 min at 65°C. Later, chloroform: isoamyl alcohol (24:1) solvent system was added to the mixture and incubated for 30 min at 37°C. The aqueous phase obtained after centrifugation at 8,000 rpm for 5 min was transferred to a fresh tube. An equal volume of ice-cold isopropanol was added to the aqueous phase, mixed by gentle inversion for 10 min at 37°C and centrifuged at 12,000 rpm, 4°C for 10 min. The white DNA precipitate obtained was washed with 1 ml of ice-cold 70% ethanol followed by another centrifugation step to remove excess ethanol. The pellet was air-dried and 100 µl of sterile H₂O or 0.2X TE buffer was added to dissolve the DNA. The same procedure was followed for all the strains to get good quality of gDNA for further amplification of 16S rRNA region.

The 16S rRNA gene segment of actinobacterial strains was amplified by polymerase chain reaction (PCR) individually in a reaction mixture containing 1X PCR buffer (ThermoFisher Scientific, USA), 2 U of *Taq* polymerase, each deoxynucleoside triphosphate at a concentration of 200 µM, 50–100 ng of gDNA, 20 µM of primer forward (5'-GAGTTTGATCCTGGCTCA -3') and 20 µM of primer reverse (5'-ACGGCTACCTGTTACGACTT -3'). The final volume of the PCR mixture was made up to 100 µl by adding distilled H₂O and the reaction mixture was overlaid with 80 µl of sterile mineral oil. Thermal cycling was carried out with a model S1000 (Bio-Rad, USA) and all the samples were subjected to an initial denaturation (3 min at 98°C) followed by denaturation (1 min at 94°C), annealing (1 min at 52°C, 28 consecutive cycles), extension (2 min at 72°C), and a final extension (5 min at 72°C) step at the end.

The amplified DNA fragment from all the strains was monitored on 1% agarose gel, eluted, and purified using

Nucleospin gel extraction kit (Macherey-Nagel, Germany). The purified PCR products were sequenced using the Big-Dye terminator kit ABI 310 Genetic Analyzer (Applied Biosystems, USA) and further recorded their accession numbers by depositing them in National Center for Biotechnology Information (NCBI) GenBank. The 16S rRNA sequences were compared with that of related sequences obtained from GenBank through NCBI BLAST search program. Nucleotide substitution rates (Knuc values) were evaluated (Kimura, 1980). Phylogenetic tree using neighbor-joining method was constructed (Saitou and Nei, 1987) along with the statistical analysis of bootstrap values by employing Molecular Evolutionary Genetics Analysis (MEGA7) software (Thompson et al., 1997).

Nutritional Parameters Affecting the Production of Antimicrobial Metabolites Growth Pattern and Effect of Incubation Time on the Production of Antimicrobial Metabolites

Growth pattern of the eight actinobacterial strains and their antimicrobial activity against Gram positive bacteria (*B. megaterium* and *S. aureus*) and Gram negative bacteria (*Enterococcus faecalis* and *P. aeruginosa*) was recorded in ISP-2 medium for 8 days. Biomass was measured as dry weight of the cell mass (mg/100 ml culture medium) and the supernatant was extracted with ethyl acetate, vacuum dried in a rotavapor followed by testing the residues (1 mg/ml) for antimicrobial activity against bacteria through agar well diffusion method using the diameter of inhibition zone (mm) (Cappuccino and Sherman, 2002).

Effect of Culture Media Composition on the Production of Antimicrobial Metabolites

Effect of growth media on the production of antimicrobial metabolites was studied by culturing the strains separately in different media viz., Arginine-glycerol (Arg-Gly), Czapek-Dox (Dox), ISP-1, ISP-2, ISP-4, Luria broth (LB), Maltose-trypotone (MT), Nutrient broth (NB), Starch-casein (SC), Yeast mannitol broth (YMB), and Yeast extract-peptic digest of animal tissue-dextrose (YPD) broths. Efficiency of the secondary metabolites of the strains was recorded as antimicrobial potential against Gram positive and Gram negative bacteria by employing agar well diffusion assay. The medium in which the strain elaborates maximum levels of antimicrobials was studied individually for all the eight strains (Kavitha and Vijayalakshmi, 2009).

Influence of Carbon and Nitrogen Sources on the Yield of Antimicrobial Metabolites

Various carbon sources such as dextrose, galactose, glycerol, maltose, mannitol, starch, sucrose, and xylose were added to the optimized media by replacing their carbon source. Likewise, the impact of different nitrogen sources on the yield of antimicrobials of the strains was studied by supplementing the nitrogen source in the medium with different nitrogen sources like ammonium chloride, aspartic acid, L-arginine, potassium nitrate, tryptone, urea, and yeast extract in the optimized carbon medium (Kavitha and Vijayalakshmi, 2009).

Detection of Possible Secondary Metabolites through Gas Chromatography–Mass Spectral Analysis (GC-MS) and Screening of Biosynthetic Gene Clusters

Analysis of Volatile Compounds by GCMS

The components of the biologically active crude extracts obtained from the strains were analyzed through Agilent GC-MS apparatus (GC: 7890A; MSD5975C). It has fused-silica HP-5 capillary column (30 m–0.25 mm, ID, film thickness of 0.25 mm) coupled directly with single quadrupole MS. Flow rate of the carrier gas, helium was maintained at 1 ml/min. Oven temperature was automated (50°C for 1 min, then 50–300°C at a rate of 5°C/min) and subsequently, held isothermally for 5 min. The temperature of injector port was kept at 250°C and that of transfer line at 300°C. MS quadrupole and MS source temperatures were maintained at 150 and 230°C, respectively (Boussaada et al., 2008). The peaks detected in GC were concluded as corresponding compounds through mass spectral data analysis software and NISTMS library data, 2008.

Preliminary Detection of Non-ribosomal Peptide Synthetases (NRPS) and Type I Polyketide Synthases (PKS-I) Biosynthetic Gene Systems

NRPS and PKS-I are the major biosynthetic gene sequences in micro-organisms involved for the production of bioactive polyketide and peptide compounds. Therefore, gDNA of the eight strains was checked for the presence or absence of these biosynthetic systems using actinobacterial specific NRPS and PKS-I PCR primers (Ayuso-Sacido and Genilloud, 2005). A 50 μl reaction cocktail containing 5 μl gDNA as template, 0.4 μM of each primer [A3F (5' GCSTACSYSATSTACACSTCSGG 3') and A7R (5' SASGTCVCCSGTSCGGTAS 3'), for NRPS; K1 (5' TAAAGTCAACATCGGBCA 3'), and M6R (5' CGCAGGTTCSGTACCAAGTA 3') for PKS-I], 0.2 mM of each of the four dNTPs, 1 U *Taq* polymerase, 10X *Taq* buffer, and 10% DMSO was prepared for all the strains separately. PCR amplification protocol for the expected product size included initial denaturation (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30 sec), annealing (59°C for 2 min—NRPS and 58°C for 2 min—PKS-I), and extension (72°C for 4 min). The amplified PCR products obtained after a final extension step at 72°C for 10 min were monitored on 1% (w/v) agarose gel electrophoresis.

RESULTS AND DISCUSSION

Enumeration of Actinobacterial Strains Isolated from Different Marine Soil Samples and Testing the Efficiency of Their Antimicrobials

A total of 73 actinobacterial strains were isolated from soil samples of East Coast of Indian marine ecosystem *viz.*, Chirala, Bapatla, Peddaganjam, Andhra Pradesh along with Kanyakumari, Tamil Nadu and Goa, Goa. As depicted in

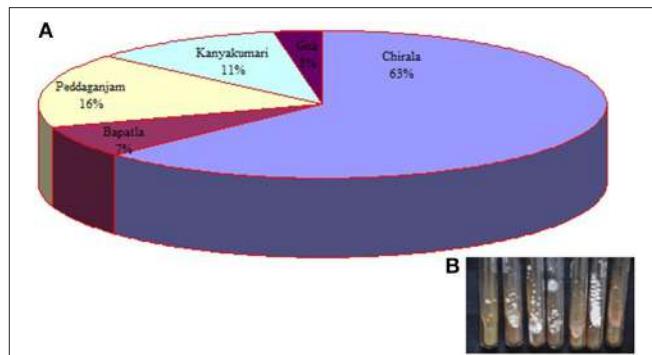


FIGURE 1 | (A) Pie diagram illustrating the percentile of actinobacterial strains isolated from marine soil samples of Chirala, Bapatla, Peddaganjam, Andhra Pradesh; Kanyakumari, Tamil Nadu and Goa, Goa, **(B)** Image of few actinobacterial strains.

Figure 1, majority of the actinobacterial strains were obtained from the marine soil samples of Chirala (63%) followed by Peddaganjam (16%) by serial dilution method on ISP-2 medium supplemented with 3% (w/v) NaCl. All the 73 actinobacterial strains isolated from the different marine soil samples were evaluated for their antimicrobial activity as described in Materials and Methods Section (**Figures 2A,B**). Among those, strains 24, 25, 26, 28, 30 (strains from Chirala region), 31 (strain from Bapatla region), 33 and 34 (strains from Peddaganjam region) exhibited high antimicrobial activity against the bacteria tested.

Marine ecosystem serves as an attractive source for the isolation and production of bioactive compounds (Blunt et al., 2015, 2016, 2017; Kamjam et al., 2017), bioactive pigments (Soliev et al., 2011), enzymes (Ramesh and William, 2012; Leipoldt et al., 2015), biofuels (Lewin et al., 2016), and also showed potential for biominerization activities along with the maintenance of nutrient web cycle, biological N₂ fixation, and environmental protection (Das et al., 2006; Biswas and Gresshoff, 2014; Alvarez et al., 2017). Around 9% actinobacteria was recorded in marine sediments (Bull et al., 2005) and suggested as a stable and prominent bioactive group of micro-organisms in marine ecology from the earlier findings (Claverias et al., 2015; Betancur et al., 2017). The present study also highlights the existence of numerous actinobacterial strains with various bioactivities from different marine coastal regions of India. Eight out of 73 strains (24, 25, 26, 28, 30, 31, 33, and 34) isolated from East Coast of Andhra Pradesh had shown pronounced bioactivity and designated them as A1, A2, A3, A4, A5, A6, A7, and A8 for further taxonomic and nutritional studies.

Cultural and Molecular Identification of Actinobacterial Strains

Growth and cultural characteristics of the eight actinobacterial strains were studied on ISP and non-ISP agar media (**Table 1**). All the strains exhibited good to moderate to poor growth patterns on different media tested. Strains A1, A3, A4, A5, and A6 showed white to pink aerial mycelia with brown substrate mycelia whereas the strains A2 and A7 exhibited creamy aerial

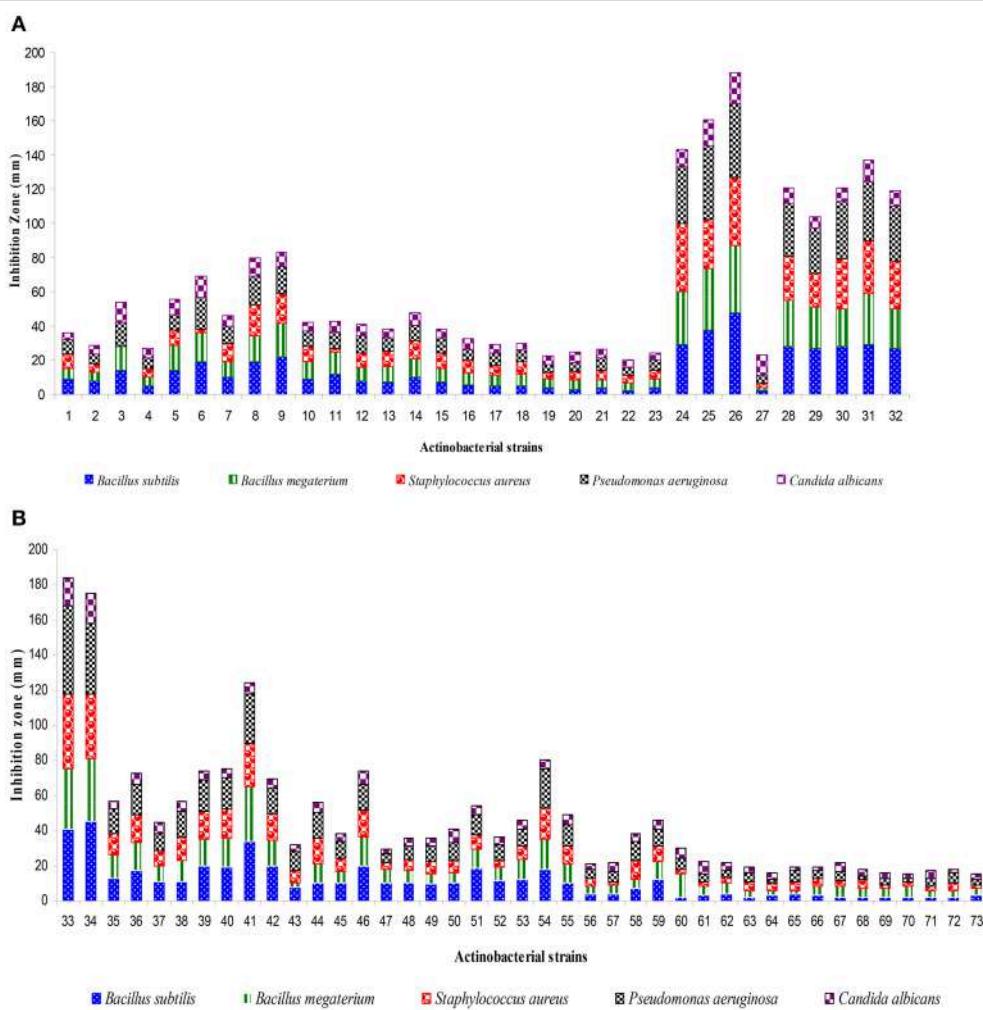


FIGURE 2 | Screening of actinobacterial strains (1–73) for antimicrobial metabolites by using agar well diffusion assay. **(A)** Strains 1–32, **(B)** Strains 33–73. Based on antimicrobial profile, actinobacterial strains viz., 24, 25, 26, 28, 30, 31, 33, and 34 were selected for further study and designated as A1–A8. Data was statistically analyzed by One-way ANOVA and found to be significant at 5% level ($P < 0.05$) between the strains.

mycelia and dark brownish substrate mycelia. Diffused yellow pigmentation was observed with only strain A8, having white to light yellowish aerial mycelia and brown substrate mycelia. Actinobacteria are ubiquitous in nature and includes a wide range of color series ranging from gray, white, yellow, red, green, blue to black (Barka et al., 2016).

Molecular identification of the strains was carried out through 16S rRNA gene fragment analysis, a powerful tool to recognize micro-organisms up to genus level (Barka et al., 2016). Figures 3A,B depicts the isolation of gDNA from all the strains and amplification with actinobacterial specific 16S rRNA gene fragment primers as described in Section Materials and Methods. The amplified products were sequenced and the phylogenetic position of the strains was analyzed through NCBI Blast search program. The results revealed that the strains viz., A3, A5, and A7 belong to genera *Dietzia*, *Kocuria*, and *Nocardiopsis* with 98, 99, and 100% similarity, respectively; while the remaining ones A1, A2, A4, A6, and A8 showed similarity index between 97 and

100% to the genus *Streptomyces* (Figure 3C). The 16S rRNA gene sequence of all the strains has been deposited in NCBI Genbank with the accession numbers KF017344 (A1—*Streptomyces* sp.), KF017345 (A2—*Streptomyces* sp.), KF006391 (A3—*Kocuria* sp.), KF896236 (A4—*Streptomyces* sp.), KC841637 (A5—*Dietzia* sp.), KF017342 (A6—*Streptomyces* sp.), KF006394 (A7—*Nocardiopsis* sp.), and KF896235 (A8—*Streptomyces* sp.).

Nutritional Parameters Affecting the Production of Antimicrobial Metabolites

Secondary metabolites especially antimicrobial compounds produced during iodiophase are the natural missiles to combat infectious diseases. Elaboration of secondary metabolites is highly dependent on the growth and nutritional parameters (Gonzalez et al., 2003; Jose et al., 2013). Growth pattern of the eight strains studied individually in ISP-2 medium for 8 days and their interesting antimicrobial profiles were tested against Gram positive and Gram negative bacteria by employing agar

TABLE 1 | Cultural characteristics of the actinobacterial strains (A1–A8) on different culture media.

Actinobacterial strains	Cultural characters on different media												
	1	2	3	4	5	6	7	8	9	10	11	12	13
A1	G	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good
	A	Cream	Cream	Dark cream	White	White	Dark cream	White	White	Light pink	Cream	Cream	Light pink
	R	Light brown	Cream	Light brown	Light brown	White	Brown	Light brown	Light brown	Creamy brown	Light brown	Light brown	Light brown
	P	—	—	—	—	—	—	—	—	—	—	—	—
A2	G	Good	Good	Good	Good	Poor	Good	Good	Good	Moderate	Good	Good	Good
	A	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream	White	Cream	Cream	Cream
	R	Creamy brown	Creamy brown	Reddish brown	Dark brown	White	White	Light brown	White	Dark brown	Creamy brown	Black	Black
	P	—	—	—	—	—	—	—	—	—	—	—	—
A3	G	Good	Good	Good	Moderate	Good	Good	Good	Good	Good	Good	Good	Good
	A	Light pink	Light pink	Light pink	White	Cream	White	Creamy brown	Dark brown	White	Light pink	Light pink	Light pink
	R	Light brown	Light brown	Creamy brown	White	Cream	White	White	Brown	Light brown	Light brown	Brown	Brown
	P	—	—	—	—	—	—	—	—	—	—	—	—
A4	G	Good	Good	Good	Good	Moderate	Good	Good	Good	Moderate	Good	Good	Good
	A	Light pink	Light pink	Light pink	Cream	White	Dark pink	Dark pink	Light pink	Light pink	Light pink	Light pink	Dark pink
	R	Brown	Dark brown	Dark brown	Cream	Light brown	White	Reddish brown	Reddish brown	Dark brown	Creamy brown	Creamy brown	Dark brown
	P	—	—	—	—	—	—	—	—	—	—	—	—
A5	G	Good	Good	Good	Moderate	Good	Good	Good	Good	Moderate	Good	Good	Good
	A	Cream	Cream	Light yellow	White	White	White	White	White	Dark pink	Light pink	Light pink	Light pink
	R	Light brown	Cream	Creamy brown	White	White	Light brown	White	White	Dark brown	Dark brown	Dark brown	Dark brown
	P	—	—	—	—	—	—	—	—	—	—	—	—
A6	G	Good	Good	Poor	No	No	Good	No	Good	No	Good	Poor	Good
	A	Light pink	Cream	Light pink	—	—	Dark cream	—	Light brown	—	Light pink	Light pink	Pink
	R	Light brown	Light brown	Dark brown	—	—	Dark brown	—	Dark brown	—	Dark brown	Dark brown	Dark brown
	P	—	—	—	—	—	—	—	—	—	—	—	—

(Continued)

TABLE 1 | Continued

Actinobacterial strains	Cultural characters on different media											
	1	2	3	4	5	6	7	8	9	10	11	12
A7	G Good	A Moderate	Cream Good	Cream Good	Cream Good	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown
	G Cream	A Cream	C Dark brown	D Dark brown	C Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown
	R Dark brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -
	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -
A8	G Good	A Moderate	C White	C White	W White	Y Yellow to light brown	Y Yellow to light brown	Y Yellow to light brown	Y Light brown	Y Light brown	Y Yellow	Y Yellow
	G White	A White	C Light brown	C Light brown	W Yellow	Y Yellow	Y Yellow	Y Yellow	Y Yellow	Y Yellow	Y Yellow	Y Yellow
	R Yellow to light brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -	R Light brown	P -
	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -	P -

G, Growth; A, Aerial mycelium; R, Reverse mycelium; P, Pigment production. Media—1 International Streptomyces Project (ISP) agar, 2 ISP-2 agar, 3 ISP-3 agar, 4 ISP-4 agar, 5 ISP-5 agar, 6 ISP-6 agar, 7 ISP-7 agar, 8 Asparagine glucose agar, 9 Czapek-Dox agar, 10 Gauze agar, 11 Maltose tryptone agar, 12 Nutrient agar, 13 Potato dextrose agar.

well diffusion assay (**Figures 4A–D**). Log phase of all the strains appeared within 48 h of growth period. Strains A2, A3, A4, and A7 entered stationary phase on 4th and 5th days of incubation and then declined while the strains A5, A6, and A8 exhibited stationary growth on 5th and 6th days. Strain A1 showed a little bit prolonged stationary phase between 5th and 7th days of growth.

In most of the actinobacterial strains, production of antimicrobials happens mostly after 4–6 days of growth (Kavitha and Vijayalakshmi, 2009; Kavitha et al., 2010) and those recorded from 5 days old culture of *Streptomyces* spp. showed best potential (Manivasagan et al., 2014). In the present study, the ethyl acetate extracts obtained from 5th day old cultures of the strains A1, A2, A3, A4, A6, and A7 exerted high antimicrobial activity against the organisms tested whereas the strains A5 and A8 produced better yields of antimicrobials after 6 days of incubation. Out of the eight strains, secondary metabolites obtained from the strain A4 (*Streptomyces* sp.) were highly active on *E. faecalis* followed by those of strain A6 (*Streptomyces* sp.). Ethyl acetate extracts of strains A8 (*Streptomyces* sp.) and A5 (*Dietzia* sp.) are more effective against *P. aeruginosa*. Other strains, A3 (*Kocuria* sp.) and A7 (*Nocardiopsis* sp.) showed more or less similar antimicrobial pattern on all the bacteria tested. Claverias et al. (2015) recorded the antimicrobial profile of marine actinobacteria including *Streptomyces* and *Dietzia* isolated from Valparaíso bay, Chile. Kamjam et al. (2017) reviewed the bioactive compounds produced by deep sea *Streptomyces* spp. and *Nocardiopsis* spp. To our knowledge, this is the first report on the antimicrobial profile of a marine strain A3 (*Kocuria* sp.).

Nutritional parameters greatly influence the biosynthesis of antimicrobial compounds which may be varied for different strains. Therefore, the secondary metabolites produced by eight strains in 11 different growth media are illustrated (**Figures 5A–D**). ISP-2 medium served as the best culture medium for the production of antimicrobial metabolites by the strains A1, A3, A4, A5, and A6. High yields of antimicrobials were observed for the strains A7 and A8 when cultured in ISP-1 medium. Starch-casein broth supported high antimicrobial activity against the test bacteria for strain A2 followed by YPD. Other media like Czapek-Dox (for strain A3), YPD (for strains A5 and A7), ISP-5 and Starch-casein (for strain A1), YMB and YPD (for strain A6), YMB and ISP-2 (for strain A8) stood next preferential ones for the production of antimicrobial metabolites. Among all, strains A2 and A4 showed potent antimicrobial activity against the bacteria tested. Earlier findings also illustrated ISP-2 (Rateb et al., 2014) and starch casein as suitable media for the production of secondary metabolites by *Streptomyces* spp. (Djinni et al., 2013).

Synthesis of antibiotics depends on the type of nutrients amended in the culture media. Especially, carbon and nitrogen sources play a crucial role on the biosynthesis of secondary metabolites by the strains both at level of activity and over expression of the genes corresponding to the enzymes involved (Sanchez et al., 2010). Initially, production of antimicrobial metabolites by the strains in the selected media supplemented with different carbon sources were tested (**Figures 6A–D**). The

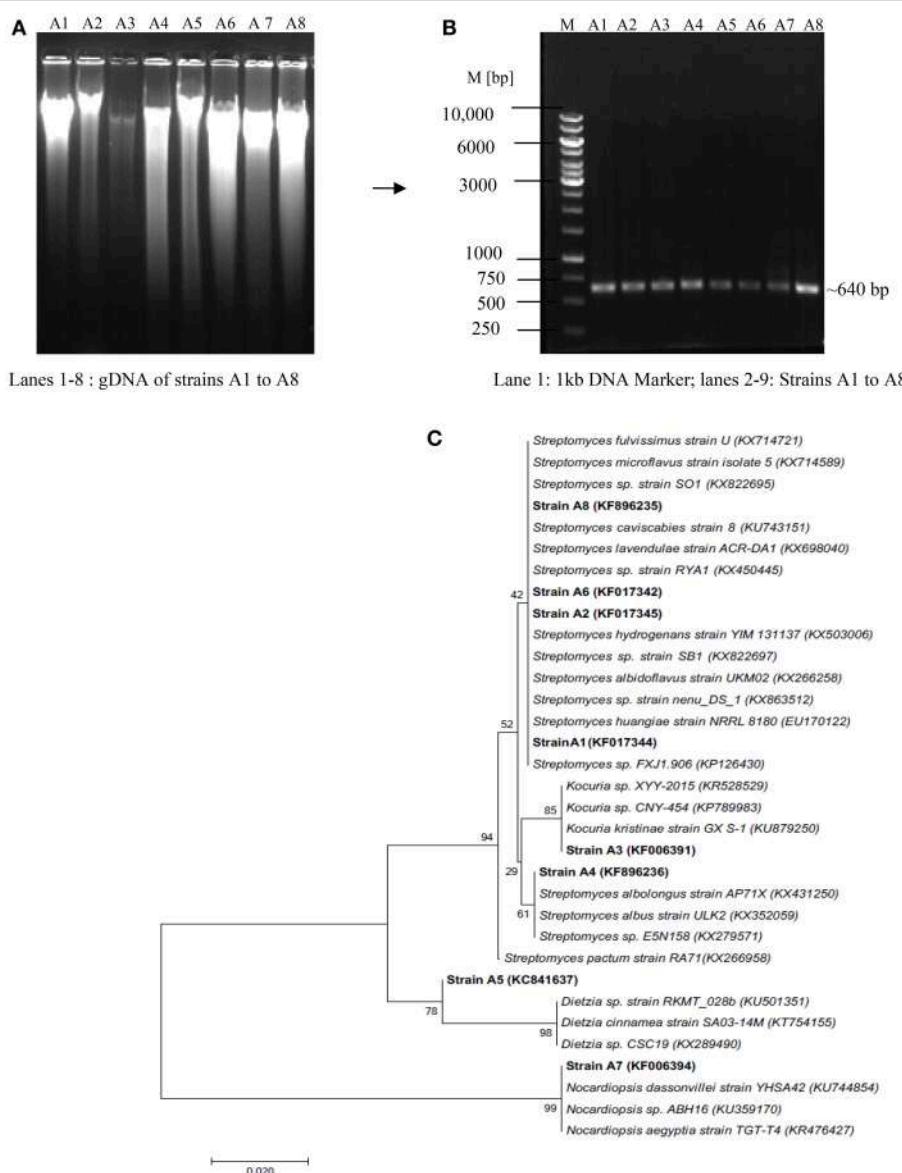


FIGURE 3 | Molecular identification of actinobacterial strains through 16S rRNA gene fragment analysis **(A)** Isolation of Genomic DNA, **(B)** PCR amplification of gDNA with Act 254F and 894R primers **(C)** Phylogenetic tree showing taxonomic position of the isolated actinobacterial strains with respect to the sequences deposited in NCBI Genbank.

secondary metabolites of the strain A1 showed promising antimicrobial activity against the bacteria tested when cultured in ISP-2 medium amended with mannitol followed by xylose, maltose, and galactose. Starch and galactose acted as promising carbon sources for the production of antimicrobial metabolites by the strain A2. Strains A3 and A7 showed optimal rates of secondary metabolites when grown in xylose enriched ISP-2 and ISP-1 media, respectively. Among the carbon sources tested, ISP-2 medium with dextrose remained as the best carbon source for the strain A4 to yield optimal antimicrobial metabolites. Strain A5 produced optimal yields of antimicrobial metabolites in the medium with starch followed by xylose. High levels of secondary

metabolites were produced by the strain A6 in ISP-2 medium incorporated with mannitol whereas dextrose in ISP-1 medium replaced with other carbon sources like galactose followed by starch and sucrose enhanced the synthesis of antimicrobials of the strain A8. Out of all the strains, strains A5 (*Dietzia* sp.), A8 and A1 (*Streptomyces* spp.) exhibited strong antimicrobial potential. Among the bacteria tested, *P. aeruginosa* showed maximum sensitivity to the secondary metabolites of the strains A5 followed by A8 and A1.

The yield of secondary metabolites varied with different carbon sources in different strains. Dextrose enriched culture medium supported the production of secondary metabolites

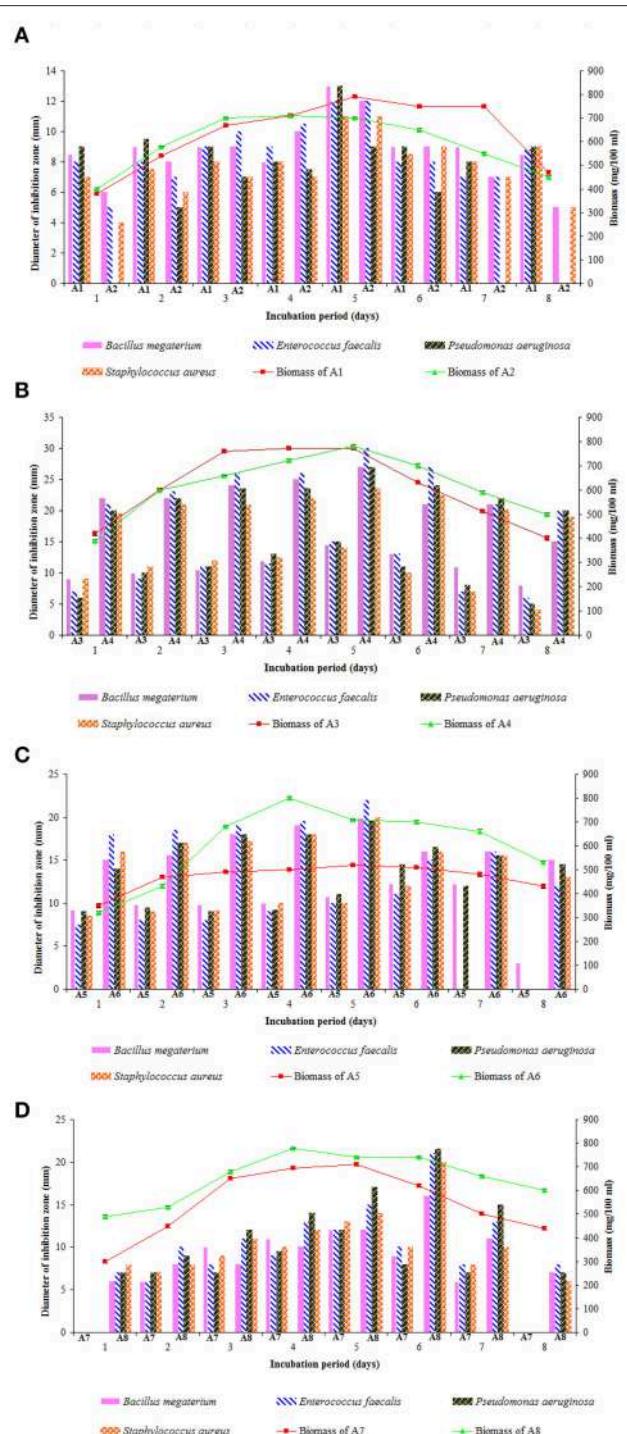


FIGURE 4 | Growth pattern of actinobacterial strains and their antimicrobial potential in ISP-2 medium **(A)** A1 and A2, **(B)** A3 and A4, **(C)** A5 and A6, and **(D)** A7 and A8. Actinobacterial strains exhibited iodophase between 4th and 5th (A2, A3, A4, and A7), 5th and 6th (A5, A6, and A8), and 5th–7th days (A1) of growth period. The secondary metabolites obtained from 5-day old cultures of A1, A2, A3, A4, A6, and A7 showed high antimicrobial activity against Gram-positive bacteria and Gram-negative bacteria while those obtained from 6 days of the strains A5 and A8 had pronounced effect. Data was statistically analyzed by Two-way ANOVA and had no significant difference between the strains.

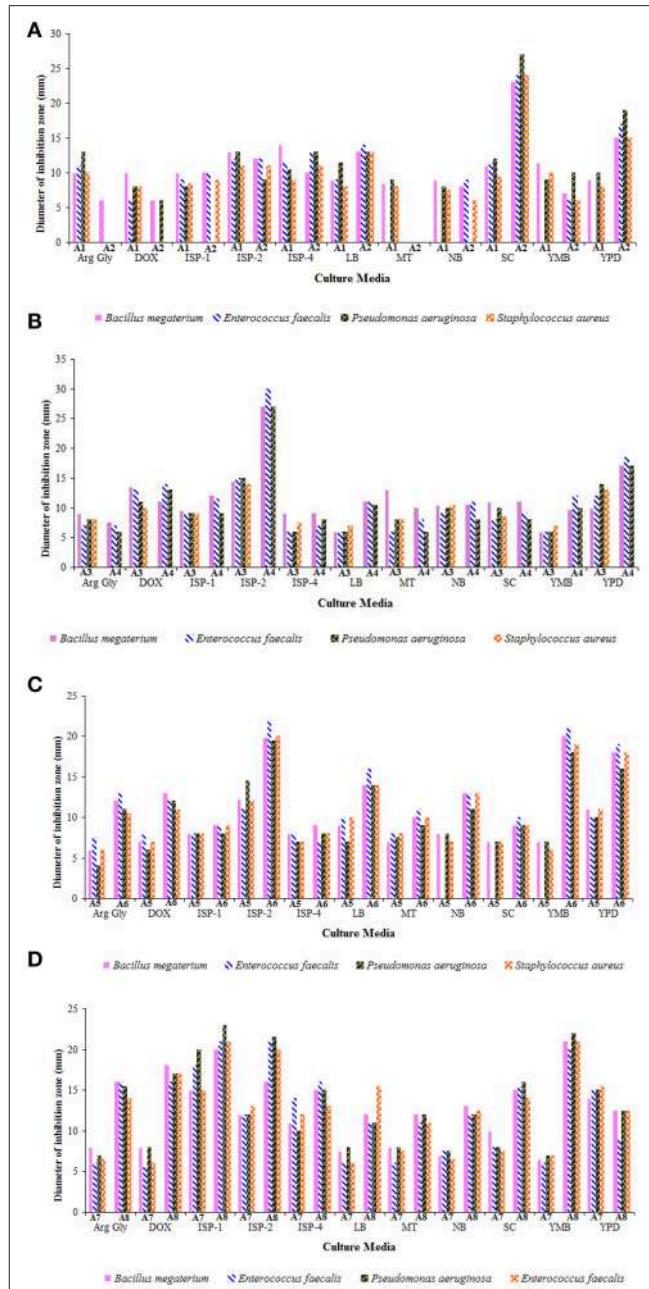
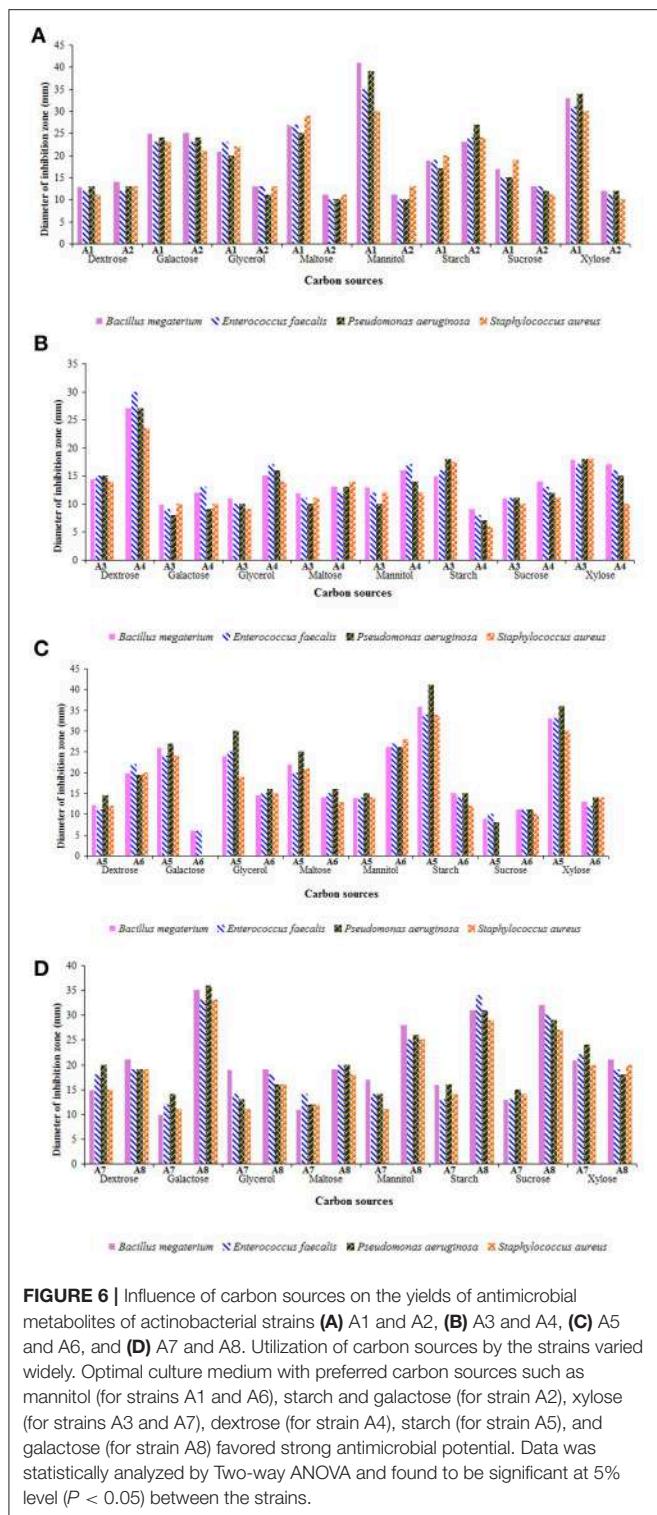


FIGURE 5 | Effect of culture media on the yields of antimicrobial metabolites of actinobacterial strains **(A)** A1 and A2, **(B)** A3 and A4, **(C)** A5 and A6, and **(D)** A7 and A8. Arginine-glycerol (Arg-Gly), Czapek-Dox (Dox), ISP-1, ISP-2, ISP-4, Luria broth (LB), Maltose-tryptone (MT), nutrient broth (NB), Starch-casein (SC), Yeast mannitol broth (YMB), and Yeast extract-peptic digest of animal tissue-dextrose (YPD) broths. ISP-2 medium supported better yields of antimicrobial metabolites for majority of the strains (A1, A3, A4, A5, and A6) whereas the strain A2 preferred starch casein broth for its antimicrobial activity. Other strains, A7 and A8 elaborated high antimicrobial activity when grown in ISP-1 medium. Data was statistically analyzed by Two-way ANOVA and found to be significant at 5% level ($P < 0.05$) between the strains.

by strain A4 in agreement with the earlier reports on marine *Streptomyces* sp. (Manikkam et al., 2015; Haque et al., 2017). But, indeed it was less efficient in increasing the yields of



secondary metabolites by most of the strains, when compared to that of other sugars. It can be inferred from these results that the simple sugars like dextrose are easily metabolized and utilized rapidly in the early log phase itself, whereas the other sugars may favor the growth of the organisms

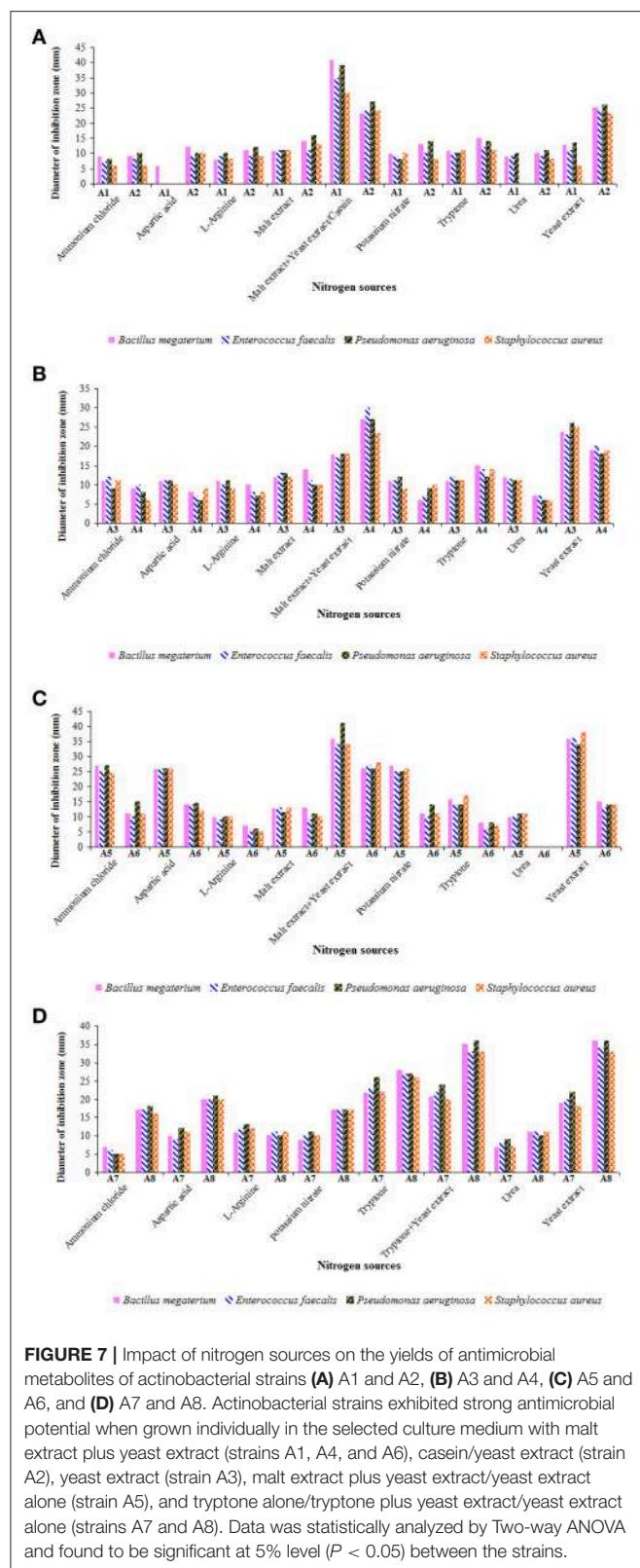
even up to stationary phase for the optimal release of secondary metabolites. Sengupta et al. (2015) also recorded that most of the actinobacterial strains isolated from Sundarbans mangrove ecosystem preferred polysaccharides (D-galactose and D-raffinose) over monosaccharides for antibiotic production. Sunita et al. (2015) observed maximum yields of antimicrobial metabolites by *Streptomyces* spp. in starch enriched medium.

Utilization of different nitrogen sources in the growth medium by actinobacterial strains are critical for secondary metabolite production (Francois and Stephane, 2001; Yao and Ye, 2016). Hence, the effect of various nitrogen sources on the yields of antimicrobial metabolites of the strains was presented in Figures 7A–D. Out of nine nitrogen sources, malt extract and yeast extract served as suitable combination in ISP-2 medium for the elaboration of antimicrobial metabolites by the strains A1, A4, and A6. Strain A2 exhibited high antimicrobial activity when cultured in starch casein medium as well as with yeast extract amendment. Strain A3 yielded optimal levels of antimicrobials in ISP-2 medium incorporated with only yeast extract (minus malt extract combination) whereas malt extract plus yeast extract and yeast extract alone favored high rates of secondary metabolites from the strain A5. Other nitrogen sources like potassium nitrate, ammonium chloride and aspartic acid also supported better yields of metabolites for the latter ones. ISP-1 media containing tryptone alone or tryptone plus yeast extract or yeast extract alone were quite suitable for the production of antimicrobial metabolites by the strains A7 and A8.

Rateb et al. (2014) isolated trirandamycin, an antifilarial drug lead from *Streptomyces* sp. 17,944 cultured in traditional ISP-2 (containing both malt extract and yeast extract) as well as under optimized conditions. Other researchers (Kavitha and Vijayalakshmi, 2009; Ripa et al., 2009) also proved yeast extract as the best nitrogen source for antimicrobial metabolites. While screening *Streptomyces* spp. for the elaboration of antimicrobial metabolites. Khaliq et al. (2013) reported tryptone enriched medium for one of the strains SK-5. In the present study, the antimicrobial metabolites of the strains A5 and A8 were highly effective against *P. aeruginosa* whereas those produced by the strain A1 are more active on *B. megaterium*. Ours is the first report on the production and optimization of antimicrobial metabolites by *Dietzia* sp. (A3) and *Kocuria* sp. (A5). Secondary metabolites of other strains (A1, A2, A4, A6, and A8) belonging to *Streptomyces* spp. and *Nocardiopsis* sp. (A7) showed strong antimicrobial potential as evident from the earlier findings (Manivasagan et al., 2014; Kamjam et al., 2017).

Analysis of Volatile Compounds from Biologically Active Crude Ethyl Acetate Extracts of the Strains by GCMS

Antimicrobial metabolites including volatile compounds were detected from actinobacteria through standard chromatographic methods (Bucar et al., 2013). Volatile compounds have been reported to exhibit diverse functions such as antibacterial, antifungal, plant growth accelerator or suppressor, infochemical molecules in inter and intra specific interactions, cell-to-cell signaling etc. (Scholler et al., 2002; Cordovez et al., 2015;



Schmidt et al., 2015; Zothanpuia et al., 2017). Therefore, the culture filtrates of actinobacterial strains obtained after fermentation were extracted with ethyl acetate and analyzed

for volatile compounds by using GCMS. A wide variety of chemical compounds were detected using GCMS library data (Supplementary Table, **Figures 8, 9**). All the strains showed an array of chemical compounds like amines, acids, pyrrolizidines, ketones, quinones, alcohols, and hydrocarbons. In the present study, production of 1, 2-benzene dicarboxylic acid by all the strains except strain A6 was recorded which may contribute for their antimicrobial potentiality. It has been reported to exhibit anti-extended spectrum β -lactamase activity (Subashini and Krishnan, 2014) as well as anticancer agent against HepG2 and VERO cell lines (Sudha and Selvam, 2012), HepG2 and MCF-7 cell lines (Krishnan et al., 2014). Phenyl ethyl alcohol of strain A2 and 3-phenyl propionic acid from strain A4 are well-known antimicrobial agents against bacteria and fungi (Narayana et al., 2008).

Antimicrobial potential of the strains A1, A3, A5, and A6 may also be due to the elaboration of 2-piperidinone and pyrrolo[1,2-a]pyrazine-1,4-dione from the strains A7 and A8. 2-piperidinone and pyrrolo[1,2-a]pyrazine-1,4-dione are characterized as one of the potential antimicrobials from *Streptomyces* sp. (Khattab et al., 2016) and fungus, *Schistidium antarctici* (Melo et al., 2014), respectively. Heterocyclic compounds like pyrazines are having two nitrogen atoms in their aromatic ring and are reported to have various bioactivities such as antimicrobial, antioxidant, anticancer, neuroprotection against ischemia/reperfusion injuries, and hypoxia (Premkumar and Govindarajan, 2005; Jia et al., 2009; Baldwin et al., 2013; Tan et al., 2015; Ser et al., 2016). Ours is the first report on the production of antimicrobial compounds viz., 2-piperidinone by *Dietzia* sp. (A3) and *Kocuria* sp. (A5) and 1, 2-benzene dicarboxylic acid by the former ones including *Nocardiopsis* sp. (A7). Further purification methods need to be standardized to characterize other interesting bioactive compounds produced by these strains.

Preliminary Detection of NRPS and PKS-I Biosynthetic Gene Systems

Apart from the isolation of antimicrobial compounds through classical extraction methods, genome-based natural product discovery also directs to most possible promising routes for searching novel secondary metabolites from various marine actinobacteria. The synthesis of antimicrobial compounds including polyketide and peptide compounds involves the biosynthetic gene clusters of NRPS and PKS-I, or even the combination of both (Undabarrena et al., 2017). Therefore, an attempt was made to check the presence of these biosynthetic systems in the gDNA of eight strains through PCR specific primers. Out of eight strains, 700 base pair nucleotide fragment specific to NRPS was recorded in the gDNA of six strains (**Figure 10**) whereas the PKS-I system (1,200–1,400 bp product) was not observed in all the strains tested.

Structurally, biosynthetic systems like NRPS and PKS consist of multifunctional polypeptides with diverse number of modules having different enzymatic properties. The modules of NRPS system constitute the activities related to condensation, adenylation, and thiolation which are mainly concerned in the recognition and condensation of the substrate. On the

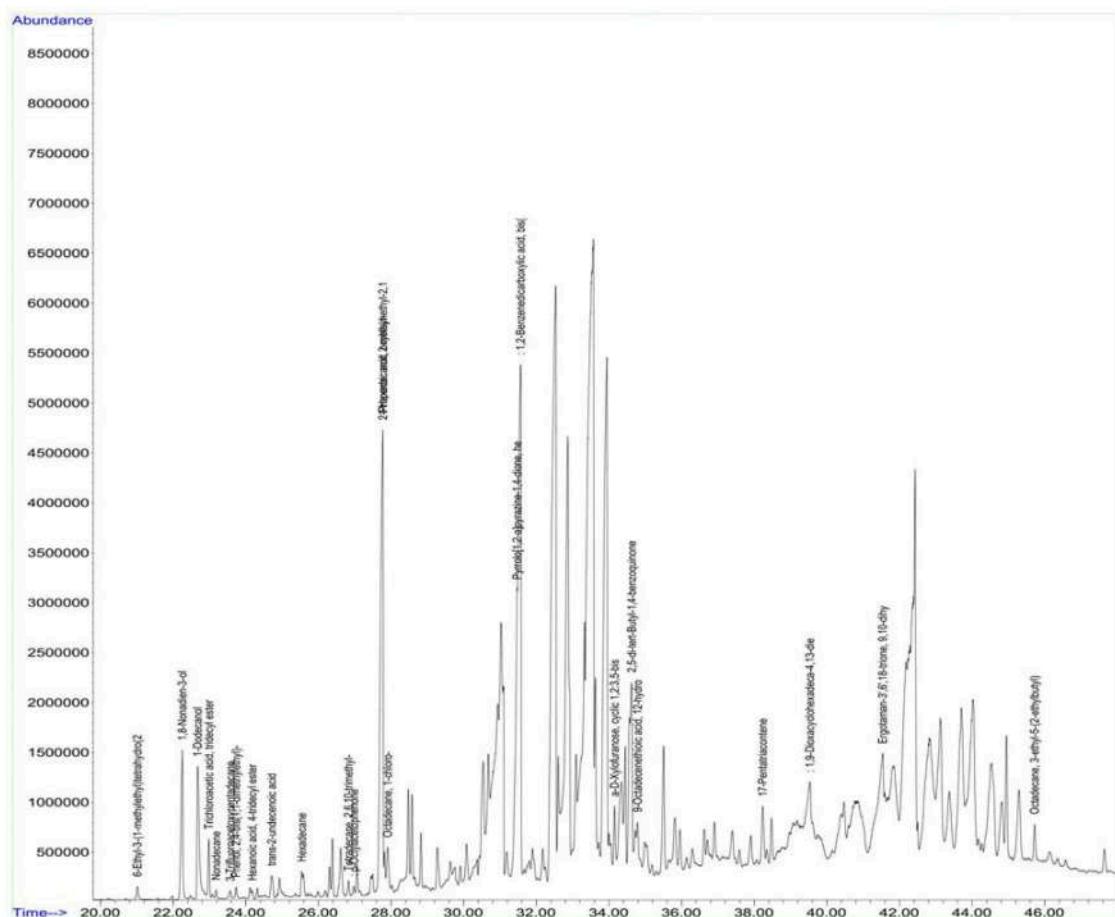


FIGURE 8 | GC chromatogram of the secondary metabolites of *Nocardiopsis* sp. (A7).

essentiality of substrate activation, elongation and modification, other domains such as heterocyclase, N-methylase, epimerase, thioesterase, and reductase are also noticed (Ayuso-Sacido and Genilloud, 2005). Degenerate primers A3F and A7R are employed to detect the conserved motif region (700 bp) of adenylation domain. Out of eight strains, six of them showed positive results for NRPS *viz.*, A2, A3, A4, A6, A7, and A8 emphasizing the possible mode of production of antimicrobial metabolites through this biosynthetic system.

Similarly, the module of other biosynthetic system, PKS-1 include three domains concerned to ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) which helps in the selection and condensation of the correct extender unit. They can further act as enoylreductase, dehydratase, and ketoreductase to undergo reduction step of β -keto group formed in the condensation. Systematic coordination of all these domains results in the production of a new polypeptide chain (Donadio and Katz, 1992; Anderson et al., 2000). In the present study, PCR amplification of gDNA of the strains by degenerate PCR primers specific to conserved motif region

of PKS-I ketosynthase and methyl-malonyl-CoA transferase (1,200–1,400 bp) was studied. But the expected gene product size was not found in the all the strains tested indicating the probable absence of this system. This may be due to insufficient complementarity of degenerate primers designed for PKS-I module or the bioactivity of the strains may correspond to the metabolites produced through other biosynthetic systems. In secondary metabolism of *Streptomyces* sp. H-KF8, Undabarrena et al. (2017) recorded 26 biosynthetic gene clusters through bioinformatics analysis tool, AntiSMASH and further grouped them into different types *viz.*, NRPS, PKS, hybrids, terpenes, RiPP, ectoine, melanine, siderophores, lantipeptides, and butyrolactones.

NRPS synthetases along with fatty acid and/or polyketide synthetases (FAS/PKS) produce different kinds of bioactive compounds including anti-infective, antimicrobial, anticancer etc. For example, pyrrole containing natural products such as prodigiosin, chlorizidine A (anti-tumor), vancomycin (antibacterial), chlorothricin (cholesterol lowering drug) are synthesized through NRPS pathway (Jaremko et al., 2015). In mixed NRPS/PKS pathway, phenyl propionic acids are

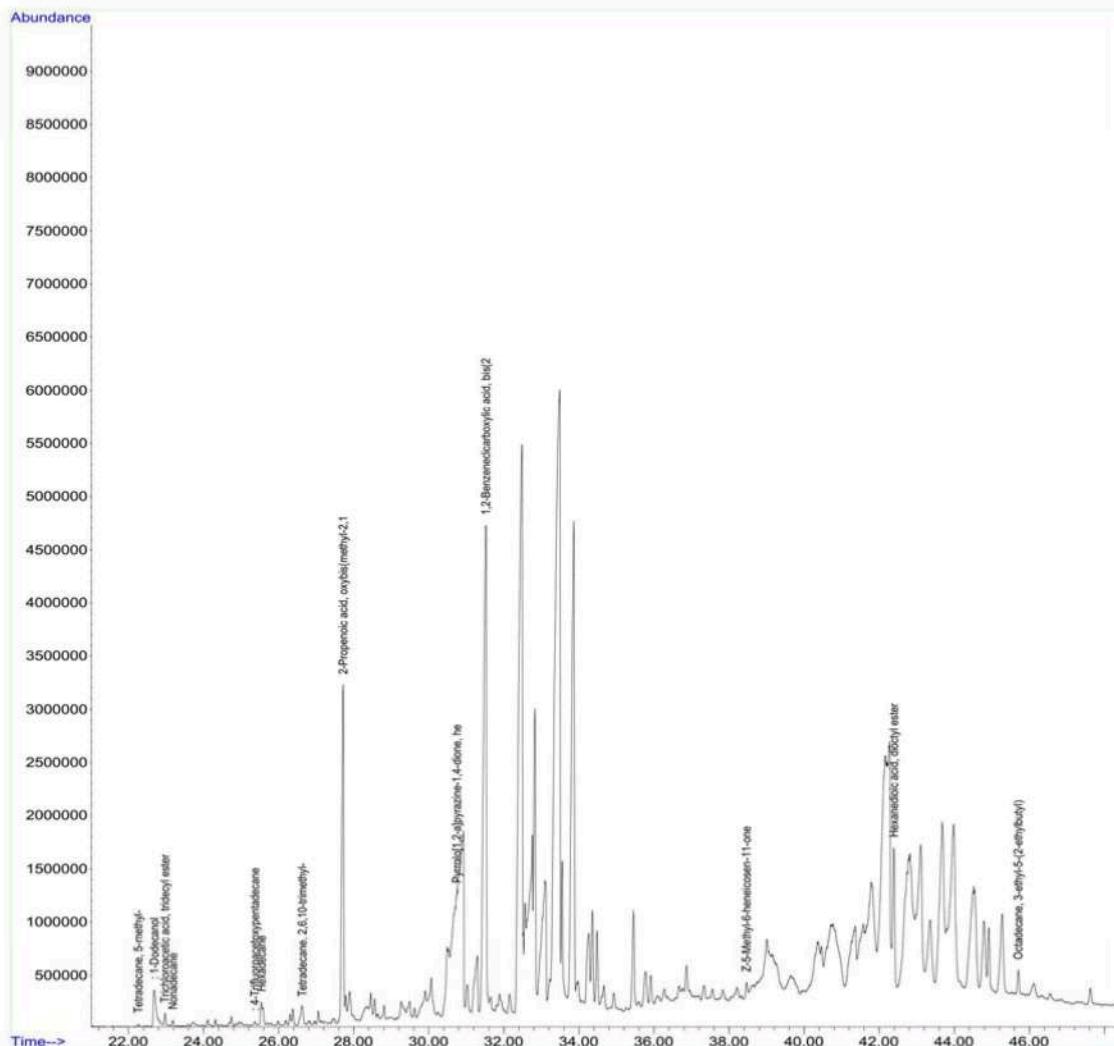
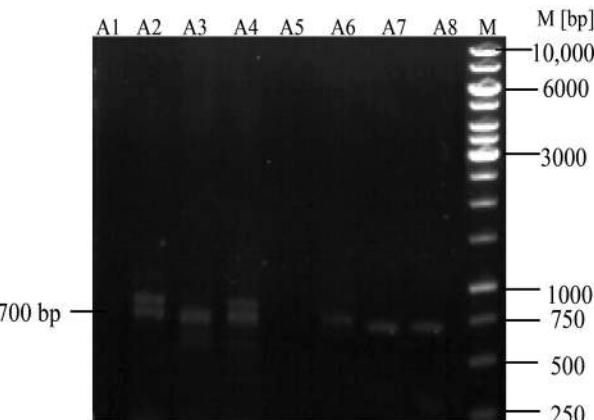


FIGURE 9 | GC chromatogram of the secondary metabolites of *Streptomyces* sp. (A8).

preferentially activated in the production of microcystins by cyanobacteria (Dickschat, 2011). Kehr et al. (2011) summarized the secondary metabolite pathways in cyanobacteria and stated the key role of NRPS modules in the biosynthesis of unusual signature moiety, 2-piperidone of depsipeptides, Anabaenopeptilides. In the present study, various volatile compounds including pyrrole, and piperidinone derivatives, phenyl propionic acid, carboxylic acids etc. were detected in the biologically active crude extracts of eight potent actinobacterial strains isolated from East Coast of Andhra Pradesh, India through GCMS which may contribute for their antimicrobial potential using NRPS biosynthetic pathway. Further investigations should be performed to purify the antimicrobial metabolites of the strains through analytical (chromatography, spectroscopy) techniques and also to elucidate their actual biosynthetic pathways.

CONCLUSION

Out of 73 marine actinobacterial strains obtained from different coastal regions of India, eight of them isolated from East Coast of Andhra Pradesh exhibited strong antimicrobial potential. Six actinobacterial strains (A1, A2, A3, A4, A6, and A7) showed high yields of antimicrobial metabolites on 5th day of incubation whereas the other two strains (A5 and A8) exhibited after 6 days. ISP-2 (for strains A1, A3, A4, A5, A6), ISP-1 (for strains A7 and A8) and Starch casein (for strain A2) supported the production of antimicrobials. Preferential utilization of carbon sources by the strains was widely varied. Maximum yields of antimicrobial metabolites were observed in optimal culture media amended with different carbon sources like xylose (for strains A1, A3, and A7), starch (for strains A2 and A5), dextrose (for strain A4), mannitol (for strain A6), and galactose (for strain A8). Nitrogen



Lanes 1-8 : strains A1 to A8; lane 9: 1kb DNA Marker

FIGURE 10 | Detection of Non-ribosomal peptide synthetases (NRPS) in actinobacterial strains. NRPS found positive in A2, A3, A4, A6, A7, A8 and negative in the remaining two strains (A1, A5).

sources such as malt extract and yeast extract served as the best candidates for antimicrobial potential of most of the strains while the strains A7 and A8 proved their efficacy in the optimal culture media with tryptone amendment. Detection of volatile compounds *viz.*, 1, 2-benzene dicarboxylic acid from most of the actinobacterial strains, 2-piperidinone, and pyrrolo[1,2-a]pyrazine-1, 4-dione from few of them through GCMS suggests that they may contribute for their major antimicrobial potential.

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- AK designed and performed whole experimental part of the work in HSS lab, Indian Institute of Science, Bangalore, India.
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Analysis of biosynthetic gene systems for the production of antimicrobials revealed the presence of NRPS in the strains A2, A3, A4, A6, A7, and A8. Further study need to be done to predict and isolate potent bioactive compounds from the actinobacterial strains of East Coast of Andhra Pradesh, India.

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AK designed and performed whole experimental part of the work in HSS lab, Indian Institute of Science, Bangalore, India.

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Inhibitory Effects of Macrotetrolides from *Streptomyces* spp. On Zoosporogenesis and Motility of Peronosporomycete Zoospores Are Likely Linked with Enhanced ATPase Activity in Mitochondria

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Effects of Macrotetrolides from
Streptomyces spp. On
Zoosporogenesis and Motility of
Peronosporomycete Zoospores Are
Likely Linked with Enhanced ATPase
Activity in Mitochondria.
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The release of zoospores from sporangia and motility of the released zoospores are critical in the disease cycle of the Peronosporomycetes that cause devastating diseases in plants, fishes, animals and humans. Disruption of any of these asexual life stages eliminates the possibility of pathogenesis. In the course of screening novel bioactive secondary metabolites, we found that extracts of some strains of marine *Streptomyces* spp. rapidly impaired motility and caused subsequent lysis of zoospores of the grapevine downy mildew pathogen *Plasmopara viticola* at 10 µg/ml. We tested a number of secondary metabolites previously isolated from these strains and found that macrotetrolide antibiotics such as nonactin, monactin, dinactin and trinactin, and nactic acids such as (+)-nonactic acid, (+)-homonactic acid, nonactic acid methyl ester, homonactic acid methyl ester, bonactin and feigrisolide C impaired motility and caused subsequent lysis of *P. viticola* zoospores in a dose- and time-dependent manners with dinactin being the most active compound (MIC 0.3 µg/ml). A cation channel-forming compound, gramicidin, and a carrier of monovalent cations, nigericin also showed similar biological activities. Among all 12 compounds tested, gramicidin most potently arrested the motility of zoospores at concentrations starting from 0.1 µg/ml. All macrotetrolide antibiotics also displayed similar motility impairing activities against *P. viticola*, *Phytophthora capsici*, and *Aphanomyces cochlioides* zoospores indicating non-specific biological effects of these compounds toward peronosporomycetes. Furthermore, macrotetrolide antibiotics and gramicidin also markedly suppressed the release of zoospores from sporangia of *P. viticola* in a dose-dependent manner. As macrotetrolide antibiotics and gramicidin are known as enhancers of mitochondrial ATPase activity, inhibition of zoosporogenesis and motility of zoospores by these compounds are likely linked with hydrolysis of ATP through enhanced ATPase activity in mitochondria. This is the first report on motility inhibitory and lytic activities of macrotetrolide antibiotics and nactic acids against the zoospores of peronosporomycete phytopathogens.

Keywords: ionophore, dinactin, zoosporocides, mitochondrial ATPase activity, biological control

INTRODUCTION

The Peronosporomycete genera such as *Plasmopara*, *Phytophthora*, *Pythium*, and *Aphanomyces* are notorious pathogens of plants, fishes and vertebrates (Agrios, 1997). Although, morphologically and physiologically they have similarities to fungi, phylogenetically they are relatives of brown algae and diatoms and thus belong to the kingdom of Straminipila (Dick, 2001). One of the unique features of the peronosporomycete pathogens is that in favorable environment they asexually produce motile zoospores from sporangia that develop at the tip of branched sporangiophores (Judelson and Blanco, 2005). Sporangia are globose to lemon-shaped containers capable of converting their cytoplasm into multiple wall-less zoospores. The release of zoospores from sporangia (zoosporogenesis) involves cleavage of the sporangial cytoplasm by nucleus-enveloping membrane networks and an assembly of two flagellae per zoospore (Hardham and Hyde, 1997). The produced zoospores are expelled from the sporangium through sporangial papillae by turgor pressure, resulting in part from high concentration of proline that accumulates in the cytoplasm of cleaving sporangia (Ambikapathy et al., 2002). The zoospores swim after their release, using an anterior tinsel-type flagellum ornamented with tripartite tubular hairs to pull the cell and a posterior whiplash-type flagellum for steering that accomplish “thrust reversal” (Islam et al., 2002b; Judelson and Blanco, 2005). The primary goal of swimming zoospores is to find potential infection sites of the host guided by host-mediated signaling cues (Islam and Tahara, 2001), followed by morphological change into round cystospores by cellular encystment and shedding of flagellae (Islam et al., 2002b, 2003). The cystospores rapidly germinate to form hyphal germ tubes, which penetrate host tissues for infection. Although, little is known about the underlying molecular mechanisms of zoosporogenesis and motility pathways, it has been found that substantial energy from reserve β -1,3-glucan (mycolaminarin) is used during zoosporogenesis and for motility of zoospores (Bimpont, 1975; Judelson and Blanco, 2005). Zoospores are highly energy demanding life stages as ATPase activity in zoospores is similar to that in contracting skeletal muscles (Holker et al., 1993; Stienen et al., 1996). Inhibition of enzymes that maintain ATP concentration, or shuttling of ATP from mitochondria to sites of high utilization such as flagellar kinetosomes of zoospores or depletion of ATP by enhancement of ATPase activity would result in impairment of motility of zoospores and suppression of zoosporogenesis (Judelson and Blanco, 2005).

Plasmopara viticola is a devastating downy mildew pathogen of grapevine, which causes severe economic losses worldwide (Agrios, 1997). This pathogen spreads by an extremely efficient cycle of asexual propagation (Kiefer et al., 2002; Riemann et al., 2002). The success of this obligate biotrophic pathogen can be attributed in part to the speed of asexual differentiation to generate biflagellate motile zoospores from airborne sporangia (zoosporogenesis). It has been observed that zoospores locate the stomata being guided by biochemical host cues followed by encystment and germination to form germ tubes to initiate infection through stomata (Kiefer et al., 2002). Disruption

of zoospore release from sporangia (zoosporogenesis) and/or motility of zoospores by any kind of inhibitor eliminates the potential for pathogenesis (Judelson and Blanco, 2005; Islam et al., 2011). Control of downy mildew in practice is difficult as host plant resistance is generally low and the polycyclic life style of the pathogen requires frequent treatments with fungicides. Novel biorational approaches may help to improve control of this notorious phytopathogen. Discovery of inhibitory chemical substances that can affect the pathways of zoosporogenesis and/or motility of zoospores might be useful for development of innovative and effective strategies for controlling the disease (Judelson and Blanco, 2005; Islam et al., 2011).

Due to the biotrophic nature, *P. viticola* is recalcitrant to cultivation on culture media and thus it is difficult to test the inhibitory potential of novel chemical compounds on zoospore release from sporangia or the motility of zoospores. We developed *in vitro* methods to produce high quantities of sporangia on excised grapevine leaves and get copious amounts of biflagellate motile zoospores in a host-free system (Islam and von Tiedemann, 2008). The released zoospores remain motile in sterilized water for 12–16 h. These *in vitro* methods allow screening secondary metabolites from antagonistic environmental microorganisms using convenient bioassay protocols (Islam et al., 2011). In this way, we recently isolated several new secondary metabolites from plants and microorganisms that suppress zoosporogenesis, inhibit motility and/or cause lysis of *P. viticola* zoospores (Abdalla et al., 2011; Islam et al., 2011; Zinad et al., 2011; Talontsi et al., 2012a,b; Dame et al., 2016). Furthermore, using a natural product, staurosporine and some further kinase inhibitors, we recently demonstrated that protein kinase C is involved in both flagellar motility and zoosporogenesis of the Peronosporomycetes (Islam et al., 2011).

Secondary metabolites from marine microorganisms especially *Streptomyces* spp. are known to possess diverse biological activities through inhibiting specific enzyme or proteins in the signaling pathways (Islam et al., 2011). In the course of screening for novel secondary metabolites from marine *Streptomyces* spp., we found that extracts of some marine *Streptomyces* spp. (such as strains Act 8970, ACT 7619) rapidly impaired motility and caused subsequent lysis of zoospores at 10 μ g/ml. We then tested all previously isolated compounds from these *Streptomyces* strains and found that macrotrichide antibiotics such as dinactin and nactic acids (Al-Refai, 2008; Mahmoud, 2008), displayed motility inhibitory and lytic activities against zoospores which were identical to the effects in crude extracts. Dinactin is a member of the macrotrichide complex produced by a range of *Streptomyces* species, which also includes nonactin, monactin, trinactin and tetranactin (Beck et al., 1962; Al-Refai, 2008; Mahmoud, 2008). These nactins are known to enhance ATPase activity in the mitochondria and cause rapid hydrolysis of ATP. They were also shown to act as monovalent cation ionophores with high selectivity for ammonium and potassium (Graven et al., 1966, 1967). The novel biological activities of macrotrichides found in this study prompted us to further test structurally related compounds of dinactin and some known ionophores to understand the structure-activity relationships as well as to get information on

the mode of action of these natural products. Therefore, the objectives of our study were (i) to screen extracts of marine *Streptomyces* spp. on motility and viability of zoospores of *P. viticola*; (ii) to test compounds previously isolated from extracts of *Streptomyces* spp. that exhibited motility inhibitory and lytic activities against zoospores; (iii) to test compounds structurally related to (+)-nonactin acid and dinactin on motility and lysis of zoospores of *P. viticola*, *Phytophthora capsici* and *Aphanomyces cochlioides*; (iv) to evaluate the effect of further known compounds such as nigericin and gramicidin having similar mode of action on zoospores; and (v) to evaluate the effects of zoospore motility inhibitors on zoosporogenesis of *P. viticola*.

MATERIALS AND METHODS

Materials and Experimental Procedure

Macrotetrolide antibiotics such as monactin and trinactin and further ionophoric compounds, nigericin and gramicidin were purchased from Tebu-bio and Sigma-Aldrich, respectively. Dinactin, nonactin, bonactin, feigrisolid, (+)-nonactin acid, (+)-homononactin acid, nonactin acid methyl ester, and homononactin acid methyl ester available in the laboratory were either previously isolated from marine *Streptomyces* spp. (Act 8970 and ACT 7619) or synthesized. All other chemicals were of at least reagent grade. Stock solutions of test compounds were prepared in small amounts of dimethyl sulfoxide (DMSO) and then diluted with water. The concentration of DMSO in the incubation medium never exceeded 1%, a condition that does not affect motility and viability of peronosporomycete zoospore (Islam et al., 2011).

Cultivation of Marine *Streptomyces* spp. and Extraction

The marine *Streptomyces* spp. strains (such as Act8970) used in this research were obtained from the collection of the Institute of Organic and Biomolecular Chemistry, University of Göttingen, Germany. These strains were pre-cultivated on M_2^+ medium (+ 50% sea water) agar plates at 28°C for 3 days. To upscale these strains, pieces of well colonized agar were added to 1 l shaker cultures. Each strain was propagated in 5 l-Erlenmyer flasks each containing 200 ml of M_2^+ (50% sea water) for 11 days at 28°C on a linear shaker with 110 rpm. After extraction of water phase and cell mass with ethyl acetate, the obtained extract was subjected to the bioassay.

Peronosporomycete Strains, Production of Zoospores and Bioassay

Sporangia of *P. viticola* were isolated from infected leaves of grapevine (*Vitis vinifera* cv. Müller-Thurgau) (Islam et al., 2011). This strain was originally gained from infected leaf materials of the grapevine cv. Riesling in 1996 and since then maintained and propagated on fresh leaves of cv. Müller-Thurgau kept on Petri dishes containing 1.5% agar at 25°C and 95% relative humidity (Islam and von Tiedemann, 2008, 2011). At day 6 of cultivation, the sporangiophores bearing lemon-shaped

sporangia were harvested into an Eppendorf vial by a micro-vacuum cleaner. The freshly harvested sporangia were separated from sporangiophores by filtration through a nylon sieve (50 µm mesh), washed twice with distilled water and then incubated in sterilized tap water (3×10^4 sporangia/mL) in the dark for 6 h at room temperature (23°C) to release zoospores. These zoospores remained motile for 10–12 h in sterilized water and were used for the bioassay (Islam et al., 2011). The bioassay for testing the effects of pure compounds on release of zoospores from sporangia was carried out as described earlier (Islam et al., 2011).

The bioassay on motility and lysis of zoospores in presence of varying doses of pure compounds was carried out as described earlier (Islam et al., 2002a, 2011). Briefly, 40 µL of sample solution was directly added to 360 µL of zoospore suspension (ca. 10^5 /mL) taken in a dish of a plant tissue culture multi-well plate to make a final volume of 400 µL and then quickly mixed with a glass rod; 1% aqueous DMSO was used as a control. The motility of zoospores was observed under a light microscope at 100-fold magnification. Quantification of time-course changes of motility and lysis of zoospores were carried out as described earlier (Islam et al., 2004). Each treatment was replicated five times. The mean value (%) ± SE (standard error) of the affected spores in each treatment was calculated.

Cultivation of *Aphanomyces cochlioides* and *Phytophthora capsici*, Production of Zoospores and Bioassay

The damping-off pathogen of sugar beet and spinach, *Aphanomyces cochlioides*, was obtained from the Sugar Beet Research Institute (IFZ) in Goettingen, Germany. The culture of this strain and protocol for production of zoospores are described elsewhere (Islam and von Tiedemann, 2011; Zohara et al., 2016). *P. capsici* was provided by Prof. W. Yuancaho of Nanjing Agricultural University, China, which was isolated from soil of Nanjing, China. This organism was cultured on V8 juice agar. Production of zoospores and bioassays were carried out following protocols reported earlier (Tareq et al., 2014; Zohara et al., 2016). Each treatment was replicated five times. The mean value (%) ± SE (standard error) of the affected spores in each treatment was calculated.

Statistical Analysis, Experimental Design/Replications

Experiments for evaluating biological activities of the pure compounds were carried out using a complete randomized design (CRD). Data were analyzed by one way analysis of variance (ANOVA) and the mean values were separated by Tukey's HSD (honest significant difference) posthoc statistic. All the analyses were performed using SPSS (IBM SPSS statistics 21, Georgia, USA). Mean value ± standard error of 5 replications were used in Tables and Figures.

RESULTS

Motility Inhibitory and Lytic Activities of Extracts of Marine *Streptomyces* spp. Strains

To see whether marine *Streptomyces* spp. produce inhibitory substances against notorious phytopathogenic peronosporomycetes, we tested crude extracts of a large number of previously studied strains on motility behavior of *P. viticola* zoospores that produce diverse bioactive secondary metabolites. Out of 89 crude extracts from different strains of marine *Streptomyces* spp. tested, strains Act 8970, B6167, B7857, ACT7619, and Gt-2005/009 displayed significantly higher ($p \leq 0.001$) motility inhibitory and subsequent lytic activities against *P. viticola* zoospores at 10 µg/ml or lower concentrations (Table 1). The antibiotic activity of the crude extracts of strains Act 8970, B6167, B7857, Act7619, and Gt2005/2009 was due to the presence of macrotricholide antibiotics as dinactin and nactic acids displayed identical motility inhibitory and lytic activities against the zoospores in a dose- and time-dependant manners. These compounds were isolated from all these strains in our laboratory (Al-Refai, 2008; Mahmoud, 2008; Rahman, 2008). Therefore, homologs of dinactin and nactic acids were used in further detailed inhibition bioassays toward zoosporegenesis and motility of zoospores of *P. viticola*. To see whether the inhibitory activities of macrotricholides and nactic acids are specific to *P. viticola* or general to other economically important phytopathogenic peronosporomycetes, we included a damping-off pathogen of sugar beet and spinach, *Aphanomyces cochlioides* and a late blight pathogen of chili and several vegetables, *Phytophthora capsici*.

Motility Inhibitory and Lytic Activities of Macrotricholide Antibiotics and Nactic Acids against *P. Viticola* Zoospores

Compounds previously isolated from strains of Act 8970 viz. dinactin, (+)-nonactic acid, (+)-homononactic acid, homononactic acid methyl ester, and nonactic acid methyl ester, were tested on motility of *P. viticola* zoospores (Table 2 and Figure 1). Among them, the macrotricholide antibiotic dinactin displayed the highest potency in arresting motility and caused subsequent lysis of *P. viticola* zoospores starting from 0.3 µg/ml (Table 2). The activities of the linear nactic acids and their methyl esters were 5–50-fold lower in inhibition of zoospore motility than of dinactin. Dinactin inhibited the motility completely and caused lysis of all stopped zoospores (100%) within 15 min exposure to the compound at 1 µg/ml. One way ANOVA revealed that zoospore motility inhibitory activities of the varying concentrations of the tested compounds varied significantly at $p \leq 0.05$. Microscopic observation revealed that in presence of dinactin, swimming of zoospores was rapidly impaired or slowed down and/or zoospores spun in tight circles for a short time (Figure 2). Finally, all affected zoospores stopped moving and most of them subsequently lysed within several minutes of treatment, depending on the concentration

of the compound. Before lysis, the cellular materials in halted zoospores rapidly became granulated and then gradually fragmented and dispersed into the surrounding water upon burst of their cell membranes (Figure 2f). In contrast, *P. viticola* zoospores in untreated control dishes exhibited the characteristic helical swimming almost following a straight line for several hours. Other nactic acids and their methyl esters also impaired motility of zoospores and caused subsequent lysis in an identical manner but at varying concentrations (Table 2). Among them, (+)-homononactic acid exhibited the strongest activity in both inhibition of motility and lysis of zoospores followed by nonactic acid methyl ester, homononactic methyl ester and (+)-nonactic acid in decreasing order. (+)-Homononactic acid inhibited motility of 100% zoospores at 5 µg/ml by 60 min of treatment, which was 10-fold stronger activity compared to the activity of (+)-nonactic acid (Table 2). The zoospore lytic activities of the tested compounds also varied significantly at $p \leq 0.05$.

Biological Activity of Compounds Structurally Related to Dinactin and Dinactic Acids

Dinactin is a member of the macrotricholide complex produced by a range of *Streptomyces* species which includes several homologs such as nonactin, monactin, and trinactin (Beck et al., 1962). Early literature reported that these compounds almost equally enhance mitochondrial ATPase activity and cause rapid hydrolysis of ATP (Graven et al., 1966). They are also known to act as monovalent cation ionophore with high selectivity for ammonium and potassium (Graven et al., 1967) and have diverse biological activities (Zizka, 1998). To get insight into the structure-activity relationships, we tested some homologs of dinactin such as nonactin, monactin and trinactin together with two linear compounds such as bonactin and feigrisolate C previously isolated from marine *Streptomyces* species (Figure 1).

All three homologs (nonactin, monactin, and trinactin) of dinactin displayed motility impairing and lytic activities against *P. viticola* zoospores in an identical fashion and dose- and time-dependent manner (Table 3). One way ANOVA revealed that zoospore motility inhibitory activities of the tested compounds and their different concentrations varied significantly at $p \leq 0.05$. The strengths of activity of all these homologs were similar. Bonactin and feigrisolate C also exhibited motility inhibitory and lytic activities in a similar manner but required almost 10-fold higher doses compared to dinactin. Time-course investigation revealed that dinactin and trinactin caused 100% inhibition of zoospore motility within 15 min exposure to the tested compounds at 1 µg/ml (Figure 3A). At the same concentration, nonactin and monactin also inhibited motility by 100% but required longer time, i.e., 30 and 60 min, respectively. All compounds showed almost similar phenomena in causing lysis of halted zoospores (Figure 3B). Initially, zoospores became paralyzed or moved very slowly in tight circles, stopped and then rapidly immobilized (Figure 2). The zoospore lytic activities of the

TABLE 1 | Motility halting and zoosporicidal activity of marine *Streptomyces* spp. extracts against the downy mildew pathogen *Plasmopara viticola*.

Name of extract	Dose ($\mu\text{g/ml}$)	Motility halting and zoosporicidal activity (% \pm SE) [†]							
		15 min		30 min		45 min		60 min	
		Halted	Burst	Halted	Burst	Halted	Burst	Halted	Burst
Act 8970	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	11 \pm 1.2	0 \pm 0
	5	3 \pm 0.6	0 \pm 0	7 \pm 2.3	0 \pm 0	17 \pm 2.9	5 \pm 2.3	48 \pm 3.5	36 \pm 2.3
	10	62 \pm 4.6	29 \pm 2.3	79 \pm 3.5	58 \pm 4.0	86 \pm 3.5	75 \pm 5.2	98 \pm 1.2	84 \pm 4.0
B6167	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	0 \pm 0	0 \pm 0	5 \pm 1.7	0 \pm 0	22 \pm 2.9	9 \pm 1.2	43 \pm 4.0	15 \pm 4.6
	10	47 \pm 3.5	23 \pm 2.9	68 \pm 2.3	37 \pm 5.2	86 \pm 2.3	69 \pm 2.9	88 \pm 1.2	77 \pm 3.5
B7857	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	0 \pm 0	0 \pm 0	9 \pm 1.2	0 \pm 0	23 \pm 2.3	18 \pm 1.7	39 \pm 3.5	21 \pm 2.3
	10	49 \pm 2.9	11 \pm 4.0	62 \pm 4.6	28 \pm 5.2	75 \pm 4.0	41 \pm 4.6	87 \pm 1.8	69 \pm 5.2
Act7619	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	0 \pm 0	0 \pm 0	7 \pm 1.7	0 \pm 0	22 \pm 2.9	9 \pm 1.2	43 \pm 4.0	30 \pm 4.0
	10	47 \pm 2.3	13 \pm 2.9	58 \pm 2.3	32 \pm 5.2	78 \pm 5.8	49 \pm 2.9	88 \pm 1.2	71 \pm 3.5
Gt-2005/009	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	0 \pm 0	0 \pm 0	7 \pm 2.3	0 \pm 0	24 \pm 3.5	16 \pm 1.7	40 \pm 4.6	22 \pm 2.3
	10	57 \pm 4.0	9 \pm 1.7	68 \pm 5.2	36 \pm 5.2	79 \pm 5.8	59 \pm 4.0	90 \pm 3.5	67 \pm 4.6
B5136	1	20 \pm 1.2	0 \pm 0	28 \pm 2.9	0 \pm 0	38 \pm 1.7	0 \pm 0	54 \pm 4.0	0 \pm 0
	5	99 \pm 0.6	9 \pm 0.6	100 \pm 0	88 \pm 4.0	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
	10	100 \pm 0	85 \pm 3.5	100 \pm 0					
B3497	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	14 \pm 1.2	0 \pm 0	38 \pm 1.7	18 \pm 2.9
	5	96 \pm 1.7	81 \pm 3.5	99 \pm 0.6	88 \pm 2.9	100 \pm 0	92 \pm 2.9	100 \pm 0	100 \pm 0
	10	99 \pm 0.6	85 \pm 4.0	100 \pm 0	93 \pm 2.9	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
B4818	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	78 \pm 3.5	52 \pm 3.5	100 \pm 0	78 \pm 2.9	100 \pm 0	95 \pm 2.3	100 \pm 0	100 \pm 0
	10	98 \pm 1.2	98 \pm 1.2	100 \pm 0					
B7798	1	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	5	78 \pm 3.5	45 \pm 2.3	88 \pm 4.6	60 \pm 3.5	95 \pm 2.9	73 \pm 4.0	100 \pm 0	100 \pm 0
	10	98 \pm 1.2	75 \pm 4.6	100 \pm 0					
B7060	5	0 \pm 0*	0 \pm 0	0 \pm 0*	0 \pm 0	45 \pm 2.3	26 \pm 1.7	93 \pm 2.9	76 \pm 3.6
	10	98 \pm 1.2	0 \pm 0	100 \pm 0	55 \pm 3.5	100 \pm 0	78 \pm 4.6	100 \pm 0	82 \pm 5.2
	50	100 \pm 0	72 \pm 4.0	100 \pm 0	81 \pm 5.2	100 \pm 0	86 \pm 5.2	100 \pm 0	92 \pm 3.5
B8774	10	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	50	99 \pm 0.6	65 \pm 5.2	100 \pm 0	75 \pm 3.5	100 \pm 0	82 \pm 4.6	100 \pm 0	99 \pm 0.6
	100	100 \pm 0	80 \pm 4.6	100 \pm 0	95 \pm 2.3	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0
B7747	10	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	50	98 \pm 1.2	35 \pm 4.6	100 \pm 0	42 \pm 2.9	100 \pm 0	60 \pm 3.5	100 \pm 0	69 \pm 3.5
	100	100 \pm 0	56 \pm 2.9	100 \pm 0	72 \pm 4.0	100 \pm 0	80 \pm 5.2	100 \pm 0	88 \pm 4.6
B4842	10	0 \pm 0	0 \pm 0	18 \pm 1.7	9 \pm 0.6	28 \pm 1.2	12 \pm 2.3	35 \pm 2.9	26 \pm 1.2
	50	100 \pm 0	10 \pm 1.2	100 \pm 0	28 \pm 2.9	100 \pm 0	43 \pm 2.9	100 \pm 0	64 \pm 3.5
	100	100 \pm 0	41 \pm 3.5	100 \pm 0	52 \pm 3.5	100 \pm 0	72 \pm 5.2	100 \pm 0	80 \pm 5.2

(Continued)

TABLE 1 | Continued

Name of extract	Dose ($\mu\text{g/ml}$)	Motility halting and zoosporicidal activity (% \pm SE) [†]							
		15 min		30 min		45 min		60 min	
		Halted	Burst	Halted	Burst	Halted	Burst	Halted	Burst
B5530	10	0 \pm 0	0 \pm 0	35 \pm 2.3	0 \pm 0	39 \pm 3.5	0 \pm 0	46 \pm 2.3	0 \pm 0
	50	100 \pm 0	45 \pm 3.5	100 \pm 0	63 \pm 3.5	100 \pm 0	78 \pm 5.8	100 \pm 0	80 \pm 3.5
	100	100 \pm 0	70 \pm 4.6	100 \pm 0	83 \pm 4.0	100 \pm 0	95 \pm 2.9	100 \pm 0	100 \pm 0
B4677	10	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	6 \pm 0.6	0 \pm 0	10 \pm 1.2	0 \pm 0
	50	100 \pm 0	48 \pm 4.6	100 \pm 0	80 \pm 5.2	100 \pm 0	88 \pm 3.5	100 \pm 0	91 \pm 4.0
	100	100 \pm 0	98 \pm 1.2	100 \pm 0	100 \pm 0				
B8300	50	60 \pm 4.0	0 \pm 0	72 \pm 2.9	0 \pm 0	88 \pm 4.6	0 \pm 0	100 \pm 0	0 \pm 0
	100	100 \pm 0	0 \pm 0	100 \pm 0	23 \pm 2.3	100 \pm 0	36 \pm 3.5	100 \pm 0	49 \pm 0
B8690	50	94 \pm 2.3	0 \pm 0	92 \pm 1.2	47 \pm 2.9	93 \pm 2.9	67 \pm 4.6	100 \pm 0	80 \pm 5.2
	100	100 \pm 0	40 \pm 3.5	100 \pm 0	69 \pm 4.6	100 \pm 0	83 \pm 3.5	100 \pm 0	100 \pm 0
B8251	50	79 \pm 2.9	0 \pm 0	88 \pm 1.2	30 \pm 4.0	98 \pm 1.2	42 \pm 3.5	100 \pm 0	55 \pm 2.3
	100	100 \pm 0	39 \pm 4.6	0 \pm 0	59 \pm 2.3	100 \pm 0	63 \pm 2.3	100 \pm 0	70 \pm 5.2
B9042	10	26 \pm 2.3	0 \pm 0	48 \pm 1.2	20 \pm 2.3	65 \pm 4.6	32 \pm 2.9	82 \pm 5.2	72 \pm 3.5
	50	67 \pm 4.6	49 \pm 4.0	87 \pm 2.3	62 \pm 1.7	94 \pm 2.3	79 \pm 4.0	100 \pm 0	93 \pm 2.3
B7936	10	0 \pm 0	0 \pm 0	18 \pm 2.3	6 \pm 0.6	49 \pm 4.0	25 \pm 2.8	72 \pm 4.0	51 \pm 4.0
	50	57 \pm 3.5	31 \pm 4.0	77 \pm 4.6	40 \pm 3.5	86 \pm 5.2	56 \pm 3.5	94 \pm 3.5	83 \pm 2.9
B8160	10	18 \pm 1.7	0 \pm 0	29 \pm 3.5	12 \pm 0.6	52 \pm 3.5	24 \pm 2.3	88 \pm 3.5	43 \pm 2.3
	50	67 \pm 2.3	41 \pm 3.5	89 \pm 2.9	57 \pm 5.1	92 \pm 2.3	72 \pm 4.0	100 \pm 0	94 \pm 2.9
B1638a	10	38 \pm 2.9	7 \pm 1.7	47 \pm 4.0	19 \pm 2.3	68 \pm 4.0	33 \pm 3.5	85 \pm 4.0	46 \pm 3.5
	50	76 \pm 4.6	53 \pm 2.9	93 \pm 3.5	68 \pm 4.0	100 \pm 0	88 \pm 2.9	100 \pm 0.0	90 \pm 2.9
B4854	10	5 \pm 0.6	0 \pm 0	19 \pm 2.3	0 \pm 0	34 \pm 2.3	0 \pm 0	51 \pm 2.9	14 \pm 1.2
	50	81 \pm 3.5	43 \pm 3.5	92 \pm 4.0	67 \pm 4.0	100 \pm 0	95 \pm 2.3	100 \pm 0	97 \pm 1.7
	100	99 \pm 0.6	90 \pm 2.9	100 \pm 0	95 \pm 2.3	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0

[†]One way ANOVA was performed and data in column varies significantly at $p \leq 0.001$. Post-hoc tests could not be performed because the number groups is more than 50. *Zoospores (100%) moved to bottom and move very slowly; We selected strains those previously found to produce antibiotics against pathogenic microorganisms.

varying concentration of the tested compounds also varied significantly at $p \leq 0.05$.

Motility of Zoospores in Presence of Gramicidin and Nigericin

To better assess whether induction of ATPase activity and hydrolysis of ATP in mitochondria or any other mechanism is associated with motility inhibitory and lytic activities of zoospores by macrotetrolide antibiotics, a channel-forming ionophore, gramicidin and a mobile carrier of cations through plasma membranes, nigericin were tested (Graven et al., 1966, 1967). Gramicidin showed the highest activity ($p \leq 0.05$) among the tested compounds, which was 2-fold stronger than those of macrotetrolides for inhibition of zoospore motility by 100% (Table 3). On the other hand, nigericin also displayed zoospore motility arresting activity but had 10-fold weaker efficacy

compared with gramicidin. In both cases, halted zoospores were lysed in a similar manner as shown by the macrotetrolide antibiotics. On the other hand, nigericin displayed almost equal strength to bonactin in arresting motility of 100% zoospores at 5 $\mu\text{g/ml}$, while the dose required for equivalent efficacy by feigrisolide was 10 $\mu\text{g/ml}$ (Table 3). Motility inhibitory and lytic activities against zoospores by gramicidin and nigericin were statistically significant ($p \leq 0.05$).

Effects of Macrotetrolide Antibiotics on Motility of *Aphanomyces cochlioides* Zoospores

To evaluate whether motility inhibitory and lytic activities of marotetrolides are common phenomena in Peronosporomycete zoospores, we tested all homologs of dinactin and other bioactive compounds evaluated on *P. viticola* against a

TABLE 2 | Motility halting and zoosporicidal activity of nactic acids and their esters and dinactin isolated from marine *Streptomyces* sp. Act 8970 against the grapevine downy mildew pathogen *Plasmopara viticola*.

Compound	Dose ($\mu\text{g/ml}$)	Motility halting and zoosporicidal activity (% \pm SE)*							
		15 min		30 min		45 min		60 min	
		Halted	Burst	Halted	Burst	Halted	Burst	Halted	Burst
(+)-Nonactic acid	10	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	0 \pm 0e	0 \pm 0f	0 \pm 0e	0 \pm 0g
	30	71 \pm 4c	56 \pm 2c	80 \pm 2bc	72 \pm 4c	82 \pm 3c	75 \pm 4bc	88 \pm 4b	82 \pm 4de
	50	90 \pm 4b	82 \pm 5b	98 \pm 1a	86 \pm 4b	100 \pm 0a	95 \pm 2a	100 \pm 0a	98 \pm 2ab
	100	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
(+)-Homonactic acid	1	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	0 \pm 0e	0 \pm 0f	0 \pm 0e	0 \pm 0
	5	91 \pm 2ab	88 \pm 4b	97 \pm 2a	90 \pm 2ab	99 \pm 1a	95 \pm 2a	100 \pm 0a	98 \pm 1ab
	10	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Nonactic acid methyl ester	5	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	0 \pm 0e	0 \pm 0f	0 \pm 0e	0 \pm 0g
	10	35 \pm 4e	19 \pm 1e	71 \pm 4c	46 \pm 4de	79 \pm 3c	70 \pm 5c	89 \pm 4b	83 \pm 4de
	20	52 \pm 3d	32 \pm 3d	79 \pm 4bc	54 \pm 3d	85 \pm 3bc	73 \pm 6bc	100 \pm 0a	88 \pm 5bd
Homonactic methyl ester	10	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	0 \pm 0e	0 \pm 0f	0 \pm 0a	0 \pm 0g
	20	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	10 \pm 1e	0 \pm 0f	25 \pm 2d	0 \pm 0g
	30	60 \pm 1d	30 \pm 2d	72 \pm 3c	42 \pm 5e	88 \pm 6bc	59 \pm 3d	100 \pm 0a	75 \pm 2e
Dinactin	0.1	0 \pm 0f	0 \pm 0f	0 \pm 0e	0 \pm 0g	0 \pm 0e	0 \pm 0f	0 \pm 0e	0 \pm 0g
	0.3	0 \pm 0f	0 \pm 0f	31 \pm 2d	19 \pm 2f	40 \pm 3d	23 \pm 6e	53 \pm 2c	42 \pm 4f
	0.5	75 \pm 2c	63 \pm 4c	83 \pm 3b	74 \pm 4c	92 \pm 4ab	82 \pm 5b	96 \pm 2ab	93 \pm 3ac
	1.0	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a

*Means within a column followed by the same letter(s) are not significantly different as assessed by Tukey's HSD (honest significance difference) post-hoc ($p \leq 0.05$).

sugar beet damping-off pathogen *Aphanomyces cochlioides* (Peronosporomycete). An almost identical phenomenon was observed when motile *A. cochlioides* zoospores were exposed to macroterolide antibiotics or other inhibitors, but surprisingly none of the compounds caused any lysis of the halted zoospores until 60 min (Table 4). One way ANOVA revealed that zoospore motility inhibitory and lytic activities of the tested compounds and their varying concentrations varied significantly at $p \leq 0.001$. Irrespective of the test compounds, all motility-impaired zoospores rapidly became round cystospores instead of lysis, however, none of them germinated until 60 min after the treatment (data not shown).

Motility Inhibitory and Lytic Activities of Dinactin and Trinactin against *Phytophthora capsici* Zoospores

Two homologs of macroterolide antibiotics, dinactin and trinactin were also tested against the zoospores of another notorious peronosporomycete phytopathogen, *Phytophthora capsici*. As expected, both compounds displayed inhibitory activity against *P. capsici* zoospores in a dose- and time-dependant manner. Trinactin showed significantly ($p \leq 0.001$) stronger inhibitory activity compared with dinactin (Figure 4). The zoospores of *P. capsici* seemed less sensitive to the macroterolides compared to *P. viticola* zoospores. The IC₅₀ values for motility inhibition of zoospores by dinactin and

trinactin were ca. 1.0 and 0.5 $\mu\text{g/ml}$, respectively. Unlike *P. viticola* zoospores, only a small fraction of motility impaired zoospores became lysed by the treatment of dinactin and trinactin. Approximately, 50% of the halted zoospores became round cystospores and failed to germinate until 60 min after the treatments.

Inhibition of Zoosporogenesis by Nonactic Acid and Macroterolides

Freshly harvested and washed *P. viticola* sporangia ($3 \times 10^5/\text{ml}$) typically release zoospores up to $1 \times 10^6/\text{ml}$ in sterilized water within 5–6 h. We tested whether macroterolide antibiotics (nonactin, monactin, dinactin, and trinactin), bonactin, feigrisolide, nigericin and gramicidin, have an effect on the process of zoospore release (i.e., zoosporogenesis). The bioassay revealed that all compounds significantly ($p \leq 0.001$) inhibited zoosporogenesis in a dose-dependent manner but in varying concentrations (Table 5 and Figure 5). Zoosporogenesis was completely blocked by monactin, dinactin, trinactin and gramicidin at 5 $\mu\text{g/ml}$. At lower doses of these compounds, the release of zoospores still occurred but most zoospores became immobilized soon after release. Nonactin also displayed a similar inhibitory effect but required a 2-fold higher concentration for equivalent activity to other macroterolides. The bonactin and feigrisolide also suppressed the release of zoospores but required several fold higher concentrations compared with dinactin.

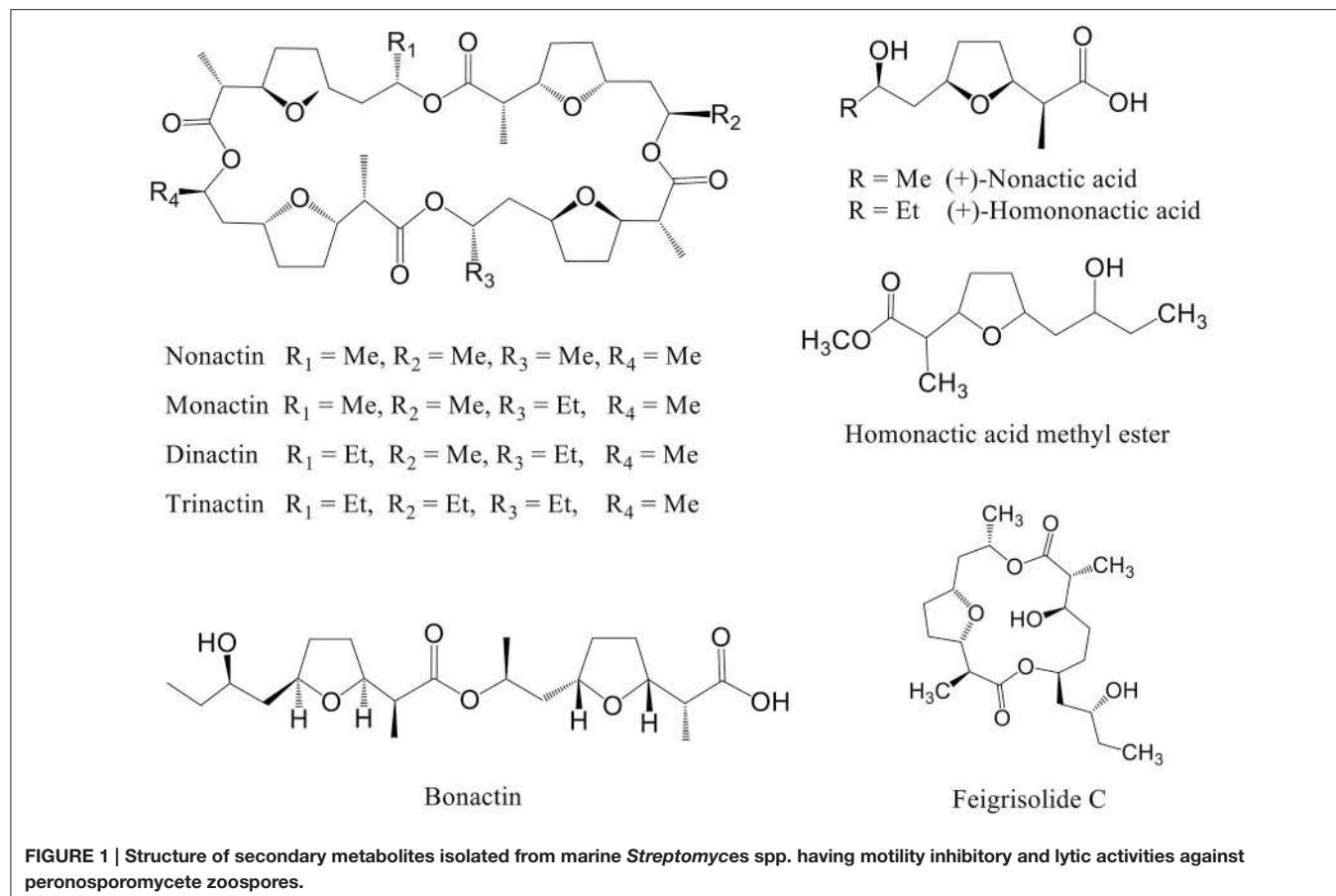


FIGURE 1 | Structure of secondary metabolites isolated from marine *Streptomyces* spp. having motility inhibitory and lytic activities against peronosporomycete zoospores.

Nigericin had weak activity (IC_{50} 10 $\mu\text{g/ml}$) in suppressing zoosporogenesis of *P. viticola*.

DISCUSSION

In this study, we demonstrated that macroterolide antibiotics such as dinactin and nactic acids isolated from marine *Streptomyces* spp. impaired motility and caused lysis of *P. viticola* zoospores that are key stages of this devastating pathogen of grapevine (Tables 2, 3). These bioactive compounds also inhibited motility of *P. capsici* and *A. cochlioides* zoospores in a similar way (Table 4) and suppressed the release of zoospores from *P. viticola* sporangia in a dose-dependent manner (Table 5). In addition, the homologs of dinactin (e.g., nonactin, monactin, trinactin), which are known as enhancers of mitochondrial ATPase activity similar to nigericin and gramicidin also suppressed zoosporogenesis, impaired motility and caused lysis of *P. viticola* zoospores (Table 5, Figures 3, 4). Taken together, our results show for the first time that macroterolide antibiotics and nactic acids from marine *Streptomyces* spp. and other bacteria suppress zoosporogenesis and impair motility of peronosporomycete zoospores. Furthermore, preliminary bioassay revealed that one of the macroterolide, dinactin suppressed the sporulation of *P. viticola* in artificially inoculated grapevine leaf disks (data not

shown) indicating its potential as a natural peronosporomicide. A further *in vivo* study is warranted to test this hypothesis. As zoosporogenesis and motility of zoospores are high energy demanding processes, the mode of action of these inhibitory activities by macroterolides is likely linked with the hydrolysis of mitochondrial ATP through enhanced ATPase activity. Motility inhibition and subsequent lysis of Peronosporomycete zoospores by various kinds of natural products such as indolocarbazole alkaloid, staurosporine (Islam et al., 2011), khatmiamycin from *Streptomyces* sp. ANK313 (Abdalla et al., 2011), isocoumarins from *Streptomyces* sp. ANK302 (Zinad et al., 2011), macrocyclic lactam antibiotics from *Lysobacter* sp. SB-K88 (Islam et al., 2005), polyflavonoid tannins from the bark of *Lannea coromandelica* (Islam et al., 2002a), anacardic acids from *Ginkgo biloba* (Begum et al., 2002), and polyketides and depsidones from the fungal endophyte *Cryptosporiopsis* sp. CAFT122-2 and *Phomopsis* sp. CAFT69, respectively (Talontsi et al., 2012a,b) have been reported previously. Impairment of motility and lysis of peronosporomycete zoospores by oligomycins and pamamycin homologs from marine *Streptomyces* have also been reported (Dame et al., 2016).

A hallmark of our finding is that all four macroterolide antibiotics (nonactin, monactin, dinactin, and trinactin) produced by marine *Streptomyces* spp. displayed qualitatively and quantitatively similar motility-impairing activities against

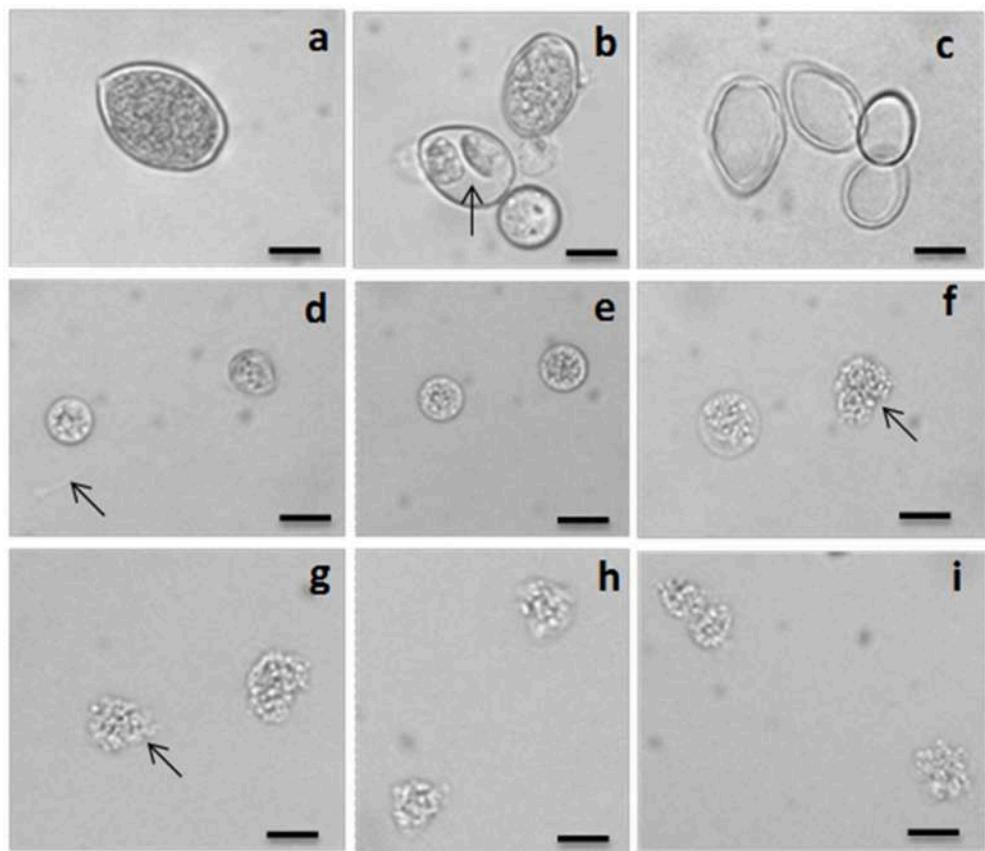


FIGURE 2 | Light micrographs showing sporangium, differentiating sporangium, empty sporangium (ghost) and inhibitory effects of dinactin, trinactin and gramicidin on zoospores of the grapevine downy mildew pathogen *Plasmopara viticola*. **(a)** A mature freshly harvested sporangium, **(b)** differentiated sporangia with developed zoospores (arrow) inside, **(c)** empty sporangia (ghosts) after release of zoospores, **(d)** two halted zoospores at the bottom of the dish just after addition of dinactin (1 µg/ml). Arrow indicates the trace of a halted zoospore, **(e)** both halted zoospores become round by 5 min after treatment with dinactin (1 µg/ml), **(f)** disruption of membrane and granulation of cell organelle and lysis (arrow) of zoospores by dinactin 10 min after treatment (1 µg/ml), **(g)** lysis of zoospores by dinactin 15 min after treatment (1 µg/ml), **(h)** lysis of zoospores by trinactin 15 min after treatment (1 µg/ml), **(i)** lysis of zoospores by gramicidin 15 min after treatment (1 µg/ml). Bar represents 10 µm.

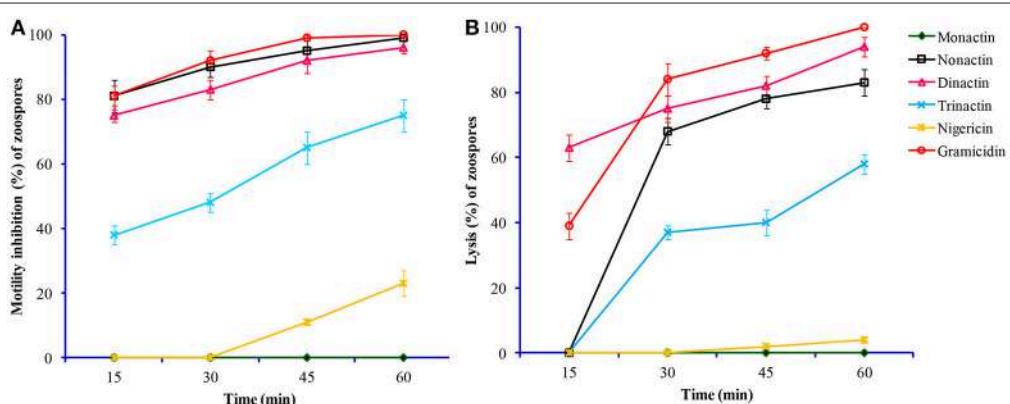


FIGURE 3 | Time-course comparative motility inhibitory (A) and lytic activities (B) of macroterolide antibiotics against zoospores of the grapevine downy mildew pathogen *Plasmopara viticola* at 0.5 µg/ml ($p \leq 0.05$).

TABLE 3 | Motility inhibitory and zoosporicidal activity of bonactin, feigrisolide C, macroterolide antibiotics (nonactin, monactin, dinactin and trinactin), nigericin and gramicidin against the grapevine downy mildew pathogen *Plasmopara viticola*.

Compound	Dose ($\mu\text{g/ml}$)	Motility halting and zoosporicidal activity (% \pm SE) [†]							
		15 min		30 min		45 min		60 min	
		Halted	Burst	Halted	Burst	Halted	Burst	Halted	Burst
Bonactin	1.0	0 \pm 0h	0 \pm 0g	43 \pm 4e	31 \pm 4g	58 \pm 2d	41 \pm 2e	71 \pm 4c	63 \pm 4d
	2.0	87 \pm 4bc	67 \pm 5d	92 \pm 3ac	88 \pm 2bc	96 \pm 3ab	93 \pm 4ab	99 \pm 1ab	97 \pm 3ab
	5.0	100 \pm 0a	85 \pm 5bc	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
	10.0	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Feigrisolide C	1.0	0 \pm 0h	0 \pm 0g	0 \pm 0g	0 \pm 0i	8 \pm 1f	4 \pm 1g	24 \pm 4f	18 \pm 3f
	5.0	27 \pm 4g	0 \pm 0g	37 \pm 4ef	12 \pm 2h	43 \pm 2e	18 \pm 3f	51 \pm 5d	41 \pm 3e
	10.0	96 \pm 2ab	88 \pm 5ab	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Nonactin	0.1 ^s	0 \pm 0h	0 \pm 0g	0 \pm 0g	0 \pm 0i	12 \pm 1f	0 \pm 0g	32 \pm 2ef	0 \pm 0g
	0.5	81 \pm 5df	0 \pm 0g	90 \pm 3ac	68 \pm 4e	95 \pm 3ab	78 \pm 3cd	99 \pm 1ab	83 \pm 4c
	1.0	95 \pm 3ac	81 \pm 6bc	100 \pm 0a	97 \pm 2ab	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Monactin	0.1 ^s	0 \pm 0h	0 \pm 0g	0b \pm 0g	0 \pm 0i	0 \pm 0f	0 \pm 0g	0 \pm 0g	0 \pm 0g
	0.5	0 \pm 0h	0 \pm 0g	0b \pm 0g	0 \pm 0i	0 \pm 0f	0 \pm 0g	0 \pm 0g	0 \pm 0g
	1.0	88 \pm 4ad	73 \pm 4cd	96 \pm 1ab	92 \pm 2ac	99 \pm 1a	98 \pm 1a	100 \pm 0a	99 \pm 1a
	2.5	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Dinactin	0.1 ^s	0 \pm 0h	0 \pm 0g	0 \pm 0g	0 \pm 0i	0 \pm 0f	0 \pm 0g	0 \pm 0g	0 \pm 0g
	0.3	0 \pm 0h	0 \pm 0g	31 \pm 2f	19 \pm 2h	40 \pm 3e	23 \pm 3f	53 \pm 2d	42 \pm 4e
	0.5	75 \pm 2ef	63 \pm 4d	83 \pm 3bc	74 \pm 4de	92 \pm 4ac	82 \pm 3bd	96 \pm 2ab	93 \pm 3ac
	1.0	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a	100 \pm 0a
Trinactin	0.1 ^s	0 \pm 0h	0 \pm 0g	7c \pm 1g	0 \pm 0i	12 \pm 2f	3 \pm 1g	39 \pm 3e	28 \pm 3f
	0.5	38 \pm 3g	0 \pm 0g	48 \pm 3e	37 \pm 2g	65 \pm 5d	40 \pm 4e	75 \pm 5c	58 \pm 3d
	1.0	100 \pm 0a	100 \pm 0a	100f \pm 0a	100 \pm 0a				
	2.5	100 \pm 0a	100 \pm 0a	100f \pm 0a	100 \pm 0a				
Nigericin	0.5	0 \pm 0h	0 \pm 0g	0 \pm 0g	0 \pm 0i	11 \pm 1f	0 \pm 0g	23 \pm 4f	0 \pm 0g
	1.0 ^d	32 \pm 4g	0 \pm 0g	63 \pm 3d	11 \pm 1hi	80 \pm 3c	72 \pm 5d	94 \pm 3ab	88 \pm 4bc
	2.5	72 \pm 5f	6 \pm 1fg	81 \pm 4c	18 \pm 2h	89 \pm 7ac	85 \pm 4bc	99 \pm 1ab	98 \pm 1ab
	5.0	82 \pm 2cf	38 \pm 3e	94 \pm 2ab	81 \pm 3cd	99 \pm 1a	89 \pm 4ac	100 \pm 0a	100 \pm 0a
Gramicidin	0.05	0e \pm 0h	0 \pm 0g	0 \pm 0g	0 \pm 0i	0 \pm 0f	0 \pm 0g	0 \pm 0g	0 \pm 0g
	0.1	70 \pm 3f	15 \pm 1f	82 \pm 5c	51 \pm 4f	84 \pm 5bc	72 \pm 4d	88 \pm 3b	86 \pm 5c
	0.5	81 \pm 3df	39 \pm 4e	92 \pm 3ac	84 \pm 5cd	99 \pm 1a	92 \pm 2ab	100 \pm 0a	100 \pm 0a
	1.0	100 \pm 0a	100 \pm 0a	100f \pm 0a	100 \pm 0a				

[†]Means within a column followed by the same letter(s) are not significantly different as assessed by Tukey's HSD (honest significance difference) post-hoc ($p \leq 0.05$).

^sStimulant and remained middle layer of water; spiral swimming.

the phytopathogenic peronosporomycete zoospores (Table 3 and Figures 3, 4). Furthermore, an ion-forming peptide gramicidin also displayed strong motility-impairing effects against the zoospores. As zoospores are unable to take up nutrients from their environment for maintenance of motility, they require steady supply of energy (ATP) from the internal cellular energy reserves (β -1,3-glucan or mycolaminarins) (Bimppong, 1975). The ATPase activity per volume of zoospores is similar to that of contracting skeletal muscles (Holker et al., 1993; Stienen et al., 1996). Therefore, disruption of energy

supply from mitochondria causes impairment of swimming behavior of the zoospores. Both macroterolide antibiotics and gramicidin have been found to enhance ATPase activity and cause rapid hydrolysis of ATP in the mitochondria (Graven et al., 1966). Therefore, the motility inhibitory effect of same macroterolide antibiotics and gramicidin shown in this study is likely to be linked to the depletion of ATP by enhanced ATPase activity in the mitochondria of treated zoospores. The underlying molecular mechanism of maintenance of zoospore motility is still poorly understood. As antibiotics have been

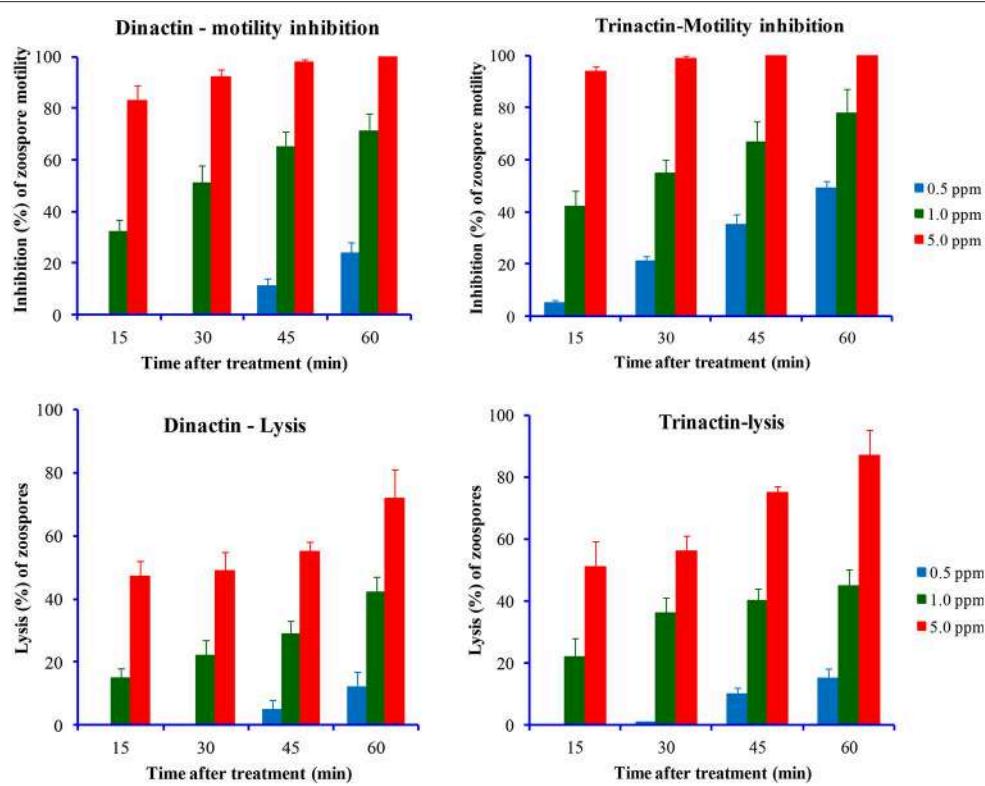


FIGURE 4 | Time-course comparative motility inhibitory (upper panel) and lytic activities (lower panel) of dinactin and trinactin against zoospores of the late blight pathogen of chili and cucumber, *Phytophthora capsici* at varying doses of tested compounds.

used as effective tools for many metabolic studies, a further quantitative study on ATPase activity in the mitochondria of zoospores treated with varying doses of macroterolide antibiotics should shed light on motility pathway of zoospores and may be used in the search for new targets for controlling this notorious class of phytopathogens. Inhibition of zoospore motility through disruption of cytoskeletal filamentous actin by microbial metabolites such as latrunculin B, 2,4-diacytetylphloroglucinol and macrocyclic lactam antibiotic, xanthobaccin A has been reported (Islam, 2008; Islam and von Tiedemann, 2011).

Another novel finding of this study was suppression of zoospore release from sporangia of *P. viticola* by macroterolide antibiotics (Table 5 and Figure 5). The cation channel-former and inducer of mitochondrial ATPase activity, gramicidin also suppressed zoosporogenesis with very high efficacy. Although, the underlying molecular mechanisms of zoosporogenesis are still poorly understood (Judelson and Blanco, 2005), cleavage of nuclei and differentiation of sporangia during zoosporogenesis require supply of energy from mitochondria. Moreover, intracellular Ca^{2+} ions play important roles in zoosporogenesis (Islam and Tahara, 2001). Therefore, suppression of zoosporogenesis by known inducers (macroterolides and gramicidin) of mitochondrial ATPase activity and ionophores suggests that depletion of ATP in mitochondria by hydrolysis of ATP in concert with translocation

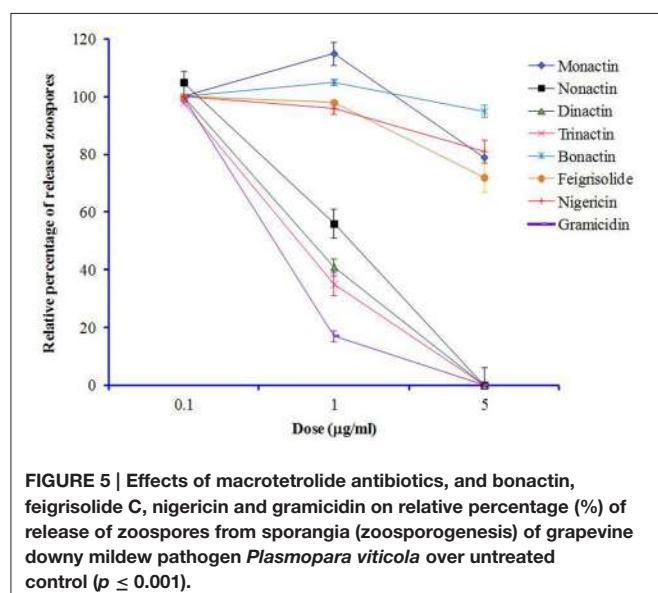
(efflux/influx) of cations from the cells might be involved in this process. Suppression of zoosporogenesis in *P. viticola* by staurosporine from a marine *Streptomyces* sp. B5136 (Islam et al., 2011) and 2,4-diacytetylphloroglucinol from the soil bacterium *Pseudomonas fluorescens* have previously been reported (Islam and von Tiedemann, 2011). Moreover, a selective inhibitor of protein kinase C (PKC), chelerythrine also suppressed zoosporogenesis which indicated the involvement of PKC in the process of zoospore release from sporangia (Islam et al., 2011).

The experimental results reported in the present study do not clarify the precise mechanism involved but they point out that induction of ATPase activity in mitochondria and/or translocation/imbalance of cations in the cells might suppress zoosporogenesis and impair motility of the zoospores. Therefore, elucidation of the role of ATPase in the swimming pattern and motility of zoospores will obviously help to advance our understanding of the biology and pathogenicity of the peronosporomycete phytopathogens. In this study, some linear tetrahydrofurans such as (+)-homonastic acid and bonactin also impaired motility of zoospores qualitatively and quantitatively similar to the macroterolide antibiotics. Therefore, naturally occurring low molecular weight inducers of ATPase might have high potential as lead compounds for designing novel effective agrochemicals against the peronosporomycete phytopathogens.

TABLE 4 | Motility inhibitory activity of nactic acids and their esters, and dinactin isolated from marine *Streptomyces* sp. Act 8970 against the sugar beet damping-off pathogen *Aphanomyces cochlioides* AC-1.

Compound	Dose ($\mu\text{g}/\text{ml}$)	Motility inhibitory activity* (60 min) (% \pm SE)
(+)-Nonactic acid	10	0 \pm 0d
	20	37 \pm 3c
	40	88 \pm 5b
	80	95 \pm 2ab
(+)-Homonactic acid	1	0 \pm 0d
	5	98 \pm 1ab
	10	100 \pm 0a
Nonactic acid methyl ester	5	0 \pm 0d
	10	89 \pm 5ab
	20	100 \pm 0a
Homonactic methyl ester	10	0 \pm 0d
	20	88 \pm 5ab
	30	100 \pm 0a
Dinactin	0.1	0 \pm 0d
	0.5	98 \pm 1ab
	1.0	100 \pm 0a

*Data are average values \pm SE of five replications in each dose of each tested compound. Dinactic acid was inactive up to 100 $\mu\text{g}/\text{ml}$. No lysis of zoospores was observed until 60 min of treatment by any of the tested compounds. Data were analyzed by one way ANOVA [$F_{1(5, 32)} = 417.3, p < 0.001$]. Mean values were separated by Tukey's HSD (honest significance difference) posthoc statistic.



One of the important findings of our study is that the zoospores of a biotrophic phytopathogen, *P. viticola* halted by macroterolide antibiotics or gramicidin or nigericin were rapidly immobilized (Figures 2, 3B). A similar phenomenon was observed when zoospores of *P. capsici* were treated with higher

concentration of dinactin and trinactin. In an earlier study, Graven et al. (1966) reported that nonactin, monactin, dinactin, trinactin and gramicidin induced swelling of mitochondria through induction of alterations in the ion translocation system. Nigericin has also been found to inhibit mitochondrial respiration by blocking the uptake of both K^+ and inorganic phosphorus (Pi) ions (Graven et al., 1967). Therefore, rapid lysis of halted *P. viticola* zoospores by the macroterolides, gramicidin, or nigericin might be associated with alteration in the ion translocation system in the mitochondria of zoospores and/or imbalance of osmotic balance in the zoospores. It appears from this study that inhibition of mitochondrial respiration by any chemical inhibitor might impair motility of zoospores. Experiments using gramicidin and nigericin ionophores, Appiah et al. (2005) demonstrated that altering potassium homeostasis during zoospore swimming significantly influenced speed, swimming pattern, and encystment of zoospores of *Phytophthora* and *Pythium* species (Appiah et al., 2005). Although, almost all *P. viticola* zoospores stopped by nactic acids or macroterolides were lysed, the stopped *A. cochlioides* zoospores by the same compounds became, however, round cystospores instead of lysing. *A. cochlioides* is a soilborne phytopathogen and hence sensitivity of zoospores to surrounding complex heterogenous signals in soils might be different compared to a biotrophic leaf pathogen such as *P. viticola*. In soilborne pathogens, those zoospores failing to find their host during their motile stage or exposed to toxic substances, rapidly become cystospores by developing a cell wall (Islam, 2011). These cystospores can regenerate secondary zoospores under favorable conditions to search the host plant. This adaptive strategy may be absent or not essential in the leaf pathogen *P. viticola* and zoospores released on a leaf can easily find stomata for infection, and hence zoospores halted by nactic acids and macroterolides are immediately lysed. Motility inhibition of *A. cochlioides* zoospores without lysis by a nonhost metabolite, nicotinamide has been reported (Islam et al., 2004).

Nonactin, dinactin, trinactin and teranactin isolated from a variety of *Streptomyces* species are cyclotetralactones derived from nonactic acid and homononactic acid as building units of ionophoric character. Nonactin and its homologs such as monactin, dinactin, trinactin, and tetranactin were isolated as bioactive compounds from *Streptomyces* spp. by many researchers (Beck et al., 1962; Dominguez et al., 1962; Meyers et al., 1965; Haneda et al., 1974; Callewaert et al., 1988; Sobolevskaya et al., 2004; Hashida et al., 2012). The precursor of nonactin and other marcotetrolides, nonactic acid is biosynthesized in *Streptomyces* spp. by a type II polyketide synthase (PKS) system (Walczak et al., 2000). In our study, both cyclotetralactones and their precursor, nonactic acid and homononactic acids displayed inhibitory effects against zoosporogenesis and motility of zoospores but in varying concentrations. Among the nactic acids, the homonactic acid showed a higher activity than nonactin. A further study is needed to establish precise structure-activity relationships of these bioactive compounds which might lead to the development of an effective biopesticide against the peronosporomycete phytopathogens.

TABLE 5 | Effects of macroterolide antibiotics, and bonactin, feigrisolide C, nigericin and gramicidin on the release of zoospores from sporangia (zoosporogenesis) of the grapevine downy mildew pathogen *Plasmopara viticola*.

Compound	Dose ($\mu\text{g/ml}$)	Relative percent of released zoospores (% \pm SE) and their motility behavior*	
		Zoospores	Behaviors or fate of released zoospores
Nonactin	0.1	105 \pm 4 ac	Normal swimming
	0.5	120 \pm 6 a	Normal swim ming
	1.0	115 \pm 5 ab	Swam faster than normal speed
	5.0	79 \pm 6 fg	No motile zoospores and 100% zoospores lysed just after release
	10.0	0 \pm 0 l	–
Monactin	0.1	100 \pm 0 bd	Normal swimming
	1.0	56 \pm 4 hi	80% of the released zoospores lysed and others swam straight with less or no turning. Speed of swimming was normal.
	5.0	0 \pm 0 l	–
Dinactin	0.1	100 \pm 0 bd	Normal swimming
	1.0	41 \pm 3 ij	50% of released zoospores lysed and others swam normally
	5.0	0 \pm 0 l	–
Trinactin	0.1	98 \pm 1 cd	Normal swimming
	1.0	35 \pm 4 j	Presence of debris at the bottom of dish, all released zoospores lysed.
	5.0	0 \pm 0 l	–
Bonactin	0.1	100 \pm 1 bd	Normal swimming
	1.0	105 \pm 1 ac	Normal swimming
	5.0	95 \pm 2 cf	Normal swimming
	10.0	85 \pm 5 dg	95% of released zoospores lysed and 5% had normal swimming
Feigrisolide C	0.1	100 \pm 0 bd	Normal swimming
	1.0	98 \pm 1 cd	95% normal swimming and 5% lysed.
	5.0	72 \pm 5 gh	80% of released zoospores lysed, others normal swimming
	10.0	7 \pm 1 kl	100% of released zoospores lysed
Nigericin	0.1	100 \pm 0 bd	Normal swimming
	1.0	96 \pm 2 cd	Normal swimming
	5.0	81 \pm 4 eg	Normal swimming
	10.0	47 \pm 5 ij	90% of released zoospores lysed. Those moving were abnormal in size (bigger) and irregular in shape. No normal zoospores.
Gramicidin	0.1	100 \pm 0 bd	Normal swimming
	0.5	75 \pm 4 g	75% released zoospores lysed and others slowly swimming
	1.0	17 \pm 2 k	100% released zoospores lysed
	5.0	0 \pm 0 l	–

*Data presented here are average values \pm SE of five replications in each dose of compounds. Data were analyzed by one way ANOVA [$F_{1(26, 60)} = 2009$, $p < 0.001$]. Mean values were separated by Tukey's HSD (honest significance difference) posthoc statistic.

Zoosporogenesis and motility of zoospores are two life stages critically important for disease cycles and also virulence of the peronosporomycete phytopathogens (Latijnhouwers et al., 2004; Judelson and Blanco, 2005; Islam et al., 2011). A motility inhibitory compound, staurosporine has recently been found to successfully suppress development of downy mildew on treated grapevine leaves (Islam et al., 2011). Macroterolide antibiotics exhibit a very wide range of effects, ranging from antimicrobial to insecticidal, acaricidal, anticancer, antiprotozoan (coccidiostatic), immunosuppressive and antiparasitic (antihelminthic) (Meyers et al., 1965; Borrel

et al., 1994; Zizka, 1998; Kusche et al., 2009). Plant growth promotion and exhibition of specific insecticidal effects of precursors of macroterolide antibiotics, nonactic and homononactic acids have been reported (Jizba et al., 1992). The zoospore motility inhibitory substances found in this study such as dinactin, and nactic acids produced by several *Streptomyces* spp. including a marine strain Act 8970 might help us to design strategies for biorational management of the notorious Peronosporomycete phytopathogens. This study reveals that macroterolide antibiotics are not only potential candidates for development of antiperonosporomycete

agrochemicals but could also be used as tools for dissecting underlying molecular mechanisms governing zoosporegenesis and motility functions of zoospores of the fungus-like peronosporomycetes.

AUTHOR CONTRIBUTIONS

MTI: Involved in conceived idea, designed and executed experiments, analyzed data and writing manuscript; AvT and HL: Involved in conceived idea, designed experiments, and critically edited manuscript.

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A Marine Actinomycete Rescues *Caenorhabditis elegans* from *Pseudomonas aeruginosa* Infection through Restitution of Lysozyme 7

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The resistance of *Pseudomonas aeruginosa* to conventional antimicrobial treatment is a major scourge in healthcare. Therefore, it is crucial that novel potent anti-infectives are discovered. The aim of the present study is to screen marine actinomycetes for chemical entities capable of overcoming *P. aeruginosa* infection through mechanisms involving anti-virulence or host immunity activities. A total of 18 actinomycetes isolates were sampled from marine sediment of Songsong Island, Kedah, Malaysia. Upon confirming that the methanolic crude extract of these isolates do not display direct bactericidal activities, they were tested for capacity to rescue *Caenorhabditis elegans* infected with *P. aeruginosa* strain PA14. A hexane partition of the extract from one isolate, designated as *Streptomyces* sp. CCB-PSK207, could promote the survival of PA14 infected worms by more than 60%. Partial 16S sequence analysis on this isolate showed identity of 99.79% with *Streptomyces sundarbansensis*. This partition did not impair feeding behavior of *C. elegans* worms. Tested on PA14, the partition also did not affect bacterial growth or its ability to colonize host gut. The production of biofilm, protease, and pyocyanin in PA14 were uninterrupted, although there was an increase in elastase production. In *lys-7::GFP* worms, this partition was shown to induce the expression of *Lysozyme 7*, an important innate immunity defense molecule that was repressed during PA14 infection. GC-MS analysis of the bioactive fraction of *Streptomyces* sp. CCB-PSK207 revealed the presence of methyl esters of branched saturated fatty acids. In conclusion, this is the first report of a marine actinomycete producing metabolites capable of rescuing *C. elegans* from PA14 through a *lys-7* mediated activity.

Keywords: *Caenorhabditis elegans*, *Pseudomonas aeruginosa*, marine actinomycetes, *Streptomyces* sp., *lysozyme 7*

INTRODUCTION

Pseudomonas aeruginosa, an opportunistic human pathogen is a principal cause of nosocomial infection, leading to morbidity and mortality in immune-compromised patients (Moy et al., 2006; Driscoll et al., 2007). Among dangers posed by *P. aeruginosa* is healthcare associated pneumonia and infection of burn patients (Rello et al., 2003; Agodi et al., 2007). The perilous emergence

of multidrug resistant *P. aeruginosa* strains is hindering the development and effectiveness of antibiotics (Hauser and Sriram, 2005; Levy, 2005; Aloush et al., 2006). To circumvent problems associated with antibiotic resistance, the search for new anti-infectives targeting bacterial virulence or host immunity have gained momentum (Clatworthy et al., 2007; Hancock et al., 2012). In comparison to traditional antibiotics which exert their effects through bactericidal activities, anti-infectives do not contribute to selection pressure which unwantedly leads to resistance development (Hamill et al., 2008).

The nematode *Caenorhabditis elegans* is readily infected with numerous human bacterial pathogens and amenable to various molecular tools, making it a reliable model for understanding different facets host-pathogen interaction such as, virulence factors and innate immunity pathways (Aballay and Ausubel, 2002). These attributes, coupled with a high degree of conservation with human innate immune signaling pathways, promote the use of *C. elegans* for drug discovery (Artal-Sanz et al., 2006; Burns et al., 2006). The co-existence of both pathogen and host in a host-pathogen relationship provides the capacity of identifying chemical entities capable of rescuing infected host. Academically, this may lead to the discovery of molecules that attenuate bacterial virulence or augment the immunity of the host (Moy et al., 2006). The use of *C. elegans* as in host-pathogen screening assays have been extended to many human pathogens, including *Enterococcus faecalis* (Moy et al., 2006), *Candida albicans* (Breger et al., 2007), *Vibrio alginolyticus* (Durai et al., 2013), *Staphylococcus aureus* (Kong et al., 2014b), *Burkholderia pseudomallei* (Eng and Nathan, 2015), and *Salmonella enteritidis* (Kulshreshtha et al., 2016).

Actinomycetes are persistent soil inhabitants with exceptional capacity to produce clinically useful secondary metabolites, having contributed to more than 50% of the microbial antibiotics discovered (Bérny, 2005). Early efforts in actinomycetes drug discovery concentrated mostly on soil isolates, due to the erroneous view that the marine environment is a poor source for this group of bacteria (Fenical and Jensen, 2006). However, the diversity of the marine environment enforces a natural selection toward an immeasurable pool of microbial secondary metabolites and may therefore offers a rich and yet unexploited source of actinomycetes, with representatives reported from seawater, intertidal zones, ocean floor, deep ocean trenches, ocean sediments, invertebrates, and plants (Bull et al., 2005). As result, a promising number of novel secondary metabolites with biological properties are constantly being reported from marine actinomycetes (Feling et al., 2003; Lam, 2006; Solanki et al., 2008; Kang et al., 2015). Compounds originating from marine microbes that attenuate virulence through inhibition of quorum sensing system without bacteriocidal activities have also been reported (Fu et al., 2013; Nail et al., 2013).

The *C. elegans*-PA14 relationship has been used to screen natural products from terrestrial plants, endophytic fungi, marine bacteria, and seaweeds, to search for compounds capable of boosting immunity of PA14 infected worms or diminishing quorum sensing and virulence factors (Zhou et al., 2011; Dharmalingam et al., 2012; Kandasamy et al., 2012; Liu et al., 2013). Elsewhere, single compounds such as, curcumin and

selenium were also reported to protect *C. elegans* during PA14 infection (Rudrappa and Bais, 2008; Li et al., 2014). Given the vast potential of marine actinomycetes as source of secondary metabolites and the robustness of the *C. elegans*-PA14 screening assay, we utilized the assay to screen for marine actinomycetes capable of producing metabolites that extend the lifespan of infected worms. An extract from *Streptomycetes* sp., conferred survival advantage to the PA14 infected *C. elegans* with a host-directed mechanism partially mediated by the up-regulation of *lys-7* gene. Major compounds in the bioactive fraction were identified as methyl esters of several saturated fatty acids.

MATERIALS AND METHODS

Bacteria and Worms

PA14 and *Escherichia coli* strain OP50 were cultured as described previously (Dharmalingam et al., 2012). *C. elegans* strain CF4059 with genotype *fer-15(b26)II; rol-6(su1006)II; fem-1(hc17)IV* which is sterile at 25°C and of the roller phenotype to avoid confounding progeny production during screening and aid in worm scoring were obtained from Cynthia Kenyon Lab (University of California, USA). *C. elegans* strain SAL105 with genotype *pha-1(e2123) III; denEx2* whose *lys-7* gene was tagged with green fluorescent protein (Alper et al., 2007) were obtained from *Caenorhabditis* Genetics Center (CGC), USA (<https://cbs.umn.edu/cgc/home>), respectively. Procedures for maintenance and handling of all worms were approved by the Universiti Sains Malaysia Animal Ethics Committee.

Microbial Sample Collection

A total of 10 sea bed soil samples were collected from waters at depths ranging from 10 to 20 m deep from Songsong Island, Yan, Kedah, Malaysia (5°48'37.2"N 100°17'47.5"E) on December 2013. The sediment samples were spread on petri plates and dried overnight in laminar flow hood (Valli et al., 2012).

Isolation of Actinomycetes

After drying, samples were heated at 70 ± 2°C for 15 min and were grinded lightly with alcohol-sterilized mortar and pestle. Ten-fold serial dilution up to 10⁻⁵ was carried out by diluting 1.0 g of sediment sample in 9.0 mL of 50% artificial sea water (ASW). Approximately, 0.1 mL of the mixture was spread on starch casein agar (SCA) supplemented with 80 µg mL⁻¹ of cycloheximide. All plates were incubated at 28 ± 2°C and observed for actinomycetes growth for 28 days (Mincer et al., 2002; Valli et al., 2012). Grown colonies were observed for morphological differences and listed as candidates for screening assays.

Preparation of Actinomycetes Extracts

The isolates were cultured in M1 medium [ingredients: 10.0 g soluble starch, 4.0 g yeast extract, 2.0 g peptone, and 1.0 L distilled water followed by autoclaving at 121°C for 20 min] and incubated with shaking at 28 ± 2°C, 200 rpm for 7–14 days. The culture broth was freeze-dried and extracted with 1:100 (w/v) methanol (MeOH). The mixture was shaken overnight

and then filtered using Whatman grade 1 cellulose filter paper with 11 μM pore size. The filtrate was concentrated using rotary evaporator at $60 \pm 2^\circ\text{C}$. The extracts were stored at $-20 \pm 2^\circ\text{C}$ and adjusted to working concentration with distilled water.

Anti-microbial Assay

Anti-bacterial screening of extracts was performed using the modified Kirby-Bauer disc diffusion method. A few colonies of *P. aeruginosa* PA14 from a culture plate incubated for 24 h were directly inoculated in 0.85% saline. The suspension was compared to a 0.5 McFarland turbidity standard and adjusted with sterile saline. A sterile cotton swab was dipped into the suspension and pressed on the wall of tubes to remove excess bacterial suspension. The swab was repeatedly streaked over the entire surface of Mueller-Hinton agar (Merck, Germany) until the entire surface was streaked. A sterile Whatman antibiotic disc with a 6 mm diameter was placed on the bacterial lawn and 10 μL of 200 $\mu\text{g mL}^{-1}$ crude extract was transferred onto each disc. The plates were inverted and incubated at $37 \pm 2^\circ\text{C}$ for 24 h. The zone of inhibition was measured and recorded after the incubation period.

Slow Killing Survival Assay

For the survival assay, *C. elegans* strain CF4059 was used. Young adult worms were age-synchronized and infected with PA14 on Pseudomonas Infection agar (PIA) as described previously (Dharmalingam et al., 2012). Final concentration of extract in each plate was 200 $\mu\text{g mL}^{-1}$ while a negative control plate contained only distilled water. Worm survival was scored every 24 h, with mortality designated as failure of worm to react by motion when prodded with platinum wire (Powell and Ausubel, 2008). A total time of 96 h was selected as the screening time-point as it gave the best resolution in identifying a potent hit candidate. Worms that crawled onto the plate wall were not included in the final survival analysis.

Molecular Characterization of Bacterial Isolate

The isolate which produced extract contributing to highest worm survival in the slow killing survival assay described above was cultured in M1 broth for 5 days at 180 rpm at $28 \pm 2^\circ\text{C}$. Genomic DNA of the isolate was extracted using Real Biotech Corporation Hi-Yield Genomic DNA Kit. Universal primers 1492R and 27F were used for the amplification of DNA polymerase chain reaction (PCR) amplification with Applied Biosystem Veriti® 96-Well Fast Thermal. The PCR process involves initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 and 40 s, and final extension at 72°C for 5 min. The PCR product was purified using QIA quick PCR Purification Kit by Qiagen. Sequences of purified DNA samples were aligned with the corresponding phylogenetic tree constructed using MEGA6 (Tamura et al., 2013). Comparison of 16S ribosomal RNA gene sequences of the isolate was done using EzTaxon (Chun et al., 2007).

Liquid-Liquid Partitioning of Bacterial Isolate

The extract of the isolate contributing to highest survival of worms was further partitioned in n-hexane, dichloromethane, ethyl acetate, and butanol. The methanol-aqueous was mixed with each solvent in 1:1 (v/v) ratio in a separator funnel and shaken vigorously. The funnel was let to stand for 15 min and the resulting layers were collected and dried with a rotary evaporator. The subsequent partitions were then employed in a slow killing survival assay. Subsequently, the partition causing highest percentage of worm survival was subjected to the assays described below.

Dose Response Assay

The CF4059 worms were exposed to the infection plate as described in the slow-killing assay with or without partition supplementation at 50, 200, 400, and 1,000 $\mu\text{g mL}^{-1}$ final concentration. Worm survival was scored every 24 h.

Pharyngeal Pumping Assay

C. elegans CF4059 was exposed to the infection plates as described in the slow-killing assay. The pharyngeal pumping of three randomly picked worms was observed for 20 s at 12 h interval using Leica Stereomicroscope M205 FA. Pumping rate was measured by counting grinder movement and contraction/relaxation cycles of the bulb (Hobson et al., 2006).

Growth of *P. aeruginosa* PA14

Method for kinetic growth study was a modification of an earlier protocol (Hall et al., 2014). A few PA14 colonies from 24 h freshly cultured plates were inoculated in 250 mL conical flask with 50 mL Mueller-Hinton broth (Merck, Germany), followed by incubation at 37°C , 180 rpm for 24 h. The culture was transferred into 50 mL centrifuge tube and centrifuged at $2,775 \times g$ for 30 min. The supernatant was discarded and remaining cell pellet was washed twice. The cell pellet was then dissolved in fresh MH broth. The treatment well contained 10 μL of bacterial cell culture, 180 μL of MH broth and 10 μL of 8 mg/mL extract. Control well only contained 10 μL of bacterial cell culture and 190 μL of MH broth. The microtiter plates were incubated in the microplate reader at 37°C for 24 h with sampling interval every 4 h at 625 nm (BioTek Synergy Mx, USA).

PA14 Biofilm Assay

Biofilm assay was carried out as described previously (O' Toole, 2011). PA14 was cultured in Luria Bertani (LB) broth overnight at 37°C with shaking at 180 rpm. PA14 was cultured in LB broth at 37°C with 180 rpm shaking overnight. A 96-well biofilm assay plate with 400 $\mu\text{g mL}^{-1}$ of actinomycete partition in LB broth was inoculated with the overnight PA14 culture at 1:100 ratio and further incubated overnight at 37°C . The cells were discarded and plate was rinsed with tap water. Biofilm formed on the wall of the plate was stained with 1% crystal violet and solubilized with 30% acetic acid in water. Optical density was measured using microtiter plate reader (SpectraMax M5) at 550 nm wavelength.

PA14 Protease, Elastase Assay, and Pyocyanin Assay

Protease and elastase assay were carried out as described elsewhere (Rudrappa and Bais, 2008). PA14 was cultured at 37°C for 24 h, with or without presence of *Streptomyces* sp. partition in LB broth. The supernatant was collected and filtered using 0.22 µM nylon filter. About 50.0 µL supernatant was added into the reaction mixture consisting of 0.8% azocasein (Sigma) in 500 µL of 50 mM K₂HPO₄ at pH7. The reaction mixture was incubated at 25°C for 3 h. The reaction was stopped by adding 0.5 mL of 1.5 M HCl into the mixture. The tubes were placed on ice for 30 min and centrifuged at 7,826 × g for 10 min. Finally, 0.5 mL of 1 M NaOH was added into the tubes and the reading was measured at 440 nm.

For elastase assay, 50.0 µL supernatant was added to 1.0 mL of 10 mM Na₂HPO₄ at pH7 and 20 mg of elastin-Congo red. The tubes were incubated for 4 h at 37°C with 180 rpm shaking. The tubes were centrifuged at 7,826 × g for 10 min and the optical density reading was taken at 495 nm.

Pyocyanin assay was carried out as described elsewhere (Essar et al., 1990). PA14 was cultured with or without presence of *Streptomyces* sp. metabolites and supernatant was collected as above. About 4.5 mL of chloroform was added to 7.5 mL of collected supernatant and vortexed for 20 s. The mixture was centrifuged at 2,880 × g for 10 min. About 3.0 mL of the resulting blue layer at the bottom of the tube was transferred into a new tube, followed by addition of 1.5 mL of 0.2 M HCl and vortexing for 20 s. Tubes were then centrifuged for 2 min at 2,880 × g and 1.0 mL of the ensuing pink layer was transferred into cuvettes and reading was taken at 520 nm (Thermo Scientific Genesys20).

Visualization of *lys-7* in *C. elegans*

Slow killing assay was carried out using the transgenic *lys-7::GFP* *C. elegans* strain SAL105. The fluorescence micrograph of worms was captured using a Leica Microsystem M205 FA following 24 h of pathogen exposure. Images were analyzed using Image J (National Institutes of Health, USA) to quantify *lys-7* fluorescent intensity.

Preparative TLC Fractionation

TLC plate (Merck TLC silica gel 60 F₂₅₄, Germany) coated with silica was used as the stationary phase. Sample of hexane partition was prepared by diluting 10 mg of extract in 1.0 mL of CHCl₃. The sample was spotted on the plates with a capillary tube. The plates were then put in developing chamber with solvent system of methanol:chloroform of 20:80. After drying, a small portion of the plate was cut, followed by staining with vanillin-sulphuric acid reagent (Yadav and Gupta, 2013). The stained plates were then air-dried for 15 min and oven-dried at 96 ± 2°C for 8 min (Maurya and Srivastava, 2013). Spots formed were aligned on the plate and marked. The marked area was scraped using a scalpel. The collected fractions were dissolved in 100% ethanol, filtered by using Whatman no.1 filter paper and rotated to dryness. The fractions were subjected for survival assay and the fraction with positive result was sent for GC-MS analysis (Agilent 6890, USA) with capillary column of 30 m × 0.25 mm × 0.25 µm (Agilent HP-5 ms, USA). The flow rate was set at 1.2 mL/min, with 10 µL sample injection. Helium gas was used and total run

was 40 min. The obtained spectrum was compared with NIST Spectral Library for compound identification.

Data Analysis

All numerical data were analyzed using GraphPad Prism 5 and StatView5.0.1 (SAS Institute, Inc) software. Values were presented as mean ± standard deviation (SD) of at least two independent experiments. Data from the killing assays were analyzed with StatView 5.0.1 and plotted using the Kaplan-Meier Cumulative Survival Plot for Time (non-parametric survival analysis). The comparison was analyzed using the GraphPad Prism 5 Log-rank (Mantel-Cox) significance test. Data from dose response assay, pharyngeal pumping assay, biofilm assay, kinetic growth of PA14 and total cell fluorescent count were analyzed with GraphPad Prism 5 unpaired t-test.

RESULTS

Isolation of Marine Actinomycetes and Antimicrobial Assay

A total of 18 morphologically different strains were successfully isolated from the marine sediment samples (Table S1). These isolates produced aerial mycelium, with four of the isolates showing pigmentation. All isolates produced mycelial clump when cultured in broth medium after incubation in shaker at 180 rpm and 28 ± 2°C. All 18 isolates were extracted and subjected to anti-microbial assay. None of these isolates caused a visible inhibition zone on the *P. aeruginosa* PA14 lawn (data not shown) which means these extracts do not possess bactericidal activities toward PA14.

Effect of Marine Actinomycetes Methanol Extracts and Partitions on Survival of PA14 Infected *C. elegans* Worms

Compared to untreated PA14 infected worms, infected worms treated with 8 of the 18 marine actinomycetes extract, respectively, (A3, A5, A22, A26, A38, A42, A48, and A50) showed improved survival rates (Figure 1). Among these, statistically significant increase in survival rate was achieved with A3, A5, and A22 (36.99 ± 2.80–57.31 ± 3.85%), with isolate A5 contributing to the highest survival rate. In tandem, A5 also delayed mortality of infected worms, as represented in TD50 value (Table S2). In addition, worms treated with the remaining extracts (A20, A24, A30, A31, A39, A40, A41, A43, A45, and A47), showed increased susceptibility to killing by PA14. Further partitioning of the isolate A5 methanol extract followed by slow killing assay revealed the hexane partition of isolate A5 to be most potent in attenuating the killing of infected worms, with survival rate of 69.65 ± 4.50% (Figure 2) and TD50 = 102.2 ± 5.54 h (Table S3).

A5 Isolate Identified as *Streptomyces* sp. CCB-PSK207

Using partial 16S analysis, the A5 isolate showed sequence identity of 99.7–99.85% with several *Streptomyces* sp. This include *Streptomyces sundarbansensis*, *Streptomyces puniceus*, *Streptomyces badius*, *Streptomyces sidensis*, *Streptomyces rubiginosohelvolus*, *Streptomyces pluricolorescens*,

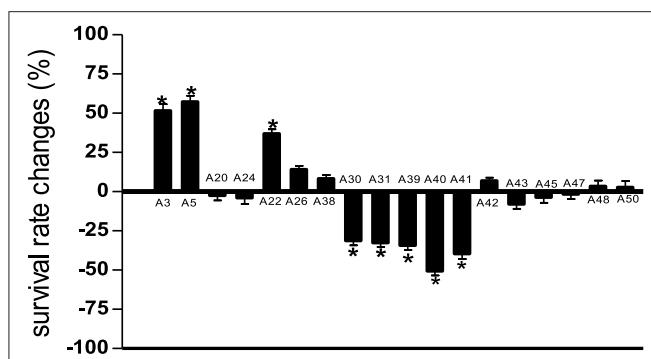


FIGURE 1 | Survival of *C. elegans* infected with *Pseudomonas aeruginosa* PA14 in the presence of marine actinomycetes crude methanolic extract. A5 crude methanolic extract resulted in significantly highest *C. elegans* survival during the PA14 killing assay. *Denotes significance in the Log-rank test in comparison to the untreated control ($p < 0.05$). Data were representative of two independent experiments.

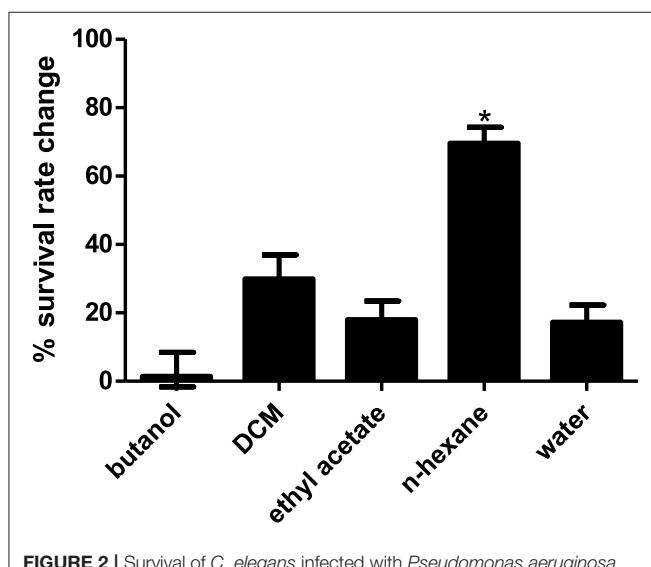


FIGURE 2 | Survival of *C. elegans* infected with *Pseudomonas aeruginosa* PA14 in the presence of different portion of A5 isolate. Hexane partition of the A5 methanolic extract resulted in significantly highest *C. elegans* survival during PA14 killing assay. *Denotes significance in the Log-rank test in comparison to the untreated control ($p < 0.05$). Data were representative of three independent experiments.

Streptomyces parvus, *Streptomyces globisporus*, and *Streptomyces cyanofuscatus* (Figure 3). The sequence was deposited in NCBI GenBank under accession number KX372372. On ISP2 agar plates, A5 isolate produced white beige aerial mycelia and brownish substrate mycelia with no pigmentation (Figure S1). A5 isolate was named as *Streptomyces* sp. CCB-PSK207.

Effect of *Streptomyces* sp. CCB-PSK207 Hexane Partition on Survival of PA14-Infected *C. elegans*

Results showed that the hexane partition of the *Streptomyces* sp. CCB-PSK207 extract promoted survival of PA14-infected worm

in a dose dependent manner with a gradual increase observed from 45.33 ± 4.32 to $72.71 \pm 4.66\%$ at concentration range of 50–400 $\mu\text{g mL}^{-1}$ (Figure 4). There was no further increase in *C. elegans* survival promotion at 1,000 $\mu\text{g mL}^{-1}$ concentration.

Effect of *Streptomyces* sp. CCB-PSK207 Hexane Partition on *C. elegans* Feeding Activities

Comparison of pharyngeal pumping rate in *C. elegans* exposed to the *Streptomyces* sp. CCB-PSK207 hexane partition with worms without presence of partition showed no significant difference for all three time points (Figure 5). This indicated that the *Streptomyces* sp. CCB-PSK207 hexane partition did not interrupt *C. elegans* feeding rate.

Effect of *Streptomyces* sp. CCB-PSK207 Hexane Partition on PA14 Growth

Similarly, the *Streptomyces* sp. CCB-PSK207 hexane partition did not impair growth kinetics of PA14, which denote that the rescue of PA14 infected worms did not occur through killing of pathogens (Figure 6).

Effect of *Streptomyces* sp. CCB-PSK207 Hexane Partition on PA14 Virulence Factor Production

Results showed no significant difference in the level of biofilm, protease, and pyocyanin production between control PA14 and those exposed to *Streptomyces* sp. CCB-PSK207 hexane partition (Figure 7). However, a significant increase in elastase production was observed in treated PA-14.

Effect of *Streptomyces* sp. CCB-PSK207 Hexane Partition on the Expression of *lys-7* in PA14 Infected *C. elegans*

As compared to worms fed with *E. coli* OP50, PA14-infected worms showed diminished fluorescent signal (Figures 8A,B). However, treatment with *Streptomyces* sp. CCB-PSK207 hexane partition appear to restore GFP expression in both PA 14 infected worms and OP50 fed worms (Figures 8C,D). Imaging-based software quantification showed that worms exposed to *Streptomyces* sp. CCB-PSK207 metabolites produced significantly highest intensity of GFP expression (Figure 8E).

Effect of *Streptomyces* sp. CCB-PSK207 Fraction on Survival of PA14-Infected *C. elegans*

Three fractions were collected from preparative TLC and further employed in the *C. elegans* slow killing survival assay. Among them, fraction A5HB showed a significant increase in worm survival rate at $71.43 \pm 4.67\%$ (Figure 9) and TD50 of 93.6 ± 1.9 h (Table S4). Fraction A5HA resulted in <20% worm survival, while A5HC increased the susceptibility of worms toward mortality caused by PA14, respectively.

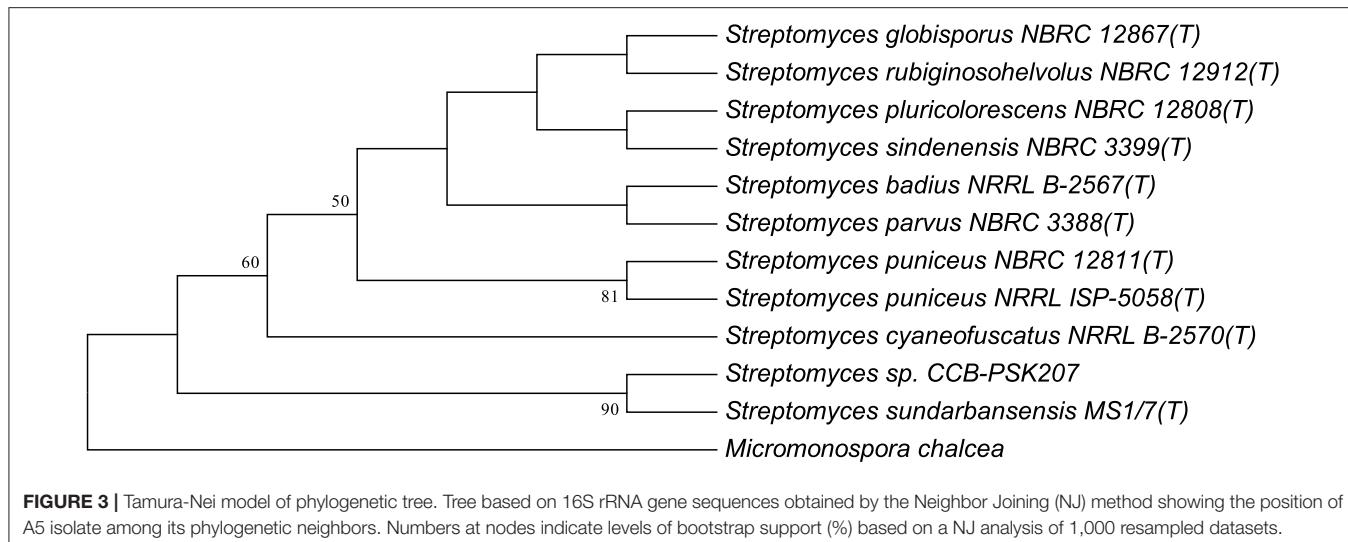


FIGURE 3 | Tamura-Nei model of phylogenetic tree. Tree based on 16S rRNA gene sequences obtained by the Neighbor Joining (NJ) method showing the position of A5 isolate among its phylogenetic neighbors. Numbers at nodes indicate levels of bootstrap support (%) based on a NJ analysis of 1,000 resampled datasets.

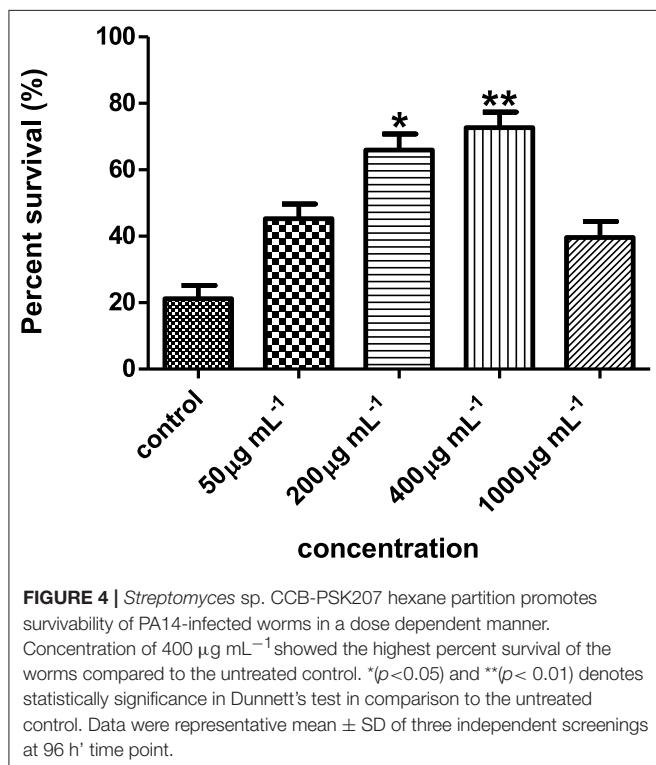


FIGURE 4 | *Streptomyces* sp. CCB-PSK207 hexane partition promotes survivability of PA14-infected worms in a dose dependent manner. Concentration of $400 \mu\text{g mL}^{-1}$ showed the highest percent survival of the worms compared to the untreated control. *($p < 0.05$) and **($p < 0.01$) denotes statistically significance in Dunnett's test in comparison to the untreated control. Data were representative mean \pm SD of three independent screenings at 96 h time point.

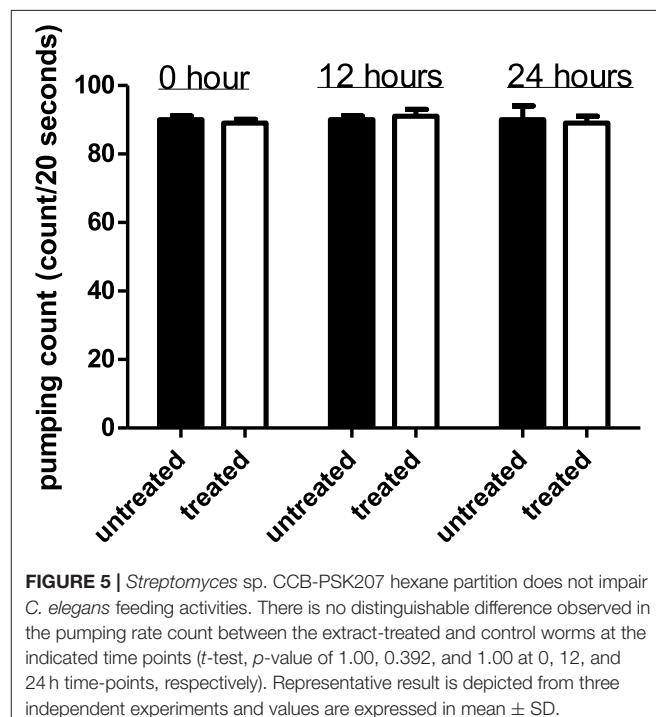


FIGURE 5 | *Streptomyces* sp. CCB-PSK207 hexane partition does not impair *C. elegans* feeding activities. There is no distinguishable difference observed in the pumping rate count between the extract-treated and control worms at the indicated time points (*t*-test, p -value of 1.00, 0.392, and 1.00 at 0, 12, and 24 h time-points, respectively). Representative result is depicted from three independent experiments and values are expressed in mean \pm SD.

Chemical Profiling of *Streptomyces* sp. CCB-PSK207 A5HB Fraction

Further chemical profiling and compound identification was done using GC-MS (Pollak and Berger, 1996). Seven main compounds were identified, Figure S2 namely (1) tetradecanoic acid methyl ester, (2) pentadecanoic acid 14-methyl methyl ester, (3) tetradecanoic acid, 12-methyl-methyl ester, (4) tridecanoic acid methyl ester, (5) hexadecanoic acid methyl ester (6) octadecanamide, and (7) 1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester (Table 1).

DISCUSSION

Given the multiple health hazards posed by *P. aeruginosa* and the rise of multi-drug resistant strains, it is essential that novel drugs with anti-infective properties are discovered (Hauser and Sriram, 2005). Marine actinomycetes are a promising target, as exemplified by consistent discovery of promising metabolites against fungal, parasitic, bacterial, and viral diseases (Rahman et al., 2010; Subramani and Aalbersberg, 2012; Manivasagan et al., 2013). The *C. elegans*-PA14 slow killing assay has been employed to search for potential immune-boosting metabolites

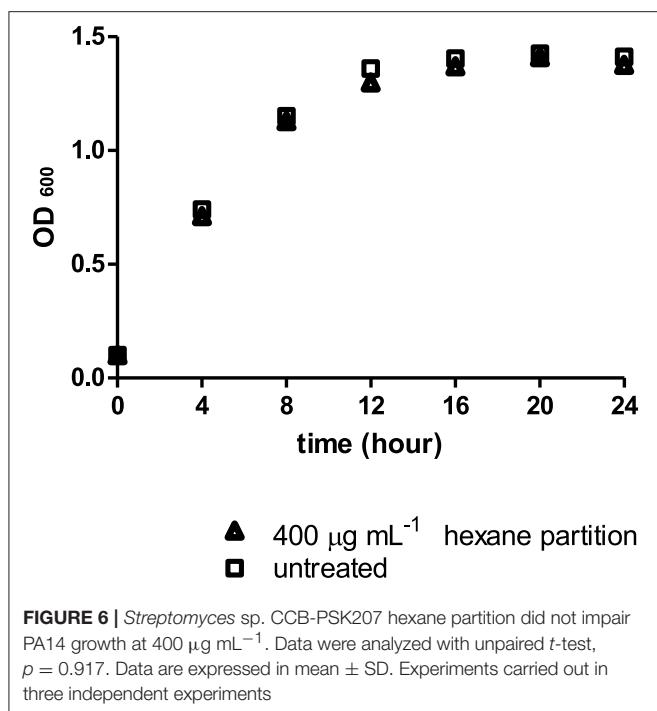


FIGURE 6 | *Streptomyces* sp. CCB-PSK207 hexane partition did not impair PA14 growth at 400 $\mu\text{g mL}^{-1}$. Data were analyzed with unpaired *t*-test, $p = 0.917$. Data are expressed in mean \pm SD. Experiments carried out in three independent experiments

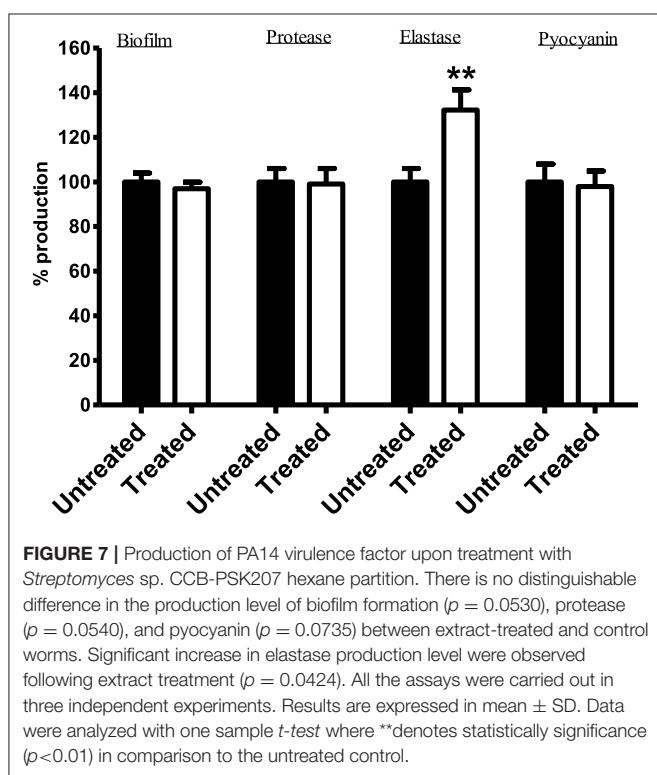


FIGURE 7 | Production of PA14 virulence factor upon treatment with *Streptomyces* sp. CCB-PSK207 hexane partition. There is no distinguishable difference in the production level of biofilm formation ($p = 0.0530$), protease ($p = 0.0540$), and pyocyanin ($p = 0.0735$) between extract-treated and control worms. Significant increase in elastase production level were observed following extract treatment ($p = 0.0424$). All the assays were carried out in three independent experiments. Results are expressed in mean \pm SD. Data were analyzed with one sample *t*-test where **denotes statistically significance ($p < 0.01$) in comparison to the untreated control.

(Adonizio et al., 2008b; Zhou et al., 2011; Durai et al., 2013; Li et al., 2014). We report here the use of this assay to screen marine actinomycetes for anti-infective properties against *P. aeruginosa*.

We first showed that the actinomycetes extracts did not directly inflict mortality on PA14. This will rule out the detection of compounds with bactericidal effects and divert subsequent discovery to isolation of lead compounds targeting immunity of host or virulence of pathogen. Using the slow killing assay, we discovered a partition from the methanol extract of an actinomycete isolated from sea sediment which significantly boost the survival in PA14-infected worms in a dose dependent manner. This increase is comparable to level of survivals observed in PA14 infected worms treated with *Swietenia macrophylla* seed extract (Dharmalingam et al., 2012) and curcumin (Rudrappa and Bais, 2008). Using 16S analysis, this isolate was shown to have >99.5% identity with several *Streptomyces* sp. and was designated as *Streptomyces* sp. CCB-PSK207. Among all actinomycetes, the *Streptomyces* group is economically valuable, giving rise to 50–55% of known antibiotics (Bérdy, 2005). However, only a small portion of marine actinomycetes have been subjected for bioprospecting of new therapeutics. Besides efficacy, having the host in the screening assay provides an added advantage of early indication of compound toxicity (Squiban and Kurz, 2011). This could plausibly explain the higher mortalities encountered by worms exposed to several of the crude methanol extracts in this present study.

Slow killing of *C. elegans* by PA14 involves the colonization and proliferation of pathogen in the host gut (Tan et al., 1999a). As such, it is important to establish if *Streptomyces* sp. CCB-PSK207 metabolites mitigate killings by diminishing gut colonization in worms. Since the colonization of PA14 in nematode gut commence with feed intake, a reliable indicator is observation of the pumping rate of *C. elegans* pharynx, a tube involved in feeding and transportation of bacteria into the gut (Avery and Shtonda, 2003). Overall, our results showed that PA14 exposed to *Streptomyces* sp. CCB-PSK207 partition could still grow and colonize gut of host after feeding. These observations were also reported with anti-infective natural products isolated from a similar screening approach (Rudrappa and Bais, 2008; Dharmalingam et al., 2012; Durai et al., 2013; Kong et al., 2014a,b).

A plausible scenario to explain the improved survival of infected worms treated with the bioactive partition is the presence of compounds with anti-virulence activities. The widespread problems associated with PA14 is principally due to the production of a series of virulence factors including protease, elastase, pyocyanin, and alginate (Lyczak et al., 2000). In addition, formation of obdurate biofilms is a crucial armory in PA14's persistency against antimicrobial therapy (Ma et al., 2009). During PA14 infection of *C. elegans*, the pathogens produce virulence-related membrane vesicles, leading to the accretion of biofilm-like material on host intestinal cells (Irazoqui et al., 2010). There have been several reports on natural product-based small molecules from marine organisms, including actinomycetes showing potency against virulence of PA14 (Hentzer et al., 2003; Fu et al., 2013; Naik et al., 2013; Yaniv et al., 2017). Our results showed that *Streptomyces* sp. CCB-PSK207 hexane partition did not subdue production of biofilm, protease, and pyocyanin in PA14. This supports our earlier observation of normal gut

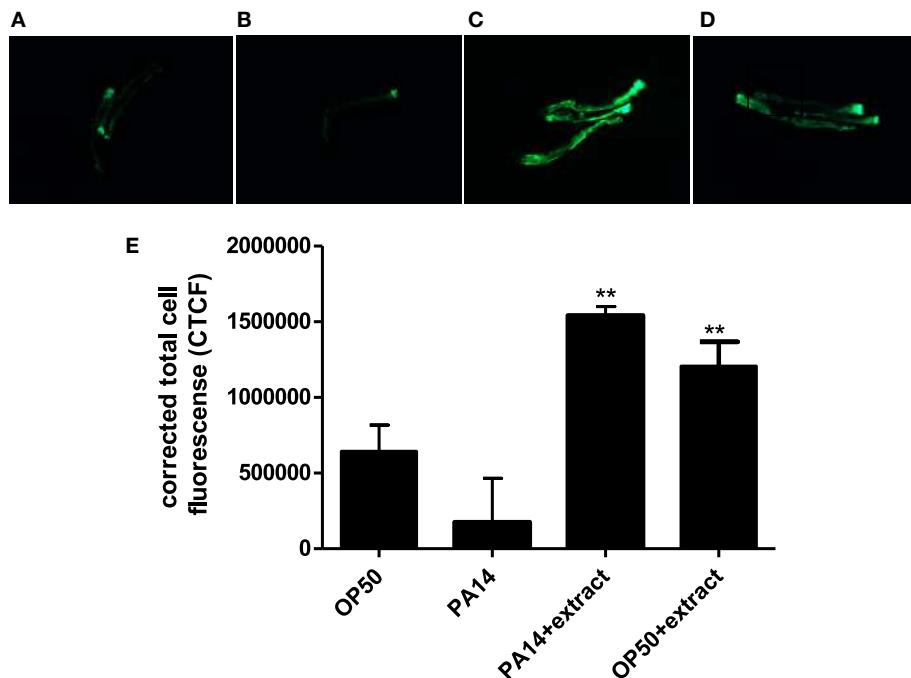


FIGURE 8 | Induced expression of *lys-7* in PA14 infected worms upon treatment with *Streptomyces* sp. CCB-PSK207 hexane partition. Representative fluorescence micrographs of worms after 24-h incubation with partition. **(A)** Worms fed on OP50, uninfected; **(B)** PA14-infected worms without extract treatment and **(C)** PA14-infected worms treated with $400 \mu\text{g mL}^{-1}$ hexane extract; **(D)** worms fed on OP50 and treated with $400 \mu\text{g mL}^{-1}$ hexane extract, uninfected. Worms were examined under Leica Microsystem M205 FA with magnification $\times 127$. “+” denotes anterior head region of the worms. **(E)** Corrected total cell fluorescence of *lys-7* micrograph A, B, C, and D. Data were analyzed with the Image J software version 1.49. **Denotes significance ($p < 0.01$, *t*-test) in comparison to the untreated control.

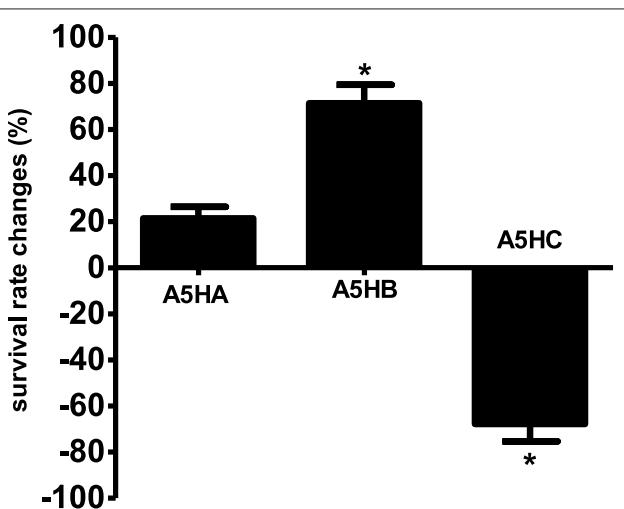


FIGURE 9 | Survival of *C. elegans* infected with *Pseudomonas aeruginosa* PA14 in the presence of different fractions in the killing assay. Fraction A5HB significantly promotes *C. elegans* survival during PA14 killing assay. *Denotes statistically significance in the Log-rank test in comparison to the untreated control ($p < 0.05$). Data were representative of two independent experiments.

colonization in nematode exposed to the bioactive extract as PA14 mutants with perturbed quorum sensing cascade are unable to colonize the gut of *C. elegans* (Tan et al., 1999b). Extracts of

several terrestrial and aquatic plant species, have been reported to rescue *C. elegans* from mortality by interfering with PA14 quorum sensing and virulence activities (Adonizio et al., 2008a,b; Rudrappa and Bais, 2008; Kandasamy et al., 2012; Husain et al., 2013; Liu et al., 2013; Sarabhai et al., 2013). Therefore, a disparity between these chemical entities and *Streptomyces* sp. CCB-PSK207 is that the latter did not seem to rescue PA14 infected worms through disruption of pathogen virulence factors. Elsewhere, similar to our results, selenite did not reduce both quorum-sensing signals and virulence factors of PA14 but was able to promote *C. elegans* survival (Li et al., 2014). Elastase or lasB is a metalloproteinase secreted by *P. aeruginosa*, with multiple roles leading toward cytotoxicity and degradation of host immune system (Kipnis et al., 2006). *C. elegans* exposed to LasB-knockout PA14 survived longer as compared to the normal virulent PA14 strain in the slow killing assay (Zhu et al., 2015). Intriguingly, our results demonstrate that actinomycete extract resulted in an increase of elastase production. Elsewhere, fatty acids have been shown to stimulate levels of elastase in *P. aeruginosa* (Kwan et al., 2011). Despite the increased levels of elastase produced by PA14 in presence of the actinomycete extract, it is worth noting that this did not translate to higher worm kills.

C. elegans possess 10 lysozyme-like proteins (*lys-1* to *lys-10*), with several of them associated with host defense (Mallo et al., 2002). Among these, *lys-7* have been shown to be immune-specific, with RNAi mediated *lys-7* knockdown worms showing

TABLE 1 | Compounds identified from fraction A5HB using GC-MS.

No.	Retention time (min.)	Compound	Formula	Molecular weight (MW)	Quality (%)
1	9.37	Tetradecanoic acid methyl ester	C ₁₅ H ₃₀ O ₂	242	96
2	9.93	Pentadecanoic acid 14-methyl-methyl ester	C ₁₆ H ₃₂ O ₂	256	94
3	9.97	Tetradecanoic acid 12-methyl-methyl ester	C ₁₆ H ₃₂ O ₂	256	89
4	10.45	Tridecanoic acid methyl ester	C ₁₄ H ₂₈ O ₂	228	96
5	10.64	Hexadecanoic acid methyl ester	C ₁₇ H ₃₄ O ₂	270	96
6	11.29	9-octadecanoic acid methyl ester	C ₁₉ H ₃₆ O ₂	296	60
7	13.60	1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester	C ₁₆ H ₂₂ O ₄	278	90

Chemical profile of compounds was compared with NIST spectral library.

increased sensitivity to pathogen killing (Mallo et al., 2002; Nandakumar and Tan, 2008; Simonsen et al., 2011). It has been reported that PA14 suppresses *C. elegans* immunity by repressing the expression of *lys-7* (Evans et al., 2008). Our results showed that the hexane partition of *Streptomyces* sp. CCB-PSK207 boosted the level of *lys-7* which was weakened during PA14 infection. Restoration of repressed *lys-7* during infection have also been reported with other single compounds and plant extracts (Dharmalingam et al., 2012; Kong et al., 2014a; Li et al., 2014). Concomitantly, uninfected worms treated with *Streptomyces* sp. CCB-PSK207 partition also showed an increase expression of *lys-7*, giving direct evidence of the presence of bioactive compounds capable of inducing *lys-7*. Taking into consideration that PA14 compromise host immunity through the suppression of various host defense molecules including *lys-7*, our results indicate that the bioactive partition have the capacity to restore this deficiency, leading to a rescue from mortality (Evans et al., 2008). While numerous reports of compounds attenuating PA14 virulence have been isolated from marine actinomycetes, to our knowledge, this is the first report of marine actinomycete metabolites inducing immunity in an infected host. A possible outcome of the induced elastase levels in PA14 in presence of the actinomycete extract is the reciprocal increase in innate immune response of host, as seen in insects (Andrejko and Mizerska-Dudka, 2011). Therefore, besides inducing *lys-7* as the defense molecule during infection, we do not rule out the possibility of an alternate stimulus of innate immunity caused by higher elastase production by PA14. A slow killing assay involving infection of *C. elegans* with LasB-knockout PA14 will be useful to endorse this possible route (Zhu et al., 2015).

GC/MS analysis of the *Streptomyces* sp. CCB-PSK207 bioactive fraction showed the presence of methyl esters of several saturated fatty acids including tridecanoic acid, tetradecanoic acid, and hexadecanoic acid. Simultaneously, methyl esters of branched-chain tetradecanoic acid, 12-methyl and pentadecanoic acid 14-methyl were also present. Some of these fatty acids have previously been reported from *Streptomyces* sp. (González et al., 2005; Ser et al., 2016). Methyl ester of tridecanoic acid, tetradecanoic acid, hexadecanoic acid, pentadecanoic acid 14-methyl, and tetradecanoic acid 12-methyl have also been reported in *C. elegans* (Henry et al., 2016). Although the actual mechanism by roles these compounds

protect *C. elegans* from PA14-induced mortality is still unclear, some hints as to their possible functions could be derived from literature pertaining to *C. elegans* immunity and fatty acids. Two 18 carbon unsaturated fatty acids, gamma-linolenic acid (GLA, C18:3n6) and stearidonic acid (SDA, C18:4n3) are pivotal for *C. elegans* defense against PA14 infection as these fatty acids regulate basal expression of immune-specific genes including *lys-7* (Nandakumar and Tan, 2008). Interestingly, hexadecanoic acid methyl ester can be elongated endogenously by worms to stearic acid (C18:0), followed by further desaturation to generate GLA or SDA, which could speculatively restore *lys-7* in PA14 infected worms (Watts and Browse, 2002). Elsewhere, 1,2-benzenedicarboxylic acid mono (2-ethylhexyl) ester (MEHP) is also reported to induce the activation of the mitogen-activated protein kinase p38 (p38 MAPK) pathway, a pivotal signaling mechanism in *C. elegans* innate immunity (Kim et al., 2002; Rakkestad et al., 2010). Obliteration of *nhr-49*, a known master regulator of lipid metabolism in *C. elegans* also resulted in higher susceptibility to pathogenic infection, giving indication to the importance of fatty acids and *C. elegans* innate immunity (Sim and Hibberd, 2016).

In conclusion, this present study revealed the rescue of PA14-infect *C. elegans* by metabolites from a locally isolated *Streptomyces* species. We also showed that this process circumvented bacterial or anti-virulence mechanisms and instead, induced worm immunity. Lastly, we showed that the bioactive molecules responsible for these observations are fatty acid methyl-esters which could hypothetically stimulate expression of *lys-7*.

ETHICS STATEMENT

Standard Operating Procedures involving *C. elegans* and living modified organisms (LMOs) were approved by the Universiti Sains Malaysia Animal Ethic Committee (AECUSM) and the Institutional Biosafety Committee (UKKP).

AUTHOR CONTRIBUTIONS

Overall approach of study was designed by AS-C. All authors were involved in designing of experiments and data analysis.

SF performed all the experiments. All authors were involved in preparation of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.02267/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deep Sea Actinomycetes and Their Secondary Metabolites

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Deep sea is a unique and extreme environment. It is a hot spot for hunting marine actinomycetes resources and secondary metabolites. The novel deep sea actinomycete species reported from 2006 to 2016 including 21 species under 13 genera with the maximum number from *Microbacterium*, followed by *Dermacoccus*, *Streptomyces* and *Verrucosispora*, and one novel species for the other 9 genera. Eight genera of actinomycetes were reported to produce secondary metabolites, among which *Streptomyces* is the richest producer. Most of the compounds produced by the deep sea actinomycetes presented antimicrobial and anti-cancer cell activities. Gene clusters related to biosynthesis of desotamide, heronamide, and lobophorin have been identified from the deep sea derived *Streptomyces*.

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INTRODUCTION

The search and discovery of novel microbes that produce new secondary metabolites can be expected to remain significant in the race against new and emerging diseases and antibiotic resistant pathogens (Berdy, 2012; Manivasagan et al., 2013). Actinomycetes are widely distributed in various marine habitats, ranging from sea sand (Hong et al., 2008), mangrove sediments (Hong et al., 2009; Hong, 2013; Azman et al., 2015), sea water (Zhang L. et al., 2012), coastal sediments (Yu et al., 2015), and deep sea sediments (Zhang et al., 2015; Chen et al., 2016). The increasing number of literature on marine actinomycetes strongly supported the view that marine environments including deep sea are significant sources for search and discovery of both diverse actinomycetes resources and secondary metabolites (Skropeta and Wei, 2014; Xu et al., 2014).

Deep sea habitats show extreme variations in available nutrients, light, oxygen concentration, pressure, salinity, and temperature. Deep-sea organisms have developed unique biochemical metabolic and physiological capabilities, which not only ensure their survival in this habitat but also provide potential for the production of novel metabolites absent in terrestrial microorganisms (Fenical, 1993; Bull et al., 2000; Skropeta and Wei, 2014). Through molecular ecology studies, actinobacterial operational taxonomic units (OTUs) have been identified from deep sea sediments. Most of those foreseeably exhibit novel species, genera and families (Stach and Bull, 2005; Chen et al., 2016). Diverse species of actinomycetes cultured from the deep seafloor surface, including the deepest sea sediment samples from the Mariana Trench, have shown great biosynthetic capacities and thus a potent source of novel natural products (Pathom-arree et al., 2006d; Abdel-Mageed et al., 2010). With the breakthrough of technological barriers associated with deep sea actinobacteria isolation strategies, such as sample collection and cultivation under standard laboratory conditions, more and more deep sea actinobacteria and their natural products have been identified. Here we review the recent progress on deep sea actinomycetes and their metabolites from literature during year 2006–2016.

DEEP SEA ENVIRONMENT AND BIODIVERSITY

The vast oceans cover 70% of the world's surface, with 95% greater than 1,000 m deep. Deep sea environments are divided into the bathyal zone (depths between 200 and 2,000 m), the abyssal (depths between 2,000 and 6,000 m) and the hadal zone (depths below 6,000 m) (Harino et al., 2009). Below sea level pressure is increased by depth, thereby in the deepest part of the trenches, the pressure varying from 10 atm at the shelf-slope interface to >1,000 atm. At bathyal depths temperatures taper off rapidly with increasing depth to 2°C. Deep-sea species must adjust their biochemical processes to survive in low temperatures, because the cold reduces chemical reaction rates. Oxygen concentration drops along with the depth, oxygen-minimum layer in mid-water, usually between 300 and 1,000 m depth. Light intensity decreases exponentially with depth in the water column. No photosynthetically useful light reaches the sea floor below about 250 m (Thistle, 2003).

Start at about 200 m depth, the deep sea is characterized by high pressure, low temperature, lack of light and variable salinity and oxygen concentration (Das et al., 2006), at the shelf break, where a clear change of fauna from shallow to deep water is observed (Thistle, 2003). According to Haefner (2003), in cold deep sea mud the diversity of life can be remarkably high with species richness rivaling that of tropical rain forest. Studying the species level of microbial diversity, finding a large number of rare species which more than half of them considered as new species and more than 95% is unidentified, furthermore the expanding of biodiversity reach to the 5,000 m in depth to abyssal which the peak amount of species at the depths of 3,000 m and beyond (Skropeta, 2008). On earth abyssal hills are the most abundant of biomass, but on wider abyss the ecological impact of the habitat heterogeneity is largely unexplored (Durden et al., 2015).

DEEP SEA ACTINOMYCETES CULTIVATION

However, so far only a few actinomycetes have been isolated from deep sea. It is because of technological barriers associated with isolation strategies. Therefore, we are in the pace to develop efficient cultivation methods to recover the actinobacteria population from extreme deep sea habitats. To achieve the task, firstly collection of samples from deep sea plays a pivotal role. In recent years several advancements have been developed in the context of sample collection from deep sea such as modified sediment grab and designer-built bounce corer (Fenical and Jensen, 2006), remote-operated submarine vehicle (Pathom-aree et al., 2006d), neuston sampling devices (Hakvåg et al., 2008), multi-core sampler (Xu et al., 2009), gravity or piston cores (D'Hondt et al., 2009), and untethered coring device (Prieto-Davó et al., 2013).

It is crucial to cultivate deep sea actinomycetes under standard laboratory conditions. There are several factors that influence the isolation, such as pre-treatment of dry heat (Shin et al., 2008), media composition (Luo et al., 2011; Pan et al., 2013;

Song et al., 2015), dilution factor (Pathom-aree et al., 2006a), seawater requirement (Song et al., 2015), artificial seawater (Pan et al., 2013; Pesic et al., 2013) and incubation time (Song et al., 2015). It has also shown the addition of different antibiotics on selective media can inhibit the growth of fungal and bacterial contamination in order to enhance the actinomycetes growth similar to those used in isolation of actinomycetes from terrestrial sample. Long term freeze storage of deep sea sediment samples at -80°C has shown to prevent the growth of fast-growing bacteria which in results enhance the actinomycetes population (Ulanova and Goo, 2015). For the initial isolation of *Streptomyces*, cultivation temperatures have also influenced the recovery from deep sea sediment samples. Optimal growth temperature generally ranging from 25 to 30°C for successful cultivation of deep sea actinomycetes (Jeong et al., 2006; Luo et al., 2011; Pesic et al., 2013).

Heat pre-treatment procedures have been used effectively for the selective isolation of members of several actinomycete taxa and also inhibited growth of bacterial and fungal colonies. Moreover, actinomycete spores and hyphae are more sensitive to wet than dry heat hence relatively low temperature regimes are used to pretreat water and soil suspensions. Although heat pretreatment procedures decrease the ratio of bacteria to actinomycetes on isolation plates, the numbers of actinomycetes may also be reduced (Williams et al., 1972; Pathom-aree et al., 2006a,b,c,d). Pathom-aree et al., isolated actinomycetes from Norwegian fjord sediments support that the numbers of actinomycetes were reduced when used heat pretreatment for isolation; fewer actinomycetes were isolated on selective media inoculated with suspensions treated at 55°C as opposed to 50°C. Similarly, higher counts were generally recorded on isolation plates seeded with non-heat pretreated suspensions (Pathom-aree et al., 2006d).

For the other method, Jensen et al., 2005 used dry and stamp method for isolation actinomycetes from tropical Pacific Ocean and found that using this method for isolation of actinomycetes showed good recovery of 44%. In addition, Ulanova and Goo (2015) found that the majority of actinomycete-like colonies were also isolated using dry stamping technique from subseafloor sediments at the Nankai and Okinawa Troughs.

NOVEL ACTINOMYCETE SPECIES

Novel actinomycete species isolated from deep sea environment between 2006 and 2016, have yielded an impressive array of novel species with the highest number found at depths of abyssal zone and deeper. Different media has been used by researchers (Table 1). It is worth to be noticed that long time culturing and low temperature were employed for some of the novel isolates (Table 1). Only one novel *Microbacterium marinum* was obtained by pretreatment at 55°C, 6 min, others were from none heat pretreated samples (Table 1). The novel deep sea actinomycete species including 21 species under 13 genera with the maximum number from *Microbacterium* ($n = 4$), followed by *Dermacoccus* ($n = 3$), *Streptomyces* ($n = 3$) and *Verrucosispora* ($n = 2$), and one novel species for each of the other 9 genera (Table 1).

TABLE 1 | New actinomycetes species (*n*= 21) isolated from deep sea environment between 2006 and 2015.

Species	Region	Depth(m)	Culture technique		References
			Extraction of act obact ria propagules/pretreatment procedure	Media	
<i>Amycolatopsis marina</i> sp. nov.	South China Sea	Not sp. cited	Not specified	SM1 with cycloheximide, neomycin sulfate and nystatin	28°C for 4 weeks Bian et al., 2009
<i>Brevibacterium oceanii</i> sp. nov.	Chagos Trench, Indian Ocean	5,904	Vortex sediment suspension in 2% NaCl for 1 min	Yeast extract/peptone (YP) agar	15°C for 15 days Bhadra et al., 2008
<i>Dermacoccus abyssi</i> sp. nov.	Mariana Trench (Challenger Deep)	10,898	Shaking sediment suspension for 30 min at 150 rpm	Raffinose-histidine agar with cycloheximide and nystatin	28°C for 12 weeks Pathom-areae et al., 2006a
<i>Dermacoccus barathri</i> sp. nov.	Mariana Trench (Challenger Deep)	10,898	Shaking sediment suspension for 30 min at 150 rpm	Raffinose-histidine agar with cycloheximide and nystatin	28°C for 12 weeks Pathom-areae et al., 2006b
<i>Dermacoccus profundii</i> sp. nov.	Mariana Trench (Challenger Deep)	10,898	Shaking sediment suspension for 30 min at 150 rpm	Raffinose-histidine agar with cycloheximide and nystatin	28°C for 12 weeks Pathom-areae et al., 2006b
<i>Microbacterium indicum</i> sp. nov.	Chagos Trench, Indian Ocean	5,904	Vortex sediment suspension in 2% NaCl for 1 min	Yeast extract/peptone (YP) agar	15°C for 15 days Shivaji et al., 2007
<i>Microbacterium marinum</i> sp. nov.	South-west Indian Ocean	2,800	Heated sediment suspension in a water bath at 55°C for 6 min	Modified DNB- seawater medium with nalidixic acid and nystatin	28°C for 1 week Zhang L. et al., 2012
<i>Microbacterium profundii</i> sp. nov.	East Pacific polymetallic nodule region	5,280	Vortex sediment suspension in sterile seawater for 15 min	Modified ZoBell medium	25°C for 2 weeks Wu et al., 2008
<i>Microbacterium sediminis</i> sp. nov.	South-west Indian Ocean	2,327	Vortex sediment suspension in sterile seawater	FJ sea water (50%) agar with rifampicin and potassiumdichromate	28°C Yu et al., 2013
<i>Modestobacter marinus</i> sp. nov.	Atlantic Ocean	2,983	Not specified	Not specified	Not specified Xiao et al., 2011b
<i>Myceligerans cantabricum</i> sp. nov.	Avilé's Canyon in the Cabrian Sea, Asturias, Spain	1,500	Not specified	1/3 tryptic soy agar and 1/6 M-BLEB sea water agar with cycloheximide and nystatin	28°C for 2 weeks Vizcaíno et al., 2015
<i>Nesterenkonia alkaphila</i> sp. nov.	Western Pacific Ocean	7,118	Not specified	Modified ISP 1 - seawater	28°C for 3 weeks Zhang et al., 2015
<i>Pseudonocardia antitumoralis</i> sp. nov.	South China Sea	3,258	Not specified	ISP 5- seawater medium	28°C for 3 weeks Tian et al., 2013
<i>Sciaciella marina</i> gen. nov., sp. nov.	Northern South China Sea	516	Not specified	Gauze No. 1 -seawater medium	28°C for 3 weeks Tian et al., 2009
<i>Serinicoccus profundii</i> sp. nov.	Indian Ocean	5,368	Not specified	Oligotrophic- seawater medium	Not specified Xiao et al., 2011a
<i>Streptomyces indicus</i> sp. nov.	Indian Ocean	2,434	Not specified	Modified HV— sea water (75%) medium	25°C Luo et al., 2011
<i>Streptomyces ranhaiensis</i> sp. nov.	South China Sea	1,632	Not specified	Humic acid-vitamin- sea water (70%) medium	28°C for 3 weeks Tian et al., 2012a
<i>Streptomyces oceanii</i> sp. nov.	Northern South China Sea	578	Not specified	10 % Nutrient seawater agar	28°C for 3 weeks Tian et al., 2012b

(Continued)

TABLE 1 | Continued

Species	Region	Depth(m)	Culture technique	Media	Incubation temperature and time	References
			Extraction of act obact ria propagules/pretreatment procedure			
<i>Verrucospora maris</i> sp. nov.	Sea of Japan	Not specified	Not specified	Colloidal chitin agar	30°C for 4 weeks	Goodfellow et al., 2012
<i>Verrucospora sediminis</i> sp. nov.	South China Sea	3,602	Not specified	Gauze No. 1 medium	22°C for 4 weeks	Dai et al., 2010
<i>Williamsia marianensis</i> sp. nov.	Mariana Trench (Challenger Deep)	10,898	Shaking sediment suspension for 30 min at 150 rpm	Raffinose-histidine agar with cycloneximide and mystatin	28°C for 12 weeks	Pathom-aree et al., 2006c

SM1*: yeast nitrogen base (67.0 g; Difco) and casamino acids (100 mg; Difco) are added to a liter of distilled water and the solution sterilized using cellulose filters (0.20 mm) prior to the addition of sterilized dipotassium hydrogen phosphate (200 ml; 1.0%, w/v); 100 ml of this basal medium was added to 900 ml of sterilized molten agar (1.5%, w/v) followed by filter sterilized solutions of D(-)-sorbitol (final concentration 1%, w/v); **YP agar***: per liter distilled water: 5 g, yeast extract, 10 g peptone, 30 g NaCl, 15 g agar; **Raffinose-histidine agar***: Raffinose 10 g, L-histidine 1 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, K₂HPO₄ 1 g, Agar 20 g, pH 7.0–7.4; **Modified DNB medium***: 0.1 g p, 0.05 g b f extract, 0.05 g NaCl, 1000 mL artificial seawater, pH 7.5; **Modified ZoBell agar***: 19.45 g NaCl, 8.89 g MgCl₂, 3.24 g Na₂SO₄, 0.169 NaHCO₃, 0.1 g tric citrate pentahydrate, 80 mg KBr, 34 mg CsCl₂, 22 mg H₃BO₃, 4.0 mg Na₂SiO₃, 2.4 mg NaF, 1.6 mg NH₄NO₃, 8.0 mg Na₃PO₄, 0.5 g p, 0.1 g yeast extract, 20 g agar (pH 5.5, adjusted with HCl); **FJ agar***: 1% glucose, 1% yeast extract, 1.5% agar, 50% seawater; **1/3 tryptic soy agar and 1/16 M-BLEB***: 1/3 tryptic soy agar (TSA, Merck) and 1/16 M-BLEB [9 g MOPS BLEB base (Ixoid) in 1 l Cantabrian Sea water/l agar; **Modified SP 1***: (1 l natural seawater, pH 10 final); 10 g glucose, 5 g peptone, 5 g yeast extract, 0.2 g MgSO₄, 7H₂O, 10 g NaHCO₃, 27 g Na₂CO₃, 10H₂O and 15 g agar; **ISP 5 medium***: L-asparagine (anhydrous basis) 1.0 g, Glycerol 10.0 g, K₂HPO₄ (anhydrous basis) 1.0 g, natural seawater 1.0 l, trace salts 0.05 g FeSO₄·x 7H₂O, 20.0 g, KNO₃ 1.0 g, NaCl 0.9 g, MgSO₄·7H₂O 0.5 g, K₂HPO₄ 0.5 g, Na₂HPO₄ 0.5 g, MgSO₄, 7H₂O 0.01 g, Agar 15.0 g, Sea water 1.0 L, Adjust pH 7.4; **Oligotrophic-seawater medium***: Oligotrophic medium (seawater, 2.0% agar); **Modified HV medium***: humic acid 1.0 g, KC1 1.7 g, FeSO₄, 7H₂O 0.01 g, Na₂HPO₄ 0.5 g, MgSO₄, 7H₂O 0.5 g, CaCO₃ 0.02 g, thiamine 0.05 mg, nicotinic acid 0.5 mg, p-aminobenzoic acid 0.5 mg, riboflavin 0.5 mg, vitamin B6 0.5 mg, inositol 0.5 mg, biotin 0.25 mg, water 250 mL seawater 750 mL, agar 18 g, pH 7.2; **Humic acid-vitamin agar***: Humic acid 2g, Asparagine 1 g, K₂HPO₄ 0.5 g, FeSO₄, 7H₂O 0.5 g, Agar 20 g, Sea-water 1000 mL, pH 7.0–7.4; **10% Nutrient agar***: Beef extract 0.03 g, peptone 0.05 g, agar 15 g, sea water 1 L; **colloidal chitin agar***: 4 g of chitin, K₂HPO₄ (0.7 g); KH₂PO₄ (0.3 g); MgSO₄·5H₂O (0.5 g); FeSO₄, 7H₂O (0.01 g); ZnSO₄ (0.001 g); MnCl₂ (0.001 g); and 200 g of agar, pH 8.0

NATURAL PRODUCTS SYNTHESIZED BY DEEP SEA ACTINOMYCETES

The numbers of novel microbial metabolites from deep sea sediment samples have been increasing, especially from deep sea streptomycetes. Eight genera of actinomycetes were reported to produce secondary metabolites, among which *Streptomyces* is the richest producer (Table 2). Earlier culture dependent studies strongly suggested that *Streptomyces* species are present in considerable number in deep sea sediment samples (Jensen et al., 2005; Pathom-aree et al., 2006d). In addition several novel species of deep sea derived *Streptomyces* strains with distinct metabolites have been reported which indicates deep sea *Streptomyces* are really worth in the context of novel natural products discovery (Pan et al., 2015; Song et al., 2015).

The deepest sea sediment samples from the Mariana Trench have been shown to possess great biosynthetic capacities. Seven dermacozines A–G were reported from the actinobacteria *Dermacoccus abyssi* sp. nov., strains MT1.1 and MT1.2 isolated from Mariana Trench sediment collected at a depth of 10,898 m. Dermacozines F and G displayed moderate cytotoxic activity against the leukemia cell line K562 with IC₅₀ values of 9 and 7 mM, respectively, whereas dermacozine C also exhibited high radical scavenger activity with an IC₅₀ value of 8.4 mM (Abdel-Mageed et al., 2010).

In recent years, South China Sea has been emerging as a potentially abundant source of novel species/genera of marine actinomycetes. Some bioactive compounds, such as pseudonocardians A–C, grincamycins B–F, and abyssomicins J–L were reported. Natural products derived from deep sea actinomycetes discovery have displayed a wide range of bioactivities, such as antitumor, antimicrobial, antifouling, and anti-fibrotic activities (Table 2).

BIOSYNTHESIS PATHWAYS FOR DEEP SEA STREPTOMYCETES NATURAL PRODUCTS

Lobophorins H and I together with three known analogs, O-β-kijanosyl-(1→17)-kijanolide, lobophorins B and F were yielded by *Streptomyces* sp. 12A35, isolated from a deep sea sediment sample collected at a depth of 2,134 m in South China Sea (Pan et al., 2013). While, lobophorins E and F, along with two known analogs lobophorins A and B were discovered from the products of the deep sea *Streptomyces* sp. SCSIO 01127, was isolated from sample collected at a depth of 1,350 m in the South China Sea (Niu et al., 2011). The gene cluster involved in biosynthesis of lobophorin was the first type I PKS gene cluster identified from the deep sea derived *Streptomyces*. Three glycosyltransferases (GTs) LobG1-LobG3 genes-inactivation mutants yielded five different glycosylated metabolites, and the result suggested that LobG3 as an iterative GT to attach two L-digitoxoses (Li et al., 2013). Desotamide B, C and D together with a known desotamide A were obtained from deep sea derived *Streptomyces scopuliridis* SCSIO ZJ46, recovered from sediment sample collected at a depth of 3,536 m in the South China

TABLE 2 | Natural products synthesized by deep sea actinomycetes.

Strain	Compounds	Region	Depth (m)	Bioactivity	References
<i>Dermacoccus abyssi</i>	Dermacozines A–G	Mariana Trench (Challenger Deep)	10,898	Moderate cytotoxic activity against the leukemia cell line K562	Abdel-Mageed et al., 2010
<i>Dermacoccus abyssi</i>	Dermacozines H–J	Mariana Trench (Challenger Deep)	10,898	Radical scavenging activity	Wagner et al., 2014
<i>Marinactinospora thermotolerans</i>	Marinacarbolines A–D, Indolactam alkaloids	South China Sea	3,865	Strong antiplasmodial activity	Huang et al., 2011
<i>Microbacterium sediminis</i> sp.nov.	Microbacterins A and B	South-west Indian Ocean	2,327	Significant inhibitory effects against a panel of human tumor cell	Liu D. et al., 2015
<i>Micromonospora</i> sp.	Levantilides A and B	Mediterranean	4,400	Anticancer	Gärtner et al., 2011
<i>Nocardiopsis alba</i> SCSIO 03039	Methoxyneihumicin	Indian Ocean	Not specified	Anticancer	Zhang et al., 2013
<i>Nocardiopsis</i> sp.	Nocardiopsins A and B	Coast of Brisbane, Australia	55	No activity	Raju et al., 2010
<i>Pseudonocardia</i> sp.	Pseudonocardians A–C	South China Sea	3,258	Anticancer, antibacterial activity	Li et al., 2011
<i>Serinicoccus profundi</i> sp. nov.	Indole alkaloid	Indian Ocean	5,368	Antibacterial activity	Yang et al., 2013b
<i>Streptomyces cavaurensis</i> NA4	Bafilomycins B1 and C1	South China Sea	1,464	Antifungal Substances	Pan et al., 2015
<i>Streptomyces drozdzowiczii</i> SCSIO 10141	Marformycins	South China Sea	1,396	Anti- infective	Zhou et al., 2014
<i>Streptomyces fungicidicus</i>	Diketopiperazines	Western Pacific	5,000	Antifouling products	Li et al., 2006
<i>Streptomyces lusitanus</i>	Grincamycins B–F	South China Sea	3,370	Anticancer	Huang et al., 2012
<i>Streptomyces niveus</i> SCSIO 3406	Marfuraquinocins	South China Sea	3,536	Cytotoxic, antibacterial activity	Song et al., 2013
<i>Streptomyces olivaceus</i> FXJ8.012	Tetroazolemycins A and B	Southwest Indian Ocean	Not specified	Metal ion-binding activity	Liu et al., 2013
<i>Streptomyces scopuliridis</i> SCSIO ZJ46	D sotamides B–D	South China Sea	3,536	Antibacterial activity	Song et al., 2014
<i>Streptomyces</i> sp.	Ammosamides A and B	Bahamas	1,618	Anticancer	Gaudêncio et al., 2008
<i>Streptomyces</i> sp.	Benzoxacystol	Atlantic	3,814	Inhibitory activity against the enzyme glycogen synthase kinase-3b	Nachtigall et al., 2011
<i>Streptomyces</i> sp.	Caboxamycin	Atlantic	3,814	Inhibitory activity against Gram-positive bacteria, anticancer	Hohmann et al., 2009
<i>Streptomyces</i> sp.	Spiroindimicins A–D	Indian Ocean	3,412	Anticancer	Zhang W. J. et al., 2012
<i>Streptomyces</i> sp.	Streptokordin	Ayu Trough	Not specified	Anticancer	Jeong et al., 2006
<i>Streptomyces</i> sp.	Streptopyrrolidine	Ayu Trough	Not specified	Anti-angiogenesis activity	Shin et al., 2008
<i>Streptomyces</i> sp. ACT232	Ahpatinin	Sagami Bay	1,174	Aspartic protease inhibitors	Sun et al., 2014
<i>Streptomyces</i> sp. SCSIO 01127	Lobophorins E and F	South China Sea	1,350	Antibacterial activity, cytotoxicity	Niu et al., 2011
<i>Streptomyces</i> sp. SCSIO 03032	Heronamides D–F	Indian Ocean	3,412	No activity	Zhang W. et al., 2014
<i>Streptomyces</i> sp. SCSIO 03032	Indimicins	Indian Ocean	3,412	Cytotoxic	Zhang W. J. et al., 2014
<i>Streptomyces</i> sp. SCSIO 04496	(6R,3Z)-3-benzylidene-6-isobutyl-1-methyl piperazine-2,5-dione	South China Sea	3,536	No activity	Luo et al., 2015
<i>Streptomyces</i> sp. SCSIO 10355	Strepsesquitriol	Indian Ocean	3,412	Inhibitory activity against lipopolysaccharide-induced TNF α production	Yang et al., 2013a
<i>Streptomyces</i> sp. SCSIO 11594	Dehydroxyaquayamycin	South China Sea	2,403	Antibacterial activity	Song et al., 2015
<i>Streptomyces</i> sp. SCSIO 11594	Marangucycline B	South China Sea	2,403	Anticancer	Song et al., 2015

(Continued)

TABLE 2 | Continued

Strain	Compounds	Region	Depth (m)	Bioactivity	References
<i>Streptomyces</i> sp. SNJ013	Sungsanpin	Jeju Island	138	Inhibitory activity to A549 with cell invasion assay	Um et al., 2013
<i>Streptomyces</i> sp. UST040711-290	12-methyltetradecanoic acid (12-MTA)	Pacific	5,774	Antifouling	Xu et al., 2009
<i>Streptomyces</i> sp. TP-A0873	Butenolids	Toyama Bay	Not specified	Peroxisome proliferator activated receptor—PPAR α agonistic	Igarashi et al., 2015; Komaki et al., 2015
<i>Streptomyces</i> sp. 12A35	Lobophorins H and I	South China Sea	2,134	Antibacterial activity	Pan et al., 2013
<i>Streptomyces</i> strain C42	Champacyclin	Baltic Sea	241	Antimicrobial activity	Pesic et al., 2013
<i>Streptomyces</i> <i>xiamensis</i> M1-94P	Xiamenmycin C and D	Pacific Ocean	2,628	Anti-fibrotic	You et al., 2013
<i>Verrucosimpora</i> sp.	Abyssomicins J–L	South China Sea	2,733	Antibacterial activity	Wang et al., 2013

Sea (Song et al., 2014). A 39 kb gene cluster governing the biosynthesis of the anti-infective desotamides has been isolated from the strain. Desotamides A and B and a new desotamide G have been obtained by heterologous expression of desotamide gene cluster in *Streptomyces coelicolor* M1152 (Li et al., 2015).

Heronamides D, E, and Fare discovered from the products of *Streptomyces* sp. SCSIO 03032, which was isolated from deep sea sediment sample collected at a depth of 3,412 m in the Bay of Bengal, Indian Ocean (Zhang W. et al., 2014). The gene cluster governing the biosynthesis of heronamide has been isolated from strain SCSIO 03032. The gene inactivation study confirmed that P450 enzyme encode HerO as an 8-hydroxylase for tailoring heronamide biosynthesis. Feeding experiments with labeled small carboxylic acid molecules confirmed the migrated double bonds in the conjugated diene-containing side chain of heronamides (Zhu et al., 2015).

Marformycins A-F were obtained from fermentation broth of deep sea sediment-derived *Streptomyces drozdzowiczii* SCSIO 1014, which was isolated from sample collected at a depth of 1,396 m in South China Sea. All compounds exerted selective anti-microbial activity against *Micrococcus luteus*, *Propionibacterium acnes*, and *P. granulosum*. Marformycins A-E displayed inhibitory activity against *M. luteus* with MICs of 0.25, 4.0, 0.25, 0.063, and 4.0 μ g/mL, respectively, while they did not display any cytotoxicity (Liu D. et al., 2015). It is suggested that these compounds may be used as promising candidates for anti-infective drug leads. The gene cluster responsible for the biosynthesis of marformycin is about 45 kb in size and has been identified from strain SCSIO 10141. The gene inactivation studies indicated that three NRPS proteins MfnC, MfnD, MfnE, a free adenylation (A) enzyme MfnK, and a free peptidyl carrier protein (PCP) MfnL were essential for the generation of the marformycin core scaffold. Further, MfnN was found to use an

intact cyclodepsipeptide intermediate as its substrate (Liu J. et al., 2015).

PERSPECTIVE

The discovery of novel actinomycete taxa with unique metabolic activity from deep sea samples, and novel compounds with the greatest biogenic, metabolic diversity and biological activities clearly illustrate that indigenous deep sea actinomycetes indeed exist in the oceans and are an important source of novel secondary metabolites. Other function of deep sea actinobacteria is also interesting such as oil degradation and biosurfactant production (Wang et al., 2014). It is worth to be noticed that no heat pretreatment, dry and stamp method and low temperature incubation were more productive for actinomycetes isolation from some deep sea samples. With the development of culture independent techniques, more productive strategy of strain isolation guided by the deep sea actinomycetes distribution or direct cloning and heterologous express the functional genes could be approached.

AUTHOR CONTRIBUTIONS

MK contribute the introduction, deep sea environment and biodiversity, actinomycete cultivation, novel taxa, and **Table 1**. PS contribute sample collection, **Table 2** and biosynthesis of secondary metabolites from deep sea streptomycetes. KH and ZD conceived the idea and revised the whole manuscript.

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Marine Sponge-Derived *Streptomyces* sp. SBT343 Extract Inhibits Staphylococcal Biofilm Formation

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Staphylococcus epidermidis and *Staphylococcus aureus* are opportunistic pathogens that cause nosocomial and chronic biofilm-associated infections. Indwelling medical devices and contact lenses are ideal ecological niches for formation of staphylococcal biofilms. Bacteria within biofilms are known to display reduced susceptibilities to antimicrobials and are protected from the host immune system. High rates of acquired antibiotic resistances in staphylococci and other biofilm-forming bacteria further hamper treatment options and highlight the need for new anti-biofilm strategies. Here, we aimed to evaluate the potential of marine sponge-derived actinomycetes in inhibiting biofilm formation of several strains of *S. epidermidis*, *S. aureus*, and *Pseudomonas aeruginosa*. Results from *in vitro* biofilm-formation assays, as well as scanning electron and confocal microscopy, revealed that an organic extract derived from the marine sponge-associated bacterium *Streptomyces* sp. SBT343 significantly inhibited staphylococcal biofilm formation on polystyrene, glass and contact lens surfaces, without affecting bacterial growth. The extract also displayed similar antagonistic effects towards the biofilm formation of other *S. epidermidis* and *S. aureus* strains tested but had no inhibitory effects towards *Pseudomonas* biofilms. Interestingly the extract, at lower effective concentrations, did not exhibit cytotoxic effects on mouse fibroblast, macrophage and human corneal epithelial cell lines. Chemical analysis by High Resolution Fourier Transform Mass Spectrometry (HRMS) of the *Streptomyces* sp. SBT343 extract proportion revealed its chemical richness and complexity. Preliminary physico-chemical characterization of the extract highlighted the heat-stable and non-proteinaceous nature of the active component(s). The combined data suggest that the *Streptomyces* sp. SBT343 extract selectively inhibits staphylococcal biofilm formation

without interfering with bacterial cell viability. Due to absence of cell toxicity, the extract might represent a good starting material to develop a future remedy to block staphylococcal biofilm formation on contact lenses and thereby to prevent intractable contact lens-mediated ocular infections.

Keywords: marine sponges, actinomycetes, *Streptomyces*, staphylococci, biofilms, contact lens

INTRODUCTION

Ocular devices such as intraocular lenses, posterior contact lenses, conjunctival plugs and orbital implants have aided in restoring and improving human vision. However, contamination of these devices with bacterial biofilms can lead to device-related ocular infections such as endophthalmitis, crystalline keratopathy, corneal ulceration, keratitis, lacrimal system, and periorbital infections (Bispo et al., 2015; Cho et al., 2015). The National Institute of Health (NIH) estimates that biofilms contribute to about 75% of the human microbial infections. Biofilms are surface-associated sessile microbial communities that are enmeshed in a self-produced extracellular matrix composed of polysaccharides, proteins, lipids and DNA (Flemming and Wingender, 2010; Oja et al., 2015). Compared to the free-living planktonic counterparts, bacteria in biofilms are 1000-fold more resistant to conventional antibiotic therapies and host immune responses (Donlan, 2001; Otto, 2009; Burmolle et al., 2010). The highly persistent and detrimental nature of biofilm-associated infections and rapid emergence of multidrug resistant strains (Barros et al., 2014; Sakimura et al., 2015) has imposed a major burden on health-care and medical settings. The current inexistence of effective biofilm-based therapeutics (Bjarnsholt et al., 2013) has necessitated the need for development of novel antibiofilm strategies for prophylaxis and/or treatment of the multitude of biofilm-associated ocular infections.

Staphylococci, particularly *S. epidermidis* and *S. aureus* are the most common causative agents of device-related infections. Infections caused by other staphylococci are far less frequent (Otto, 2009). *S. epidermidis* and *S. aureus* are commensal Gram positive bacteria found on human skin and nares, causing a wide range of indwelling medical device-related infections (Rogers et al., 2009; Uribe-Alvarez et al., 2016). The biofilm-based lifestyle of *S. epidermidis* and *S. aureus* on medical devices is a hallmark of the sub-acute and chronic recalcitrant infections caused by them (Reiter et al., 2014). Biofilm formation in *S. epidermidis* and *S. aureus* is facilitated predominantly by the synthesis of the homopolymer polysaccharide intercellular adhesion (PIA) by the enzymes coded by the *ica* locus. PIA-independent biofilm formation, mediated by surface proteins such as biofilm associated protein (Bap/Bhp) and accumulation associated protein (Aap), eDNA release, autolysins and cell sortase-anchored proteins have also been reported in several staphylococcal strains (Otto, 2009; Archer et al., 2011; McCarthy et al., 2015).

Marine actinomycetes represent an untapped reservoir of a broad range of biologically active compounds of pharmaceutical importance (Subramani and Aalbersberg, 2012; Zotchev, 2012;

Lee et al., 2014; Azman et al., 2015). Particularly, the marine sponge-associated actinomycetes are well documented for their intrinsic chemical repertoire (Abdelmohsen et al., 2014a; Santos-Gandelman et al., 2014; Reimer et al., 2015). Novel secondary metabolites with discrete biological activities have been reported from sponge-associated actinomycetes (Abdelmohsen et al., 2014a; Grkovic et al., 2014; Cheng et al., 2015). These include antimicrobial (Hentschel et al., 2001; Eltamany et al., 2014), antiparasitic (Dashti et al., 2014; Viegelmann et al., 2014), immunomodulatory (Tabares et al., 2011), antichlamydial (Reimer et al., 2015), antioxidant (Abdelmohsen et al., 2012; Grkovic et al., 2014), anticancer (Vicente et al., 2015) and anti-biofilm (Oja et al., 2015; Park et al., 2016) activities. The extreme and dynamic conditions offered by the oceans (differences in temperature, pH, pressure, light intensities etc.) are the potential reasons often linked to the production of secondary metabolites by marine actinomycetes (Abdelmohsen et al., 2017). The frequent rediscovery of bioactive compounds and redundancy of sample strains from terrestrial environment has further made the marine actinomycetes as hotspots for discovery of new compounds (Dalisay et al., 2013). Among actinomycetes, the genus *Streptomyces* are considered to be the most prolific producers of secondary metabolites for medical, agriculture and veterinary usage (Tan et al., 2016). Over two-thirds of natural products isolated to date are from *Streptomyces* which indicates their huge biosynthetic potential and chemistry profiles. The rich genetic and metabolic diversity, and the ability to catabolize a wide range of compounds, has made the genus *Streptomyces* to be probed for discovery of novel compounds that could be translated to clinical applications (Hassan et al., 2016; Ser et al., 2016; Yang and Sun, 2016; Perez et al., 2016; Zhao et al., 2016).

In our continuing effort for discovery of anti-biofilm agents, we employed a crystal violet-based screening method to identify anti-biofilm activity of organic extracts generated from marine sponge-derived actinomycetes. The biofilm forming reference strain *S. epidermidis* RP62A was employed as a model for screening. Herein we report the inhibitory effects of an organic extract from marine sponge-associated *Streptomyces* sp. SBT343 against the biofilm formation of *S. epidermidis* RP62A on polystyrene, glass and contact lens surfaces. The potential anti-biofilm activity was tested on two other strains of *S. epidermidis*, four different *S. aureus* strains and two different *P. aeruginosa* strains. The results obtained highlighted that the extract exhibited potent anti-biofilm effects on all the staphylococcal strains tested but did not exert any effect on the *Pseudomonas* strains. Preliminary evaluations on the physico-chemical characterization of active component(s) in the extract suggested their heat stable and non-proteinaceous nature.

MATERIALS AND METHODS

Pathogenic Strains and Growth Conditions

Bacterial strains used in the study are listed in **Table 1**. All strains for the biofilm study were propagated in Tryptic Soy Broth (TSB; Becton Dickinson) (17.0 g/l pancreatic digest of casein, 3.0 g/l papaic digest of soybean meal, 5.0 g/l sodium chloride, 2.5 g/l dipotassium hydrogen phosphate, 2.5 g/l glucose) and incubated at 37°C.

Fermentation Conditions and Extract Preparation

Streptomyces sp. SBT343 was cultivated from the Mediterranean sponge *Petrosia ficiformis* that was collected offshore Pollonia, Milos, Greece (N36.76612°; E24.51530°) in May 2013 (Cheng et al., 2015). Briefly, 1% (v/v) inoculum of a well grown culture of *Streptomyces* sp. SBT343 was inoculated in 150 mL modified ISP2 medium (2.5 g/l malt extract, 1.0 g/l yeast extract, D-mannitol 25 mM, in artificial sea water) in a 250 mL conical flask and the strain was subjected to batch fermentation (incubation at 30°C with shaking at 150 rpm) for 10 days. After harvesting, the filtrate of the fermented culture was extracted twice with 250 mL ethyl acetate for each time. The extract was generated by evaporating the solvent in a rotational evaporator (Heidolph Laborota, 4001, Büchi Vacuum Controller V-805). The modified ISP2 broth medium control ensured the purity of the fermentation and was also extracted separately in a similar manner and this served as the medium control for the bioactivity testing. Extracts were dissolved in DMSO (final concentration 3.75% on the cells) and used for anti-biofilm assays.

Quantification of Biofilm Formation

Quantification of biofilm formation was performed according to Weisser et al. (Weisser et al., 2010). Briefly, overnight culture of bacterial strains were diluted with TSB medium to OD₆₀₀ of 0.05, and incubated statically in a 96-well flat bottom polystyrene plate (Greiner bio-one, GmbH, Germany) with or without the varying concentrations of the *Streptomyces* sp. SBT343 extract (0, 31.25, 62.5, 125, and 250 µg/ml) at 37°C (for *S. epidermidis*

and *P. aeruginosa*) or 30°C (for *S. aureus*) for 24 h. Extract from the modified ISP2 medium control (250 µg/ml) served as the control for the experiment. For biofilm quantification, planktonic bacteria were discarded, and the wells were rinsed carefully with sterile 1X phosphate buffered saline (PBS) twice, and the adherent cells in the plate were heat-fixed at 65°C for 1 h. This was followed by staining with 0.3% crystal violet for 5 min. The stained biofilm was washed with sterile water thrice. Plates were dried in an inverted position and OD₄₉₂ readings were measured to compare the extent of biofilm inhibition in the extract treated sets vs. the modified ISP2 medium control treated set. The biofilm-negative *S. epidermidis* (ATCC12228) and *S. carnosus* TM300 served as the negative controls in the experiment.

Effect of the Extract on Existing Biofilms

To study the effect of the *Streptomyces* sp. SBT343 extract on the existing biofilm, biofilms were established by incubating the bacteria (inoculation OD₆₀₀ 0.05) in TSB medium in a 96-well flat bottom polystyrene plate at 37°C or 30°C for 24 h. Planktonic bacteria were discarded by inverting the plate on paper stacks. This was followed by addition of fresh TSB medium with varying concentration of the *Streptomyces* sp. SBT343 extract and the plate was again incubated statically at 37°C or 30°C for 24 h. The effect of the extract in eradication of preformed biofilm by the extract was measured by the crystal violet assay as explained above. Extract from the modified ISP2 medium control (250 µg/ml) and sodium metaperiodate (40 mM) served as the negative and positive controls in the experiment.

Growth Measurements

The effect of *Streptomyces* sp. SBT343 extract on the growth of staphylococcal strains was evaluated (Nithya et al., 2010) by growth curve analysis. The growth curves were determined up to 24 h. The extract at 50% biofilm inhibitory concentration (BIC₅₀) and the highest tested concentration (125 µg/ml) (non-toxic to cell lines), was added to a conical flask containing bacteria at an OD₆₀₀ of 0.1. The flasks were incubated 37°C under shaking conditions at 200 rpm. TSB medium without the bacteria served as negative control. Growth medium with the modified ISP2 medium extract (125 µg/ml) and bacteria served as the

TABLE 1 | Strains used in this study.

Strain	Description	Reference and/or source
<i>S. epidermidis</i> ATCC 12228	Non-infection associated strain	ATCC collection
<i>S. carnosus</i> TM300	Meat starter culture	Rosenstein et al., 2009
<i>S. epidermidis</i> RP62A	Reference strain isolated from intra-vascular catheter associated sepsis	ATCC collection
<i>S. epidermidis</i> O-47	Clinical isolate from septic arthritis	Heilmann et al., 1996
<i>S. epidermidis</i> 1457	Clinical isolate from a patient with infected central venous catheter	Mack et al., 1992
<i>S. aureus</i> SH1000	MSSA; <i>rsbU</i> derivative of 8325-4 <i>rsbU</i> ⁺	Horsburgh et al., 2002
<i>S. aureus</i> RN4220	Restriction-deficient transformation recipient; originally derived from NCTC 8325-4	Kreiswirth et al., 1983
<i>S. aureus</i> Newman	MSSA isolate from osteomyelitis patient	Lipinski et al., 1967
<i>S. aureus</i> USA300	CA-MRSA isolate from a wrist abscess	McDougal et al., 2003
<i>P. aeruginosa</i> PAO1	Clinical isolate from wound	Dr. Vinay Pawar, Braunschweig, Germany
<i>P. aeruginosa</i> PA14	Clinical isolate from burn wound	Dr. Vinay Pawar, Braunschweig, Germany

control. The reading was observed continuously up to 24 h at 2 h intervals. The experiment was carried out with three independent cultures.

Investigation on the Appearance of Biofilm-Switches Upon Extract Treatment

In order to study the appearance of biofilm-switches [PIA-dependent to PIA-independent (protein-mediated) biofilms] in the presence of the extract, the residual biofilms after the extract treatments were challenged with metaperiodate (40 mM NaIO₄) for PIA-dependent and proteinase K (1 mg/ml in 100 mM Tris-Cl) for PIA-independent (protein-mediated) modes of biofilm formation with the procedure described elsewhere (Wang et al., 2004).

Scanning Electron Microscopy

In order to evaluate the effect of the extract on the biofilm formation on soft contact lens (Proclear®, CooperVision® Lensbest, Germany), round pieces (diameter of 6 mm) were punched out of each contact lens using a sterile puncher. Each piece was washed twice with sterile 1XPBS and placed in a 24 well plate (Greiner bio-one, GmbH, Germany) containing 1 mL of *S. epidermidis* RP62A (OD₆₀₀ 0.05) in TSB with the extract (at concentrations of 62.5, 125, and 250 µg/ml). For testing the effect of the extract on the biofilm formation on glass surface, sterile glass cover slips (diameter of 12 mm) were placed in a 24 well plate containing 1 mL *S. epidermidis* RP62A (OD₆₀₀ 0.05) in TSB with the extract (at concentrations of 62.5, 125, and 250 µg/ml). In each case, bacteria treated with extract (250 µg/ml) from modified ISP2 medium served as the control, while wells with the TSB and the contact lens or cover slips alone served as sterile controls, respectively. Plates containing the contact lens and cover slips sets were then incubated statically at 37°C for 24 h. Samples were then washed carefully with sterile 1XPBS twice, and fixed overnight with 6.25% gluteraldehyde (in 50 mM phosphate buffer pH 7.4). After overnight fixation, samples were washed 5 times with Sörenson buffer (100 mM KH₂PO₄ and 100 mM Na₂HPO₄) and transferred to the electron microscopy unit, where they were dehydrated and then coated with gold by a low vacuum sputter coating, and scanned by scanning electron microscopy.

Confocal Microscopy

Samples on the contact lens and the cover slips were prepared and treated as above. After overnight incubation in 24 well plates, samples were subjected to a rapid epifluorescence staining method employing the bacterial viability kit Live/Dead Bac Light, Invitrogen Ltd., Paisley, UK. The kit employs Syto 9 green and propidium iodide red fluorescent nucleic acid stains for distinguishing live and dead cells, respectively. The dye was prepared and added to the samples according to the manufacturer's specifications. Samples with the dye were incubated in the dark for 15 minutes and then assessed by a confocal microscope. The final step comprised of acquiring

photographs of the samples with a Leica Microsystems (Leica TCS SP5, Leica Microsystems, Germany) at excitation levels of 488 nm for Syto 9 and 543 nm for propidium iodide. Images were acquired at 1.5 µm intervals. Further, images representing two dimensional [compressed z series (x-y sides) and compressed (x-z) views] and three dimensional views of the biofilm were acquired with IMARIS v 8.1.2. The thickness of the biofilms was also calculated with IMARIS v 8.1.2.

Cytotoxicity Studies

Human Epithelial Corneal Cells (HCEC) were cultivated in DMEM/Ham's F12 (Invitrogen Life Technologies, USA), supplemented with 5% (v/v) FBS, 1% (w/v) L-glutamine, 0.4% (w/v) antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin), insulin (5 mg/l) and EGF (10 µg/l) (PAA Laboratories GmbH, Austria). To assess the cytotoxicity on HCEC, vitality assay was performed with slight modifications (Schmitt et al., 2002). Briefly, vitality staining was performed with different concentrations of SBT343 extract for 24 h. 3.5 × 10⁵ cells were seeded in 6-well plates for 24 h in a control medium. After treatment, cells were collected, and 70 µl of the cell suspension was stained with 30 µl staining solution [Gel RedBiotrend (Köln, Germany) and fluorescein diacetate (Kreiswirth et al., 1983)]. Twenty microliter of this mixture was applied to the slide, and the fractions of green and red cells in a total of 200 cells were counted at a 500-fold magnification with a fluorescence microscope. Macrophage (J774.1) and mouse fibroblast (NIH/3T3) cell lines were cultured in RPMI 1640 (1X)+Glutamax™-I and DMEM (1X)+Glutamax™-I (Life Technologies™, USA) supplemented with 10%FCS, without antibiotics. Cytotoxicity on J774.1 and NIH/3T3 was assessed employing alamar blue assay (Huber and Koella, 1993). 1 × 10⁵ cells/ml were seeded in 96-well plates containing the extract at different concentrations (ranging from 31.25 to 500 µg/ml) and were incubated for 24 h at 37°C with 5% CO₂. After incubation, 20 µL alamar blue (Thermofischer scientific, USA) was added to each well and the plates were further incubated for 24 h at 37°C with 5% CO₂. Finally, the OD₅₅₀ values of the plates were measured and normalized with OD₆₃₀ values. The extent of cytotoxicity was determined by comparing the extract treated sets with the control. The final DMSO concentration on the cells was 1%.

Physico-chemical Characterization of Anti-biofilm Component(s)

To understand the nature of the active component(s), the extract was subjected to thermal and enzymatic (proteinase K and trypsin) treatments. Briefly, the extract was subjected to heat treatments at 50, 75, and 100°C for 1 h and cooled on ice. For the enzymatic treatment, proteinase K or trypsin (at a final concentration of 1 mg/ml) was added to the extract (at a final concentration of 0.125 mg/ml) and the reactions were incubated for 1 h at 37°C. As controls, extracts were incubated for 1 h at 37°C without the enzymes, a treatment that did not impair the anti-biofilm effect. For each of the above tests, the

biofilm inhibitory effects of treated and untreated extracts were compared using the microtitre plate assay (for biofilm formation) against all the staphylococcal strains tested. Each data point is composed of three independent experiments performed in quadruplicate.

In parallel, the activity of proteinase K and trypsin (1 mg/ml each) in the presence of DMSO were independently assessed employing the quantitative azocasein assay (Hasegawa et al., 2008).

Quantitative RT-PCR

After complementation of *S. epidermidis* RP62A with SBT343 extract at 62.5 (BIC₅₀) and 250 µg/ml for 24 h at 37°C, total RNA was isolated from planktonic bacteria and those in biofilm employing Trizol reagent (Invitrogen, Paisley, UK) and FastPrep® disrupter (Thermo Savant, Qbiogene, Inc., Cedex, France). Firstly, 1 ml of cells (from planktonic and biofilm states; the biofilm was gently removed from 24 well plate using a sterile scraper and re-suspended in 1 ml of fresh TSB medium) were centrifuged at 13000 rpm for 10 min at 4°C and pellets were re-suspended in 1 ml Trizol reagent. This suspension was briefly homogenized in a Lysing Matrix E tube (MP Biomedicals Germany, GmbH, Eschwege, Germany) in the FastPrep® cell disrupter and subjected to chloroform-based RNA extraction method. Purity and concentration of the extracted total RNA was spectrophotometrically assessed using a NanoDrop ND-1000 (peqLab Biotechnologie, GmbH). The A₂₆₀/A₂₈₀ values of (range 1.8–2.0) indicated the purity of the RNA samples and the mean RNA yield obtained was 182.63 µg/ml. All the RNA samples were digested with DNase I (Thermo Scientific). Briefly, 1 µg of RNA was digested with 1 µl of DNase by incubation at 37°C for 30 min. This was followed by addition of 1.5 µl of DNase stop solution (50 mM EDTA) and incubation at 70°C for 10 min. After quality assessment of RNA after DNase treatment, about 50 ng/µl of RNA was used as template for qPCR experiment.

cDNA synthesis and qPCR amplification was performed simultaneously by employing the power SYBR® Green RNA-to-C_T™ 1-step kit (Applied Biosystems, GmbH, Germany). Primers used in the study were designed according to the literature (Reiter et al., 2014), and were commercially produced (Eurofins MWG Synthesis GmbH, Germany). These primers were chosen based on their thermodynamic and sequence parameters. The reaction mixture for qPCR contained 1 µl of RNA template, 5 µl of power SYBR® Green RTPCR mix, 1 µl each of forward and reverse primers (10 µM), 0.08 µl of reverse transcriptase provided by the manufacturer and 1.92 µl of RNase free water. qPCR was performed using the Bio-Rad C1000 Touch™ thermal cycler with the following cycle parameters: holding stage of 48°C for 30 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and then 55°C for 1 min, with a final melting curve determination. Experiment was performed with three technical and biological replicates each. A difference of ≥7 Ct (cycle threshold) between the cDNA sample and no-template PCR control was considered negligible for relative quantification analysis. Finally, the relative expression of the target genes (*IcaA* and *IcaR*) in the presence of SBT343 extract

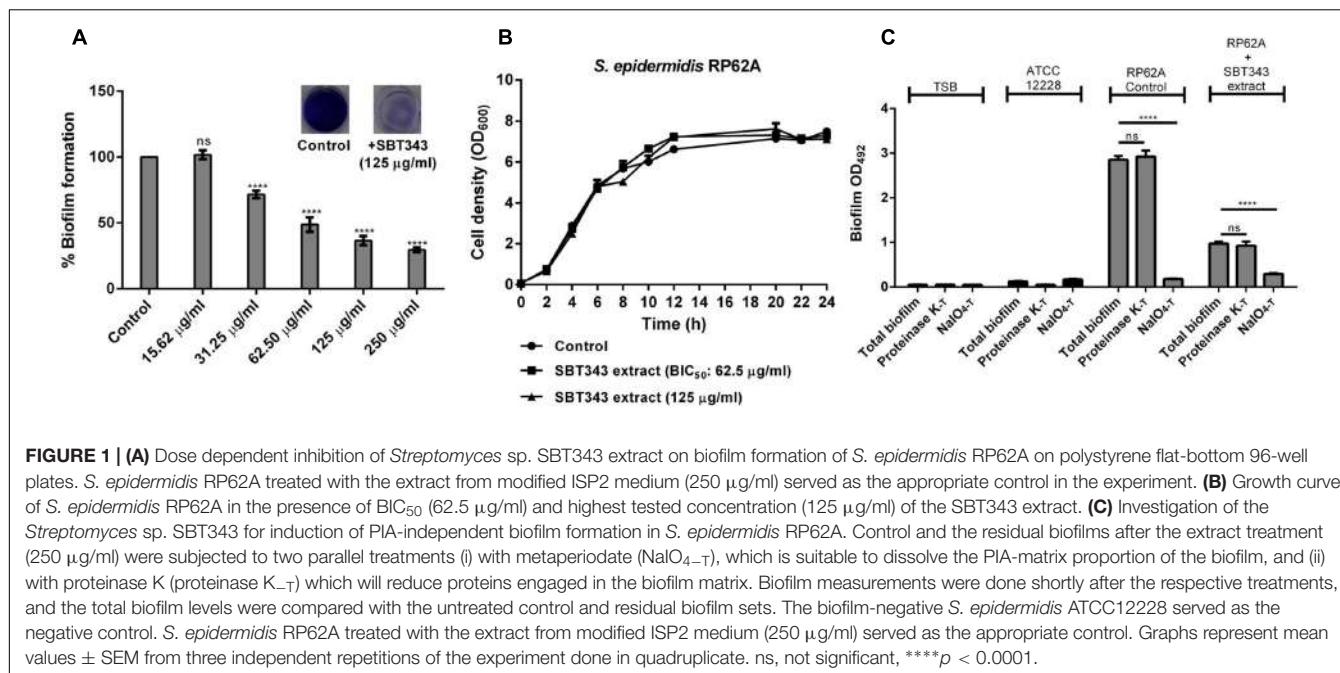
in relation to the modified ISP2 extract treated control was determined using the comparison with the expression level of the reference gene, DHFR (the expression of DHFR gene stayed constant in the conditions tested). The significance of the relative quantification was assessed by Student's *t*-test (GraphPad Prism® version 6.01).

Statistical Analysis

Experiments were repeated at least three times in quadruplicates and the data were expressed as mean ± standard error mean. The Student's *t*-test was used and *p* < 0.05 was considered as statistically significant. GraphPad Prism® version 6.01 was used for statistical analysis of the experimental data.

LC-MS Analysis

Analytical grade reagents, Methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN), and formic acid were purchased (Fisher Scientific, Hemel Hempstead, UK). In-house HPLC grade water was used from a direct Q-3 water purification system (Millipore, Watford, UK). Samples and medium control samples were prepared at a concentration of 1 mg/mL in 80:20 MeOH: DCM with a solvent blank. Experiments were performed with an Exactive mass spectrometer with an electrospray ionization source attached to an Accela 600 HPLC pump with Accela autosampler and UV/Vis detector (Thermo Scientific, Bremen, Germany). The mass accuracy was set to less than 3.0 ppm. The Orbitrap mass analyzer can limit the mass error within ±3.0 ppm. The instrument was calibrated to maintain a mass accuracy of ±1.0 ppm by applying the lock mass function. The instrument was externally calibrated per the manufacturer's instructions before the run and was internally calibrated during the run using lock masses. Mass spectrometry was carried out over a mass range of 100–2000 *m/z* in positive and negative ionization modes with spray voltage of 4.5 kV and capillary temperature at 270°C. Ten microliter were injected from each vial, at a flow rate of 300 µL/min. The column used was an ACE5 C18 column (5 µm × 75 mm × 3 mm) (Hichrom Limited, Reading, UK). A binary gradient method was utilized. The two solvents were A (water and 0.1% formic acid) and B (MeCN and 0.1% formic acid). The gradient was carried out for 10 min and the program followed; at zero minutes A = 90% and B = 10%, at 30 min A = 0% and B = 100% at 36 min A = 90% and B = 10% until end at 45 min. The UV absorption wavelength was set at 254 nm, the sample tray temperature was maintained at 4°C and the column maintained at 20°C. The samples were run sequentially, with solvent and media blanks analyzed first. LC-MS data was acquired using Xcalibur version 2.2 (Thermo Scientific, Bremen, Germany). LC-MS chromatograms were subsequently obtained using MassLynx v 4.10. This was followed by dereplication strategy. Since, the modified ISP2 medium is a complex mixture of constituents and could interfere with the identification of secondary metabolites in the SBT343 extract, a medium blank was analyzed together with the bacterial extract and obtained features were regarded as interference and subtracted for detection of true bacterial secondary metabolites.



Finally, the *m/z* values were searched for possible hits in the MarinLit® database (Abdelmohsen et al., 2014b; Macintyre et al., 2014).

RESULTS

Anti-biofilm Effect of the *Streptomyces* sp. SBT343 Organic Extract

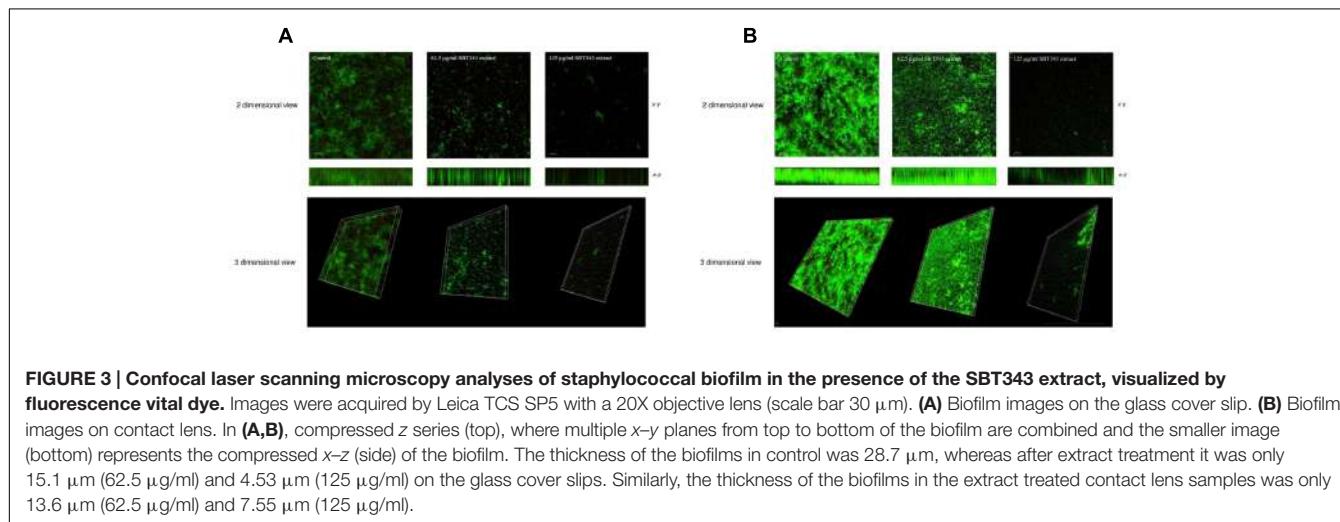
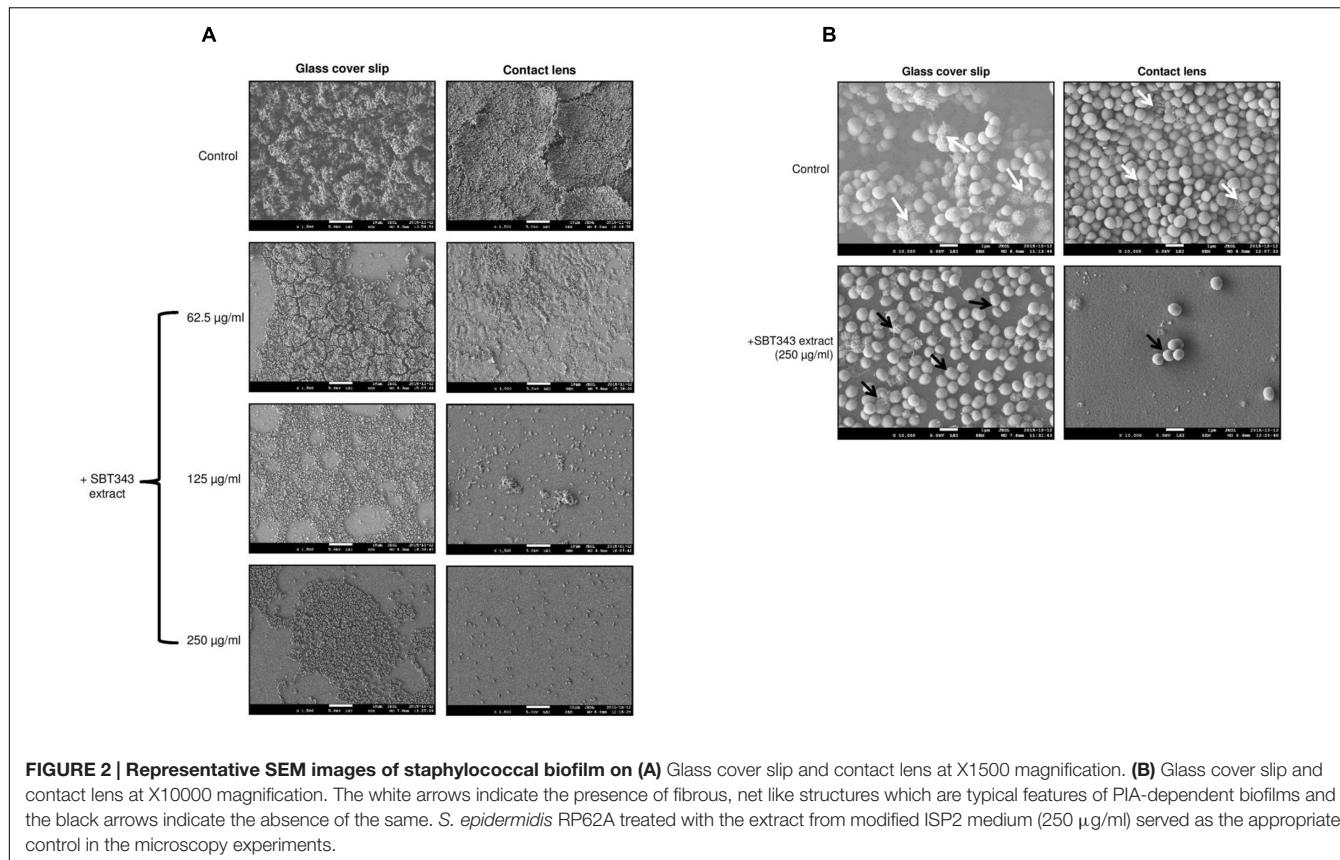
Our continuing effort for discovery of anti-biofilm agents from marine sponge-derived actinomycetes against the model isolate *S. epidermidis* RP62A, led to identification of the anti-biofilm *Streptomyces* sp. SBT343 extract. The presence of the extract at 31.25 µg/ml during the bacterial growth caused a significant ($p < 0.0001$) reduction in the biofilm formation after 24 h of growth. At 62.5 µg/ml about 50% of the biofilm formation was inhibited and this was designated as the Biofilm Inhibitory Concentration (BIC₅₀). The anti-biofilm activity of the extract was dose-dependent, leading to 71.35% inhibition of biofilm formation at the maximum concentration (250 µg/ml) tested (Figure 1A). Even after the addition of extract at BIC₅₀ and highest tested concentration, the growth of *S. epidermidis* RP62A was at the same level as that of the control. These results confirm that the biofilm inhibition by the extract is not due to growth effect (Figure 1B). SBT343 extract showed no effect on detaching/dispersing the bacteria from preformed biofilm at any of the tested concentrations (data not shown).

As the strain *S. epidermidis* RP62A is known as a strong PIA matrix biofilm producer, SBT343-mediated biofilm inhibition strongly suggests interference with PIA-mediated biofilm formation. Since, staphylococci are known to switch from PIA-dependent to PIA-independent biofilm formation under

different conditions; the presence of these spontaneous switches in the presence of the extract was assessed. The ineffectiveness of proteinase K treatment on residual biofilms highlight that there is no spontaneous switch from PIA-dependent to PIA-independent (protein-mediated) biofilm formation in the presence of SBT343 extract (Figure 1C).

SEM Analysis

The biofilm inhibition potential of the SBT343 extract was studied on glass and contact lens surfaces using microscopic techniques. Electron microscopy of biofilm formation on glass cover slips and contact lenses in the presence of 62.5, 125, and 250 µg/ml SBT343 extract confirmed the results obtained by the *in vitro* crystal violet assay (Figure 2). In the control glass cover slips, mushroom shaped, three-dimensional biofilm was observed, whereas, multi-layered biofilm was observed on control contact lenses. In the glass cover slips and contact lenses incubated with the SBT 343 extract (62.5, 125, and 250 µg/ml), a dose-dependent reduction in the biofilm was clearly seen (Figure 2A). The reduction of biofilm with the extract was even pronounced in the contact lens model suggesting its possible application in contact lens solution and storage systems. Surfaces were distinctly seen between sporadic microcolonies in the glass cover slip and the contact lens incubated with higher concentrations of the extract. Representative SEM images at higher magnification indicated that the extract did not affect the morphology of the staphylococcal cells. Further, the presence of fibrous, net-like structures in the biofilm matrix was greatly reduced in both the glass cover slips and contact lenses incubated with SBT343 extract (Figure 2B). These findings suggest that the extract possibly works by altering the biofilm matrix composition or interferes with the production of extracellular matrix.



Confocal Microscopy Analysis

For the confocal microscopy studies, effect of the SBT343 extract on the biofilm formation was also examined with two systems, first in the cover slips, where the control sample showed compact and condensed biofilm, while the treatment with the extract attenuated the formation of the biofilm in a dose dependent manner. The same observations were obtained in the contact lens as a second system for examination. The ability of the

extract to inhibit the biofilm formation was stronger in the coverslips experiments in comparison to the contact lens, but in both systems the inhibition was significant in comparison to the control (Figure 3). Noteworthy, very few non-viable bacteria (stained red) were spotted in the experiment both in the treated and the untreated sets. This further confirms that the extract does not interfere with bacterial cell viability. As a control, cells in biofilm were exposed to 75% ethanol for 5 mins and stained

with propidium iodide and SYTO green. In this case a large population of dead cells were spotted in the microscope (data not shown). Images representing compressed x - y and x - z (side) views (**Figure 3**) indicated that the number of the bacteria in the biofilm and the total biofilm thickness in the extract treated cover slips and contact lens sets were greatly reduced in comparison with the compact and condensed biofilm control. These images further corroborated the results that the reduction in fluorescence in the presence of the extract, was primarily due to the repression of biofilm formation, and has no negative effects on bacterial cell viability.

Anti-biofilm Effect of SBT343 Extract on Other Pathogens

The anti-biofilm activity of the SBT343 extract was investigated with other biofilm-forming *S. epidermidis*, *S. aureus*, and *P. aeruginosa* strains (**Table 2**). SBT343 extract (125 $\mu\text{g}/\text{ml}$) significantly reduced the biofilm formation of all the *S. epidermidis*, MSSA and MRSA strains tested, while the biofilm formation of *P. aeruginosa* was unaffected (**Figure 4A**). A dose dependent reduction in biofilm formation of the staphylococcal strains was observed upon extract treatment (**Figure 4B**), while the growth of the strains were unaltered in the presence of the extract (**Figure 4C**). Further, the extract had no effects on the preformed biofilms of all the strains tested at the highest concentration tested (data not shown). This indicates selectivity of the extract in inhibiting staphylococcal biofilms.

Cytotoxicity Analysis

We further investigated the cytotoxicity profile of the SBT343 extract against mouse fibroblast (NIH/3T3), macrophage (J774.1) and human corneal epithelial cells (HCEC). Results from vitality test and alamar blue assay demonstrated that cells did not suffer from significant toxicity after 24 h with effective concentrations of the extract (in the range of 31.25–125 $\mu\text{g}/\text{ml}$). The highest concentration 500 $\mu\text{g}/\text{ml}$ displayed moderate to high cytotoxic effects on the cell lines tested (**Table 3**).

TABLE 2 | Biofilm formation of the investigated bacterial strains employing crystal violet assay.

Strain	Biofilm (OD 492 nm)
<i>S. epidermidis</i> ATCC 12228	0.124 \pm 0.010
<i>S. carnosus</i> TM300	0.204 \pm 0.013
<i>S. epidermidis</i> RP62A	2.861 \pm 0.143
<i>S. epidermidis</i> O-47	1.387 \pm 0.044
<i>S. epidermidis</i> 1457	2.165 \pm 0.069
<i>S. aureus</i> SH1000	0.994 \pm 0.112
<i>S. aureus</i> RN4220	1.177 \pm 0.092
<i>S. aureus</i> Newman	0.691 \pm 0.149
<i>S. aureus</i> USA300	0.659 \pm 0.031
<i>P. aeruginosa</i> PAO1	1.337 \pm 0.101
<i>P. aeruginosa</i> PA14	1.331 \pm 0.067

Each data point is composed of three independent experiments performed in quadruplicate. Standard errors are reported.

Physico-chemical Characterization of Anti-biofilm Component(s)

Preliminary physical and chemical characterization of the biofilm inhibiting component(s) in the extract was assessed by subjecting the extract to physical (heat) and chemical (proteinase K or trypsin) treatments. Both the physical and chemical treatments, did not reduce the activity of the extract against all the staphylococcal strains (**Figure 5**). However, a slight increase in the activity was observed upon heat treatment, in *S. epidermidis* RP62A and 1457, and *S. aureus* SH1000 and RN4220 strains. Similarly, a slight increase in the anti-biofilm activity was observed upon enzyme treatment in *S. aureus* SH1000 and RN4220 strains (**Figure 5**). This suggests that the active component(s) in the extract is thermo-stable and non-proteinaceous in nature, and the extract contains compound(s) with anti-biofilm activity that could work similarly on different staphylococcal strains. DMSO at the tested concentrations did not influence proteinase K and trypsin activities (Supplementary Figure 1).

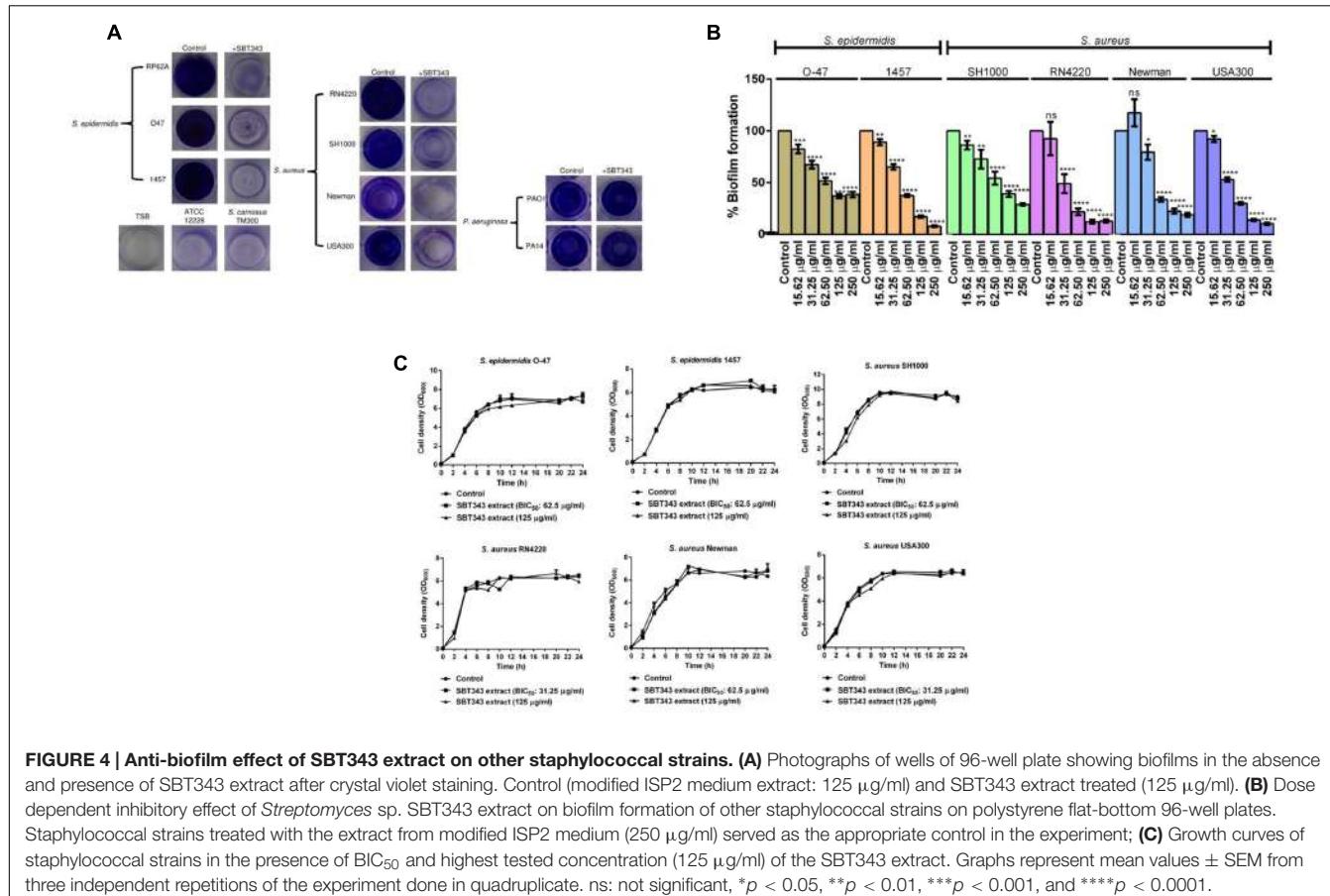
LC-MS Analysis

To identify the putative bioactive secondary metabolites in the SBT343 extract, the LC-UV/MS signature of the extract generated by high resolution Fourier Transform mass spectrometry was compared with the MarinLit database (a database for marine natural products). The dereplication of the chemical profile of SBT343 extract by comparison of HRMS data with MarinLit, led to identification of several known and unknown metabolites which were previously isolated from the genus *Streptomyces*. Both the positive and negative modes of electrospray ionization spectral data were used for dereplication purposes. The total ion chromatogram of the SBT343 extract showing the distribution of known and unknown compounds is depicted in **Figure 6A**. Further, the known compounds detected from the dereplication strategy are mentioned in **Table 4** and their structures are depicted in **Figure 6B**.

DISCUSSION

Biomaterials used in clinical and medical settings are ideal niches for formation of microbial biofilms (Shanmugapriya et al., 2012). Even though a number of natural and synthetic anti-biofilm agents have already been discovered (Richards and Melander, 2009; Gupta et al., 2016; Miquel et al., 2016; Tran et al., 2016), none of them has entered the market, owing to obstacles in translational research and lack of interest by pharmaceutical and biomedical companies (Romero and Kolter, 2011). Hence, there is a large unmet need for development of anti-biofilm formulations to tackle the problem of biofilms.

Various culture dependent and independent techniques have revealed the bacterial biofilm diversity in contact lens-related corneal diseases (Hall and Jones, 2010; Wiley et al., 2012). Staphylococci, particularly *S. epidermidis*, are frequent contaminants of a range of ocular devices. It was estimated by Hou et al. (2012) that many ophthalmic isolates of *S. epidermidis* could form biofilms *in vitro*. Strong biofilm formation by



S. epidermidis has been observed on various intraocular and contact lens (IOLs) materials (Okajima et al., 2006). Bacteria in the biofilms of contact lenses are frequently resistant to antimicrobials in the soft contact lens care products (Szczotka-Flynn et al., 2009). This has sharpened the need to develop new anti-biofilm-based contact lens products for combating ocular infections. The anti-biofilm potential of natural product based preparations, extracts, and compounds have increasingly been reported in *in vitro* biomaterial-based models (Pacheco da Rosa et al., 2013; Nair et al., 2016). Several researchers have tried to assess the anti-biofilm aspects of marine bacteria (Nithya et al., 2011; Gowrishankar et al., 2012; Papa et al., 2015; Wu et al., 2016), particularly, marine actinomycetes (Oja et al., 2015; Saleem et al., 2015; Park et al., 2016).

Leshem et al. (2011) and Cho et al. (2015) have previously reported the inhibitory effect of natural product based extract and several compounds on staphylococcal biofilm formation on contact lens and contact lens cases. We have shown that the organic extract from marine *Streptomyces* sp. SBT343 exhibits similar anti-biofilm effects at much lower concentrations (62.5–250 µg/ml). The extract had no adverse effects on the contact lens material at the highest concentrations tested (data not shown). At the same time, the lowest effective concentration of the extract did not show apparent cytotoxic effects on the three different cell lines tested which indicates the possibility of using

the extract for the human subjects. The selective anti-biofilm effect of the SBT343 extract against different staphylococcal strains without interference with the bacterial cell growth suggests the less possible appearance of resistant mutants with the usage of this extract. However, further experiments are needed to prove this hypothesis. The basis of employing TSB medium as the growth medium in the study is to provide optimal growth conditions at which the effects of SBT343 extract could be determined in short periods.

Under certain conditions, *S. epidermidis* is known to switch between the PIA-dependent and independent modes of biofilm lifestyle (Rohde et al., 2005; Hennig et al., 2007). Our findings

TABLE 3 | Cytotoxic evaluation for SBT343 extract.

Cell line	% reduction in cell viability		
	500 µg/ml	250 µg/ml	31.25–125 µg/ml
HCEC	90.75 ± 1.23****	20.66 ± 5.10*	NC
NIH/3T3	21.56 ± 2.43****	NC	NC
J774.1	33.83 ± 2.27****	NC	NC

Each data point is composed of three independent experiments performed in quadruplicate. Standard errors are reported. Differences in mean were compared to untreated control and considered statistically significant when p (*p < 0.05, ****p < 0.0001) as per Student's t-test. NC, not cytotoxic.

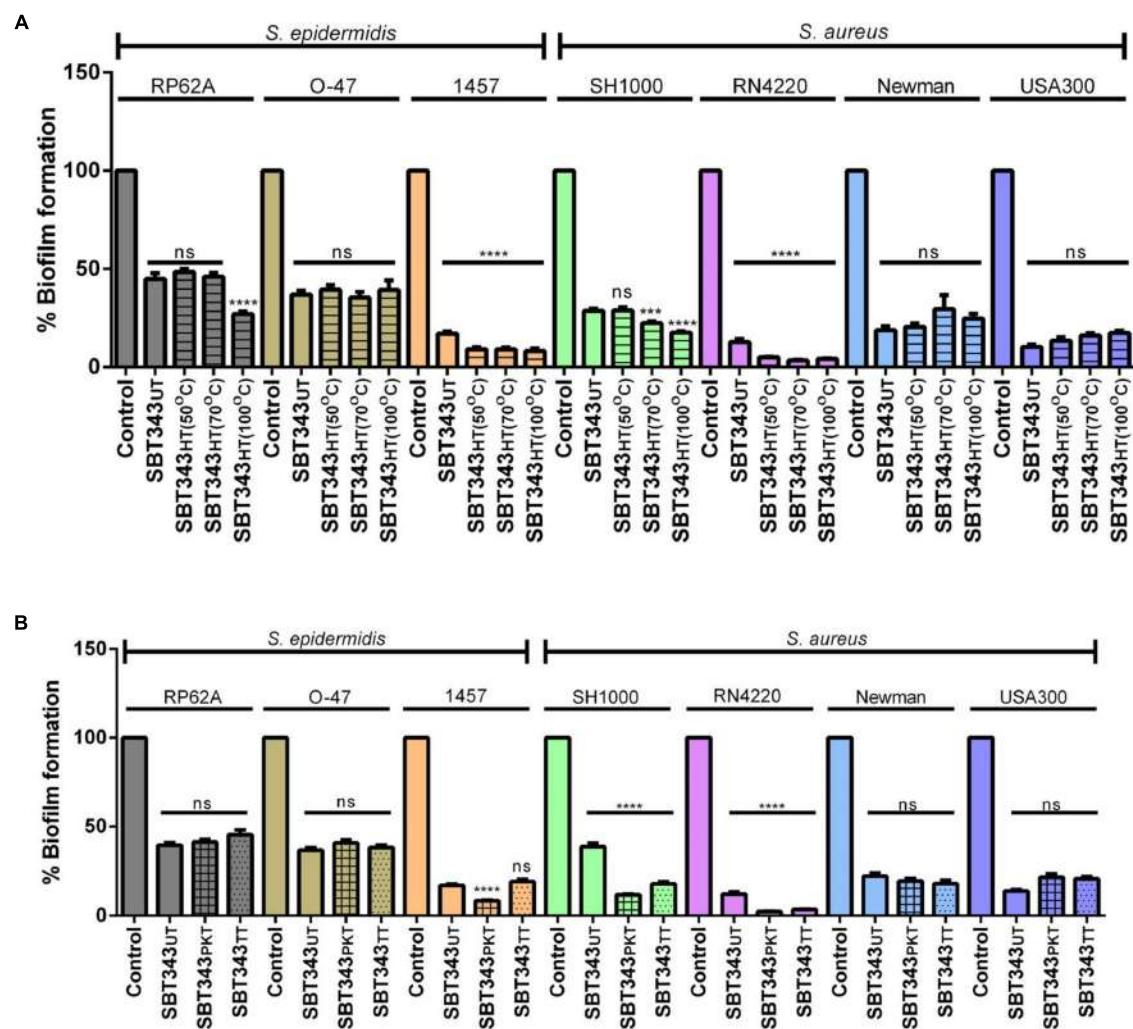
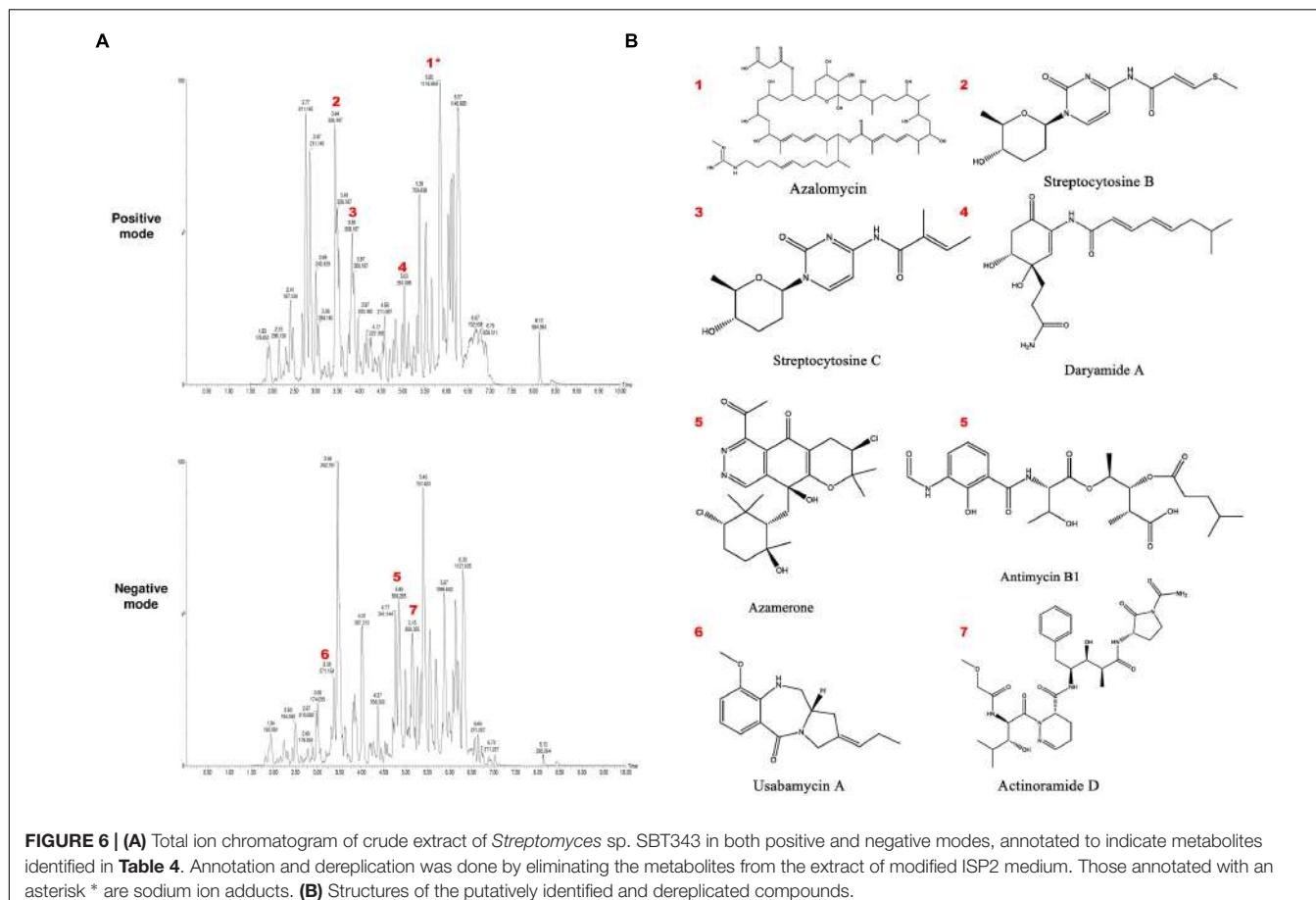


FIGURE 5 | Physico-chemical characterization of anti-biofilm component(s). (A) Influence of heat treatment on anti-biofilm activity. UT, untreated; HT, heat treated. **(B)** Influence of enzyme treatment on anti-biofilm activity. UT, untreated; PKT, proteinase K treated; TT, trypsin treated. ns, not significant; *** $p < 0.0001$.

suggest that there are no switches in the biofilm lifestyle of the organism in the presence of the extract (Figure 1C). For a better understanding of the mechanism of biofilm inhibition by the extract, the relative mRNA expression of *icaA* and *icaR* in *S. epidermidis* RP62A (after 12 and 24 h) was determined in the planktonic and the biofilm cells in the presence of the extract. However, no significant changes in the expression levels of *icaA* and *icaR* were noted in the extract treated planktonic and biofilm cells (data not shown). These results indicate that the extract possibly works by an alternate mechanism and a global gene expression analysis would assist in deciphering the exact mode of action of the extract. The universal anti-biofilm activity against different staphylococcal strains with no effects on preformed biofilm and low cytotoxicity of this extract suggests its potential usage in contact lenses storage cases to prevent contact lens-associated ocular infections. Further studies are necessary to determine the anti-biofilm effect of SBT343 extract on different contact lens and storage cases materials.

The preliminary physical and chemical characterization of the anti-biofilm component(s) in the extract indicates that the active component(s) is of thermo-stable and non-proteinaceous nature and could act similarly on different staphylococcal strains tested. Dereplication strategies are often used in the natural products-based research for rapid identification of secondary metabolites in the crude bacterial extracts (Abdelmohsen et al., 2014b; Cheng et al., 2015). Currently, several analytical methods and tools are available for dereplication of metabolites in complex mixtures. Comparison of the HRMS data at positive and negative modes with the MarinLit database resulted in identification of several putative compounds in the *Streptomyces* sp. SBT343 extract. It is clear from the chromatogram that several peaks were not identified by comparison to the database, including some of the major components that showed strong peak intensity and good resolution. The high number of unidentified compounds highlights the chemical potential of this strain as a source of new natural products. Most of the compounds



identified in the extract were previously isolated from marine *Streptomyces*. An extensive literature search on the biological activities of these compounds revealed that none of them have been tested/reported to have anti-biofilm effects, while several of the putative compounds identified are known to possess anti-fungal, anti-cancer, anti-mycobacterial and anti-malarial effects.

TABLE 4 | Putatively identified and dereplicated compounds from the high-resolution mass spectral data sets of the crude ethyl acetate extract of *Streptomyces* sp. SBT343 using MarinLit® database with a precision of ±0.1–1.0.

Peak ID	ESI Mode	m/z*	Rt (min)	Hits (m/z**)
1	P	1095.624	5.85	Azalomycin (1095.682)
2	P	325.197	3.44	Streptcytosine B (325.109)
3	P	307.187	3.83	Streptcytosine C (307.153)
4	P	350.098	5.03	Daryamide A (350.184)
5	N	510.265	4.85	Azamerone (510.168) Antimycin B1 (510.221)
6	N	272.154	3.38	Usabamycin A (272.152)
7	N	659.355	5.15	Actinoramide D (660.738)

P, positive ESI mode; N, negative ESI mode; Rt, retention time; * indicates the neutral m/z values of peaks found in the crude ethyl acetate extract of *Streptomyces* sp. SBT343; ** indicates the m/z values of the corresponding hits identified with the MarinLit® database.

This further highlights the novelty in discovery of compound(s) with specific anti-biofilm effects from the SBT343 extract. Up-scaling of the fermentation process and consequent bio-assay guided fractionation would help in isolation and identification of active compound(s) in the extract. In conclusion, our results show that the chemically rich *Streptomyces* sp. SBT343 extract has the potential to prevent the staphylococcal biofilm formation on polystyrene, glass, and contact lens surface without exhibiting toxic effects on bacterial and mammalian cells. Future characterization of lead compounds in this extract may yield novel anti-biofilm compound(s) of pharmaceutical interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: UA, TÖ, UH, WZ. Performed the experiments: SB, EO. Analyzed the data: SB, EO, TÖ, UA. Manuscript preparation: SB, EO, DK, UA, HS, UH, WZ, TÖ. Manuscript revision: SB, EO, HS, UA, UH, WZ, TÖ. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmcb.2017.00236/full#supplementary-material>

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***Streptomyces colonosanans* sp. nov., A Novel Actinobacterium Isolated from Malaysia Mangrove Soil Exhibiting Antioxidative Activity and Cytotoxic Potential against Human Colon Cancer Cell Lines**

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Streptomyces colonosanans MUSC 93J^T, a novel strain isolated from mangrove forest soil located at Sarawak, Malaysia. The bacterium was noted to be Gram-positive and to form light yellow aerial and vivid yellow substrate mycelium on ISP 2 agar. The polyphasic approach was used to determine the taxonomy of strain MUSC 93J^T and the strain showed a range of phylogenetic and chemotaxonomic properties consistent with those of the members of the genus *Streptomyces*. Phylogenetic and 16S rRNA gene sequence analysis indicated that closely related strains include *Streptomyces malachitofuscus* NBRC 13059^T (99.2% sequence similarity), *Streptomyces misionensis* NBRC 13063^T (99.1%), and *Streptomyces phaeoluteichromatogenes* NRRL 5799^T (99.1%). The DNA-DNA relatedness values between MUSC 93J^T and closely related type strains ranged from 14.4 ± 0.1 to 46.2 ± 0.4%. The comparison of BOX-PCR fingerprints indicated MUSC 93J^T exhibits a unique DNA profile. The genome of MUSC 93J^T consists of 7,015,076 bp. The DNA G + C content was determined to be 69.90 mol%. The extract of strain MUSC 93J^T was demonstrated to exhibit potent antioxidant activity via ABTS, metal chelating, and SOD assays. This extract also exhibited anticancer activity against human colon cancer cell lines without significant cytotoxic effect against human normal colon cells. Furthermore, the chemical analysis of the extract further emphasizes the strain is producing chemo-preventive related metabolites. Based on this polyphasic study of MUSC 93J^T, it is concluded that this strain represents a novel species, for which the name *Streptomyces colonosanans* sp. nov. is proposed. The type strain is MUSC 93J^T (=DSM 102042^T =MCCC 1K02298^T).

Keywords: *Streptomyces colonosanans*, actinobacteria, mangrove, antioxidant, cancer

INTRODUCTION

The discovery of new and useful compounds is constantly in need for the prevention and/or treatment of diseases. Nature has been an interesting source of many useful compounds that have important applications in various fields such as pharmacy, medicine, and biochemistry (Burja et al., 2001; Karikas, 2010). Researchers have been exploring natural sources such as plants and microorganisms for the discovery of novel drugs. Microorganisms have gained increasing attention in drug discovery and many studies revealed that microorganisms from different ecosystems have shown some potentials for human use as many interesting compounds have been derived from them (Burja et al., 2001; Chin et al., 2006).

In the field of microbial drug discovery, *Actinobacteria* strains have been greatly explored due to their ability to produce diverse bioactive secondary metabolites; accounting for 45% of all discovered bioactive microbial metabolites (Sharma and Shah, 2014). Particularly, the dominant genus of this phylum which is *Streptomyces* have a significant contribution to mankind (Azman et al., 2015). The genus *Streptomyces* is proposed by Waksman and Henrici (1943) and it is a group of Gram positive bacteria comprised ~780 species with validly published names (<http://www.bacterio.cict.fr/>). Members of this genus are producers of more than 75% of the naturally occurring antibiotics (Kinkel et al., 2014; Lee et al., 2014e; Ser et al., 2015a). Other than antibiotics, *Streptomyces* bacteria are prolific producers of various compounds with important biological activities such as antifungal, anticancer, antioxidant, and immunosuppressive activities (Kino et al., 1987; Rashad et al., 2015; Ser et al., 2016a; Law et al., 2017). It is known that exploring new taxa is one of the successful strategies that can lead to the discovery of therapeutic agents (Williams, 2009; Ser et al., 2016c). In previous drug screening programs, it is unfortunate that the screening of novel *Actinobacteria* from terrestrial source have resulted in inefficient rediscovery of known bioactive compounds (Ser et al., 2016c). Therefore, this highlighted the need to discover novel *Actinobacteria* from new or under explored area such as the mangrove environments.

Mangrove environments consists of special woody plant area mainly located in intertidal zones of estuaries, deltas, lagoons, backwaters, creeks, marshes, tropical, and subtropical coastal regions (Mangamuri et al., 2012; Ser et al., 2015a). Mangrove is one of the world's most dynamic environments which occupies millions of hectors across the world coastal areas and it has been a habitat to various flora and fauna of terrestrial, freshwater, and marine species (Mangamuri et al., 2012; Lee et al., 2014e). According to the report by Giri et al. (2011), the largest extent of mangroves is found in Asia; and Malaysia is one of the most mangrove-rich countries in Asia. Additionally, one of the least disturbed mangrove areas in Malaysia is situated at the state of Sarawak, in which most of its mangrove forests are still in pristine condition (Ashton and Macintosh, 2002). Hence, this provides a great opportunity to explore the actinobacterial population present in these mangrove forests.

Owing to the presence of various microbial enzymatic and metabolic activities, the mangrove ecosystem is highly rich in

nutrient and organic matter that in turn facilitates the rapid development of species diversity in response to environmental variation (Satheeja and Jebakumar, 2011; Mangamuri et al., 2012). Furthermore, this ecosystem experiences constant fluctuations in salinity and tidal gradient that could trigger metabolic pathway adaptations and possibly lead to the production of pharmaceutically important metabolites. Hence, there are growing interests in the utilization of mangrove microorganism resources and this have subsequently led to the discovery of novel *Streptomyces* (Hong et al., 2009; Lee et al., 2014d,e).

In recent studies, researchers have successfully identified a number of novel *Streptomyces* from mangrove environments in different countries. For examples, *Streptomyces avicenniae* (Xiao et al., 2009), *Streptomyces xiamensis* (Xu et al., 2009), *Streptomyces sanyensis* (Sui et al., 2011), and *Streptomyces qinglanensis* (Hu et al., 2012) from mangrove environments in China, *Streptomyces sundarbansensis* (Arumugam et al., 2011) from mangrove environments in India, and *Streptomyces pluripotens* (Lee et al., 2014d), *Streptomyces mangrovisoli* (Ser et al., 2015b), *Streptomyces humi* (Zainal et al., 2016), *Streptomyces antioxidantans* (Ser et al., 2016c), and *Streptomyces malaysiense* (Ser et al., 2016b) from mangrove environments in Malaysia. In addition, several studies also reported that mangrove *Streptomyces* are capable of producing antioxidant and anticancer agents (Ser et al., 2015a, 2016b,c; Tan et al., 2015). Thus, this prompted the investigation of *Streptomyces* from underexplored mangrove forest in Sarawak.

As a matter of fact, the current global burden of cancer is increasing continuously and this is mainly due to increasing urbanization, followed by the changes in environmental conditions and lifestyle (Karikas, 2010; Ser et al., 2016b; Siegel et al., 2016). Over the years, natural compounds play a relevant role in cancer therapy and prevention (Nobili et al., 2009). Several anticancer agents that have been successfully derived from *Streptomyces* include aclarubicin, bleomycin, doxorubicin, mitomycin C, and pentostatin (Tan et al., 2006, 2015). Besides, the knowledge acquired throughout years of research conducted in cancer biology has emphasized the cancer initiation and progression is mainly associated with oxidative stress- a condition characterized by elevated amounts of free radicals (Reuter et al., 2010; Ser et al., 2016b). Oxidative stress is known to cause modification or damage to important cellular macromolecules including DNA which could dramatically increase the risk of cancer (Reuter et al., 2010; Ser et al., 2015a, 2016b; Tan et al., 2015). Meanwhile, antioxidant is acknowledged to play important role in biological system. It exerts its scavenging ability to neutralize the free radicals and thus preventing deleterious effects of excessive free radicals during occurrence of oxidative stress (Ser et al., 2015a, 2016b). In view of the importance of antioxidant, efforts have been made to search for effective natural antioxidants. A number of antioxidants have been derived from *Streptomyces* such as carazostatin (Kato et al., 1989), antiostatins A₁ to A₄ and B₂ to B₅ (Mo et al., 1990), carbazoquinocins A to F (Tanaka et al., 1995), and benthocyanins A, B, and C (Shin-ya et al., 1991; Shinya et al., 1993).

This study explores novel *Streptomyces* strains present in soil samples collected from the mangrove forest located at Kuching, Sarawak. A novel strain, MUSC 93J^T was discovered and polyphasic approach demonstrated that MUSC 93J^T represents a novel species of the *Streptomyces* genus, for which the name *Streptomyces colonosanans* sp. nov. is proposed. With the advancement of next generation sequencing (NGS) technology, the genome of MUSC 93J^T was analyzed in this study. The study also aim to investigate the antioxidant and anticancer properties of MUSC 93J^T. Furthermore, gas chromatography-mass spectrometry (GC-MS) was conducted for chemical analysis of MUSC 93J^T extract in order to reveal the active compounds present in the extract. To the best of our knowledge, there is no literature reported so far in regards to the exploration of biological properties of *Streptomyces* isolated from Sarawak mangrove environments. Therefore, the outcome of this study provides a further in depth understanding on bioprospecting potential of *Streptomyces* from under-explored region of Malaysia and at the same time granting the solid foundation to support for a further in depth molecular studies on chemopreventive property possessed by *Streptomyces colonosanans* sp. nov.

MATERIALS AND METHODS

Isolation and Maintenance of Strain

Strain MUSC 93J^T was isolated from a soil sample collected at site KTTAS 1 ($1^{\circ}41'48.57''N$ $110^{\circ}11'15.30''E$), situated in the mangrove forest of Kuching, state of Sarawak, Malaysia, in June 2015. Samples of the upper 20 cm topsoil layer (after the top 2–3 cm of soils removed) were collected using an aseptic metal trowel, placed in sterile Eppendorf tube and stored in $-20^{\circ}C$. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at $50^{\circ}C$; Takahashi et al., 1996). One gram of air-dried soil was mixed with 9 mL sterilized water and then the suspension was spread onto an isolation medium ISP 2 (Shirling and Gottlieb, 1966) supplemented with cycloheximide (50 mg/L) and nalidixic acid (20 mg/L), and incubated at $28^{\circ}C$ for 14 days. Pure cultures of strain MUSC 93J^T were obtained and maintained on ISP 2 agar slants at $28^{\circ}C$ and stocked in glycerol suspensions (20%, v/v) at $-20^{\circ}C$.

Genomic and Phylogenetic Analyses

Genomic DNA extraction for PCR was performed as described by Hong et al. (2009). PCR amplification of the 16S rRNA gene was conducted according to the protocol described by Lee et al. (2014d). The 16S rRNA gene sequence of strain MUSC 93J^T was aligned with representative sequences of related type strains in the genus *Streptomyces* retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al., 1997). The alignment was first verified manually and adjusted, followed by construction of phylogenetic trees with neighbor-joining (Saitou and Nei, 1987; Figure 1) and maximum-likelihood algorithms (Felsenstein, 1981; Figure S1), utilizing the MEGA version 6.0 (Tamura et al., 2013). For neighbor-joining algorithm, the evolutionary distances were computed using the Kimura's two-parameter model (Kimura,

1980). The calculations of level of sequence similarity were performed by EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>; Kim et al., 2012). Bootstrap based on 1,000 resampling method of Felsenstein (1985) was used to analyze the stability of the resultant tree topologies.

For DNA-DNA hybridization, the extraction of genomic DNA of strain MUSC 93J^T, *Streptomyces malachitofuscus* JCM 4493^T, *Streptomyces misionensis* NBRC 13063^T and *Streptomyces phaeoluteichromatogenes* DSM 41898^T were conducted according to the protocol described by Cashion et al. (1977). DNA-DNA hybridization was performed by the Identification Service of the DSMZ, Braunschweig, Germany based on the procedure as described by De Ley et al. (1970) with slight modifications according to Huss et al. (1983). The G + C content of strain MUSC 93J^T was determined by HPLC (Mesbah et al., 1989).

BOX-PCR fingerprint analysis was performed for the characterization of strain MUSC 93J^T and the closely related strains with the use of primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3'; Versalovic et al., 1991; Lee et al., 2014b). The BOX-PCR cycling parameters were performed as described by Lee et al. (2014a) and the PCR products were visualized using 2% agarose gel electrophoresis.

Chemotaxonomic Characteristics

Biomass and freeze-dried cells for chemotaxonomic studies were obtained after growing in TSB at $28^{\circ}C$ for 5 days on a rotary shaker. The analyses of peptidoglycan amino acid composition and sugars of strain MUSC 93J^T were performed by the Identification Service of the DSMZ using published protocols (Schumann, 2011). Analysis of fatty acids (Sasser, 1990), polar lipids (Kates, 1986), and respiratory quinones were performed by the Identification Service of the DSMZ. Major diagnostic whole cell sugars of strain MUSC 93J^T were obtained according to the description by Whiton et al. (1985) and analyzed by TLC on cellulose plates (Staneck and Roberts, 1974).

Phenotypic Characteristics

The cultural characteristics of strain MUSC 93J^T was determined following growth on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, and ISP 7 agar (Shirling and Gottlieb, 1966), actinomycetes isolation agar (AIA; Atlas, 1993), starch casein agar (SCA; Küster and Williams, 1964), *Streptomyces* agar (SA; Atlas, 1993), and nutrient agar (Macfaddin, 2000) at $28^{\circ}C$ for 14 days. The morphology of strain MUSC 93J^T was observed after incubation on ISP 2 agar plate at $28^{\circ}C$ for 7–14 days (Figure 2), using Light microscopy (80i, Nikon) and scanning electron microscopy (JEOL-JSM 6400). The designations of colony colors were made according to the ISCC-NBS color charts. Gram staining was carried out by standard Gram reaction and confirmed by using KOH lysis (Cerny, 1978). The pH range for growth and NaCl tolerance were evaluated using tryptic soy broth (TSB). The pH range tested was between pH 4.0 and 10.0 at an interval of 1 pH unit. The concentration of NaCl was tested at a range of 0–10% (w/v) at intervals of 2%. The effects of temperatures on growth was examined on ISP 2 agar. The temperature range tested for growth was between 4 and $44^{\circ}C$ at intervals of $4^{\circ}C$. The growth responses to pH, NaCl, and temperature were

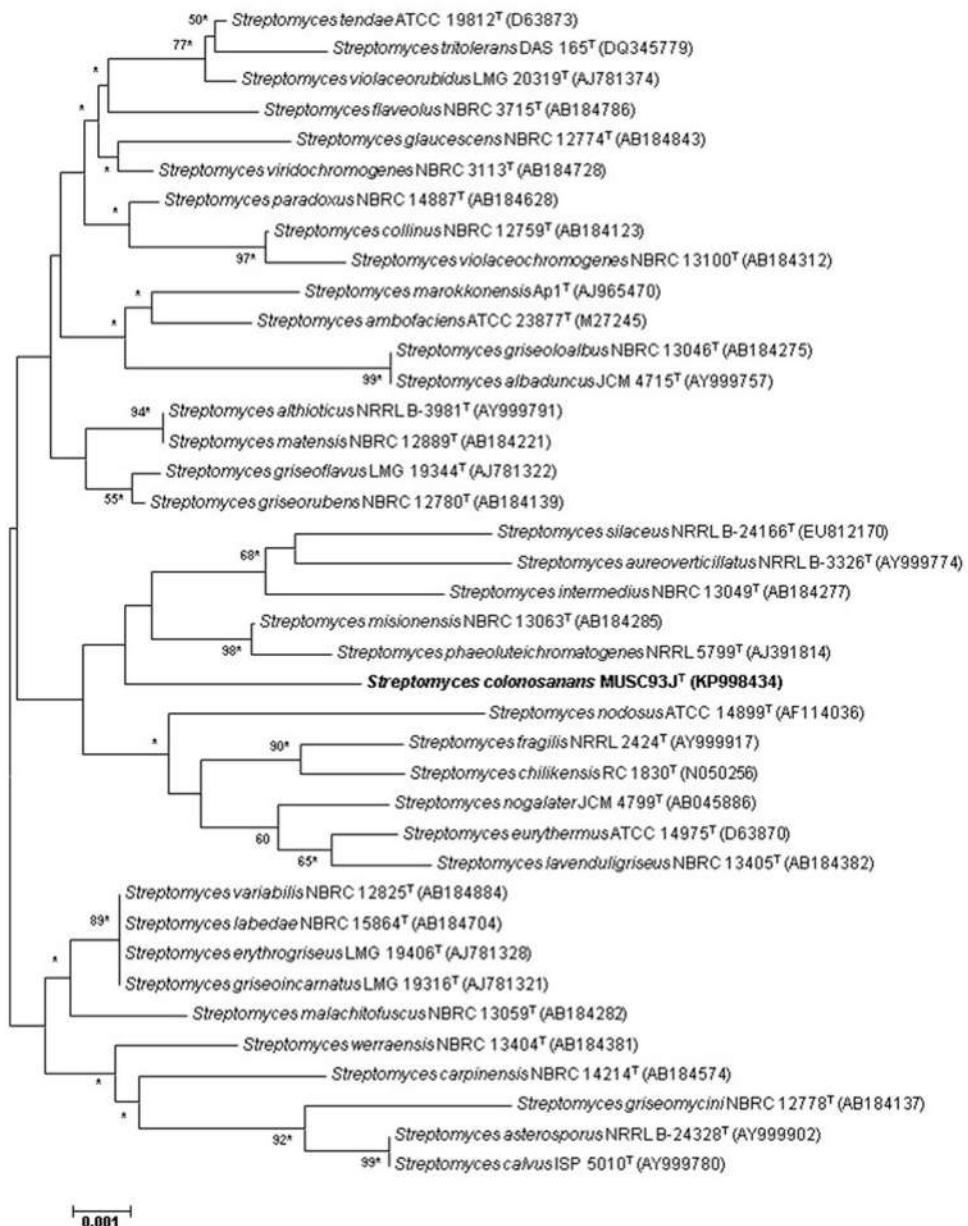


FIGURE 1 | Neighbor-joining phylogenetic tree based on almost complete 16S rRNA sequences (1,490 nucleotides) showing the relationship between strain MUSC 93J^T and representatives of some other related taxa. Numbers at nodes indicate percentages of 1,000 bootstrap re-samplings, only values above 50% are shown. Bar, 0.002 substitutions per site. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm.

observed for 14 days. The production of melanoid pigments was examined using ISP 7 medium following protocol described by Lee et al. (2014c). Hemolytic activity was examined on blood agar medium containing 5% (w/v) peptone, 3% (w/v) yeast extract, 5% (w/v) NaCl, and 5% (v/v) horse blood (Carrillo et al., 1996). Plates were examined for hemolysis after incubation at 32°C for 7–14 days. Amylolytic, lipase, cellulase, chitinase, catalase, protease, and xylanase activities were determined by growing cells on ISP 2 medium following protocol as described by Lee

et al. (2014c). The presence of clear zones around colonies indicates the potential of isolates for surfactant production. The carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates (Biolog, USA) according to the manufacturer's instructions.

All of the phenotypic assays mentioned were performed concurrently for strain MUSC 93J^T, *Streptomyces malachitofuscus* JCM 4493^T, *Streptomyces misionensis* NBRC 13063^T, and *Streptomyces phaeoluteichromatogenes* DSM 41898^T.

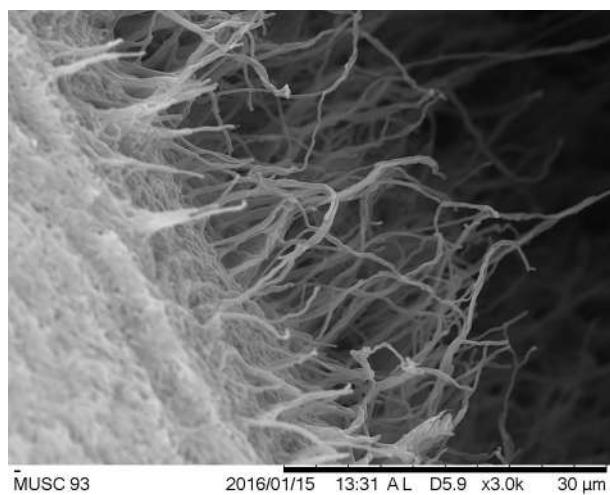


FIGURE 2 | Scanning electron microscope of *Streptomyces colonosanans* MUSC 93^T.

Percentage of SOD activity (%)

$$= \frac{((\text{Absorbance of blank 1} - \text{Absorbance of blank 3}) - (\text{Absorbance of sample} - \text{Absorbance of blank 2}))}{(\text{Absorbance of blank 1} - \text{Absorbance of blank 3})} \times 100\%$$

Extract Preparation of MUSC 93^T

Before fermentation process, strain MUSC 93^T was grown in TSB (Biomerge, Malaysia) as seed medium for 14 days. The fermentation medium, Han's Fermentation Media 1 (HFM1) was autoclaved at 121°C for 15 min prior to experiment (Hong et al., 2009; Lee et al., 2012). The fermentation was conducted in 500 mL Erlenmeyer flask containing 200 mL HFM1 (Biomerge, Malaysia) with 200 µL seed media added into it and shaking at 200 rpm for 7–10 days at 28°C. The resulting Han's Fermentation medium was recovered by centrifugation at 12000 g for 15 min. The supernatant was filtered and collected, then subjected to freeze drying process. The freeze-dried sample was extracted with methanol for 72 h. The methanol-containing extract was filtered and collected, then subjected to re-extraction under same condition for twice at 24 h interval. The collected extract was concentrated with extracting solvent evaporated by rotary vacuum evaporator at 40°C. The final concentrate extract of MUSC 93^T was collected and suspended in dimethyl sulfoxide (DMSO) as vehicle reagent prior to bioactivity screening assays.

Determination of Antioxidant Activity of MUSC 93^T Extract Using Different Assays

The 2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) Assay

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was conducted according to the protocol described by Miser-Salihoglu et al. (2013) with some modifications. ABTS radical cation (ABTS[·]) was produced via reacting ABTS stock

solution (7 mM) and potassium persulfate (2.45 mM) for 24 h before the assay. The absorbance was measured at 743 nm and the change in radical amount was indicated by the reduction in absorbance value.

Superoxide Anion Scavenging/Superoxide Dismutase (SOD)

Superoxide anion scavenging /superoxide dismutase (SOD) activity was determined using SOD assay Kit—WST (Sigma-Aldrich), a commercially available colorimetric microtiter plate method, according to the protocol given by the manufacturer. SOD activity of the extract was determined colorimetrically at 450 nm as the reduction of the Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by superoxide anion, O₂^{·-}. Sample extract solution (20 µL) at different concentrations were added to the 96-well-plate, respectively. The reaction solutions were added as described in the protocol and then the plate was incubated at 37°C for 20 min prior to measurement of absorbance at 450 nm using a microplate reader. The percentage of SOD activity (percentage of WST-1 reduction) was calculated as follows (Tan et al., 2015):

Metal Chelating

Metal Chelating activity was examined by measuring the formation of Fe²⁺-ferrozine complex as described in previous study conducted by Manivasagan et al. (2013) with slight modification. FeSO₄ (2 mM) was added into the extract followed by the addition of ferrozine (5 mM) to initiate the reaction prior to measurement of absorbance at 562 nm using spectrophotometer.

Maintenance and Growth Condition of Human Cell Lines

The human normal colon CCD-18Co cells were maintained in DMEM media supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO₂ in air at 37°C (Ser et al., 2016c). All of the human derived cancer cell lines evaluated in this study were maintained in RPMI (Roswell Park Memorial Institute)-1640 (Gibco) supplemented with 10% fetal bovine serum and 1x antibiotic-antimycotic (Gibco) in a humidified incubator with 5% CO₂ in air at 37°C (Tan et al., 2015).

Investigation of Cytotoxicity Activity of MUSC 93^T Using 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium-Bromide (MTT) Assay

For evaluation of cytotoxicity, the human normal colon CCD-18Co cells were included in this study, while the human derived cancer cell lines included were colon cancer cell lines: HCT-116, HT-29, Caco-2, and SW480. The cytotoxic activity of MUSC 93^T extract was examined using MTT assay following the protocol

previously described by Williams (1989). The cell viability was determined spectrophotometrically at 570 nm (with 650 nm as reference wavelength) using a microplate reader. The percentage of cell viability was calculated as follows:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\%$$

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed according to previously developed method with slight modification (Supriady et al., 2015). The analysis was conducted using Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), with HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m × 250 μm × 0.25 μm and helium as carrier gas at 1 mL/min. The column temperature was programmed initially at 40°C for 10 min, followed by an increase of 3°C/min to 250°C and was kept isothermally for 5 min. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those from NIST 05 Spectral Library.

Genome Sequencing and Bioinformatics Analysis of MUSC 93J^T

Genomic DNA of MUSC 93J^T was extracted using MasterpureTM DNA purification kit (Epicentre, Illumina Inc., Madison, WI, USA) followed by RNase (Qiagen, USA) treatment (Ser et al., 2015c, 2016d). Subsequently, the DNA quality was examined using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit version 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). DNA library construction was performed using NexteraTM DNA Sample Preparation kit (Nextera, USA) and the library quality was validated by Bioanalyzer 2100 high sensitivity DNA kit (Agilent Technologies, Palo Alto, CA). Paired-end sequencing was carried out on MiSeq platform with MiSeq Reagent Kit 2 (2 × 250 bp; Illumina Inc., Madison, WI, USA). The paired-end reads were then trimmed and *de novo* assembled with CLC Genomics Workbench version 7 (CLC bio, Denmark). Gene prediction was carried out using Prodigal version 2.6, whereas rRNA and tRNA were predicted using RNAmmer and tRNAscan SE version 1.21 (Lowe and Eddy, 1997; Lagesen et al., 2007; Hyatt et al., 2010). The assembly was annotated using Rapid Annotation using Subsystem Technology (RAST) and by the NCBI Prokaryotic Genomes Annotation Pipeline (Angiuoli et al., 2008; Aziz et al., 2008). Results of genome sequencing and bioinformatics analysis were presented in the description of *Streptomyces colonosanans* sp. nov.

Statistical Analysis

Experiments involved the investigation of antioxidant and cytotoxic activities were done in quadruplicate. Data analysis was performed with SPSS statistical analysis software and the results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by

appropriate *post-hoc* test (Tukey) was performed to determine the significant differences between groups. A difference was considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Genomic and Phylogenetic Analyses of Strain MUSC 93J^T

The nearly complete 16S rRNA gene sequence was determined for strain MUSC 93J^T (1490 bp; GenBank/EMBL/DDBJ accession number KP998434) and manual alignment of the sequence was performed with the corresponding partial 16S rRNA gene sequences of the type strains of representative members of the genus *Streptomyces* retrieved from GenBank/EMBL/DDBJ databases. Phylogenetic trees were constructed based on the 16S rRNA gene sequences to determine the phylogenetic position of this strain (Figure 1 and Figure S1). Phylogenetic analysis exhibited that closely related strains include *Streptomyces misionensis* NBRC 13063^T (99.1% sequence similarity) and *Streptomyces phaeoluteichromatogenes* NRRL 5799^T (99.1% sequence similarity), as they formed a distinct clade (Figure 1). The analysis of 16S rRNA gene sequence for strain MUSC 93J^T exhibited highest sequence similarity to strain *Streptomyces malachitofuscus* NBRC 13059^T (99.2%), *Streptomyces misionensis* NBRC 13063^T (99.1%) and *Streptomyces phaeoluteichromatogenes* NRRL 5799^T (99.1%); sequences similarities of <99.0% were obtained with the type strains of other species of the genus *Streptomyces*.

The DNA-DNA relatedness values between strain MUSC 93J^T and *Streptomyces malachitofuscus* JCM 4493^T (14.4 ± 0.1%), *Streptomyces misionensis* NBRC 13063^T (46.2 ± 0.4%) and *Streptomyces phaeoluteichromatogenes* DSM 41898^T (20.7 ± 1.0%) were significantly below 70%, the threshold value for the delineation of bacterial species (Wayne et al., 1987).

The BOX-PCR results indicated that strain MUSC 93J^T exhibited a unique BOX-PCR fingerprint compared with closely related type strains: *Streptomyces malachitofuscus* JCM 4493^T, *Streptomyces misionensis* NBRC 13063^T, and *Streptomyces phaeoluteichromatogenes* DSM 41898^T (Refer to Figure S2). These results are in line with results of phylogenetic analysis and DNA-DNA hybridizations, which demonstrate that strain MUSC 93J^T represents a novel species in the genus *Streptomyces*.

Chemotaxonomic Analyses of Strain MUSC 93J^T

The fatty acids profiles of strain MUSC 93J^T and closely related type strains are presented in Table 1. The major cellular fatty acids in MUSC 93J^T were identified as anteiso-C_{15:0} (23.1%), C_{16:0} (18.6%), and iso-C_{16:0} (15.1%). The fatty acids profile of MUSC 93J^T is consistent with those of closely related phylogenetic neighbors such as *Streptomyces malachitofuscus* JCM 4493^T, *Streptomyces misionensis* NBRC 13063^T, and *Streptomyces phaeoluteichromatogenes* DSM 41898^T, which contain anteiso-C_{15:0} (12.6–40.1%) and iso-C_{16:0} (14.4–18.3%) as major fatty acids (Table 1). However, the fatty acid profile of MUSC 93J^T was quantitatively different from those

TABLE 1 | Cellular fatty acid composition of strain MUSC 93J^T and its closely related *Streptomyces* species.

Fatty acid	1	2	3	4
iso-C ₁₂ :0	0.1	0.1	–	–
C ₁₂ :0	0.1	–	–	–
iso-C ₁₃ :0	0.3	0.4	0.1	0.2
anteiso-C ₁₃ :0	0.3	0.2	0.3	–
iso-C ₁₄ :0	5.4	2.5	1.8	4.8
C ₁₄ :0	1.1	0.4	0.2	0.2
iso-C ₁₅ :0	9.6	17.5	7.2	12.3
anteiso-C ₁₅ :0	23.1	12.6	40.1	35.5
C ₁₅ :1 w6c	0.2	0.1	–	–
C ₁₅ :0	2.3	1.9	0.7	1.6
iso-C ₁₆ :1 H	0.6	2.0	1.6	1.3
iso-C ₁₆ :0	15.1	18.3	14.4	17.7
C ₁₆ :1 Cis 9	–	–	1.3	0.7
C ₁₆ :0	18.6	7.9	4.0	3.4
iso-C ₁₇ :1 w9c	1.3	7.9	–	–
anteiso-C ₁₇ :1 w9c	1.1	2.6	–	–
anteiso-C ₁₇ :1 C	–	–	4.1	2.8
iso-C ₁₇ :0	3.4	8.6	2.4	3.5
anteiso-C ₁₇ :0	7.6	8.8	19.3	13.4
C ₁₇ :1 w8c	0.7	1.2	–	–
C ₁₇ :1 Cis 9	–	–	0.1	0.2
C ₁₇ :0 CYCLO	0.9	0.2	0.4	0.3
C ₁₇ :0	1.6	1.2	0.2	0.2
C ₁₇ :0 10-Methyl	–	0.3	–	–
iso-C ₁₈ :1 H	–	0.3	–	–
iso-C ₁₈ :0	0.3	0.2	–	–
C ₁₈ :1 w9c	–	0.2	–	–
C ₁₈ :1 w7c	0.2	0.2	–	–
C ₁₈ :0	0.6	0.1	–	–

Strains: 1, *Streptomyces colonosanans* MUSC 93J^T; 2, *Streptomyces malachitofuscus* JCM 4493^T; 3, *Streptomyces misionensis* NBRC 13063^T; 4, *Streptomyces phaeoluteochromatogenes* DSM 41898^T. –, <0.1% or not detected. All data are obtained concurrently from this study.

of these type strains; for instance, the anteiso-C₁₅:0 (23.1%) was found to be predominant in strain MUSC 93J^T, but the amount of anteiso-C₁₅:0 was much lesser (12.6%) in *Streptomyces malachitofuscus* JCM 4493^T (Table 1).

Based on the results of the polar lipids analysis, strain MUSC 93J^T showed the presence of aminolipid, diphosphatidylglycerol, lipid, phospholipid, phosphatidylinositol, phosphatidylethanolamine, and phosphoglycolipid. The differences in polar lipid profiles showed that MUSC 93J^T is different from related type strains; for example, strain MUSC 93J^T contain two aminolipids (Figure S3A), while *Streptomyces malachitofuscus* JCM 4493^T only contain one aminolipids (Figure S3B).

The chemotaxonomic analyses also demonstrated that the cell wall of strain MUSC 93J^T is of cell-wall type I as it contains LL-diaminopimelic (Lechevalier and Lechevalier, 1970). Many other species of the genus *Streptomyces* were also found to

have LL-diaminopimelic (Lee et al., 2005, 2014d; Xu et al., 2009; Hu et al., 2012; Ser et al., 2015b,d). The predominant menaquinones of strain MUSC 93J^T were identified as MK-9(H₈) (42%) and MK-9(H₆) (35%). This finding is in agreement with the report of the study conducted by Kim et al. (2003), in which the predominant menaquinones of *Streptomyces* are MK-9(H₈) and MK-9(H₆). The cell wall peptidoglycan was determined to contain LL-diaminopimelic acid. The whole cell sugars were found to be glucose, mannose, and ribose. The G + C content of strain MUSC 93J^T was found to be 69.9 mol%; this is within the range of 67.0–78.0 mol% described for species of the genus *Streptomyces* (Kim et al., 2003).

Phenotypic Analyses of Strain MUSC 93J^T

Strain MUSC 93J^T exhibited good growth on ISP 2 agar, ISP 6 agar, and *Streptomyces* agar after 7 days at 28°C, moderate growth on starch casein agar, weak growth on nutrient agar, and no growth on actinomycetes isolation agar, ISP 3, ISP 4, ISP 5, and ISP 7 agar. The 15-day-old culture of strain MUSC 93J^T formed light yellow aerial and vivid yellow substrate mycelium on ISP 2 agar (Table 2). These morphological characteristics are consistent with grouping of the strain to the genus *Streptomyces* (Williams, 1989). The NaCl tolerance, temperature ranges, and pH for growth of strain MUSC 93J^T occurred at 0–4% (optimum 0–2%), 24–36°C (optimum 0–2%), and pH 6.0–7.0 (optimum pH 6.0), respectively. The cells of MUSC 93J^T were positive for catalase and haemolytic activities. Hydrolysis of soluble starch, casein and tributyrin (lipase) were positive; but negative for hydrolysis of chitin, carboxymethylcellulose, and xylan. Based on a range of phenotypic properties, strain MUSC 93J^T can be differentiated from closely related members of the genus *Streptomyces* (Table 2). In chemical sensitivity assays, cells are resistant to 1% sodium lactate, D-serine, rifamycin RV, minocycline, lincomycin, niaproof 4, tetrazolium violet, tetrazolium blue, nalidixic acid, potassium tellurite, aztreonam, sodium butyrate, and sodium bromate.

According to the outcomes of genomic, phylogenetic, chemotaxonomic and phenotypic analyses, strain MUSC 93J^T merits assignment to a novel species in the genus *Streptomyces*, for which the name *Streptomyces colonosanans* sp. nov. is proposed.

Antioxidant Activity of Strain MUSC 93J^T Extract

Oxygen free radicals, also known as reactive oxygen species (ROS) are products of a normal cellular metabolism process in an organism (Valko et al., 2006). Oxidative stress occurs when there is an overproduction free radicals and deficiency of antioxidants, resulting in the accumulation of free radicals (Valko et al., 2006; Reuter et al., 2010). This condition may cause damage to DNA, proteins, and lipids which has been associated with the development of age-related diseases such as cancer, arthritis, and neurodegenerative disorders in living organisms (Valko et al., 2006; Tan et al., 2015). For that reason, antioxidants are required as they may play an important role in preventing the deleterious effects of free radicals and thus they are regarded as potential bioactive agents against cancers in human (Kawanishi

TABLE 2 | Differentiation characteristics of strain MUSC 93J^T and type strains of phylogenetically closely related species of the genus *Streptomyces*.

Characteristic	1	2	3	4
MORPHOLOGY (ON ISP 2):				
Color of aerial mycelium	Light yellow	Pale greenish yellow	Yellowish white	Light yellow
Color of substrate mycelium	Vivid yellow	Vivid greenish yellow	Yellowish gray	Brilliant Greenish yellow
GROWTH AT				
28°C	+	(+)	(+)	+
36°C	(+)	+	+	+
pH 6	+	(+)	(+)	(+)
2% NaCl	+	(+)	+	(+)
Catalase	+	+	+	+
Hemolytic	+	-	-	-
HYDROLYSIS OF				
Casein (protease)	+	-	-	+
Tributyrin (lipase)	+	-	+	+
Starch (amylolytic)	+	+	+	+
Carboxymethylcellulose (cellulase)	-	+	+	+
Xylan (xylanase)	-	-	+	-
CARBON SOURCE UTILIZATION				
D-maltose	-	+	+	+
Sucrose	-	+	+	+
D-turanose	-	+	+	+
D-raffinose	+	-	+	-
α-D-lactose	-	+	+	+
β-methyl-D-glucoside	-	+	-	+
D-salicin	-	+	-	+
N-acetyl-D-glucosamine	-	+	+	+
D-fructose	-	+	+	+
L-rhamnose	-	+	-	+
D-sorbitol	-	+	+	+
myo-inositol	-	+	+	+
Pectin	-	+	+	+
Methyl pyruvate	-	+	+	+
D-lactic acid methyl ester	-	+	+	+
Citric acid	-	+	+	+
α-keto-glutaric acid	-	+	+	+
D-malic acid	-	+	+	+
L-malic acid	-	+	+	+
Bromo-succinic acid	-	+	+	+
Propionic acid	-	+	+	+
Acetic acid	-	+	+	+
Formic acid	-	+	+	+

Strains: 1, *Streptomyces colonosanans* MUSC 93J^T; 2, *Streptomyces malachitofuscus* JCM 4493^T; 3, *Streptomyces misionensis* NBRC 13063^T; 4, *Streptomyces phaeoletichromatogenes* DSM 41898^T. All data were obtained concurrently in this study. +, Positive; -, negative; (+), weak.

All strains are positive for utilization of Dextrin, D-trehalose, D-cellulose, Gentiobiose, α-D-glucose, D-mannose, D-galactose, glycerol, D-glucose-6-PO4, D-fructose-6-PO4, Gelatin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, Glucuronamide, p-hydroxy-phenylacetic acid, L-lactic acid, Tween 40, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-keto-butyric acid, and acetoacetic acid. All strains are negative for assimilation of 3-methyl glucose.

et al., 2005; Reuter et al., 2010; Ser et al., 2016b). For example, a trial conducted by Blot et al. (1993) in China suggested that combination of antioxidants of beta carotene, vitamin E, and selenium may decrease the risk of gastric cancer. Besides, several studies and meta-analysis of the epidemiological literature have shown that increased intake of lycopene, a potent antioxidant present in tomatoes, is associated with reduced risk of prostate cancer (Gann et al., 1999; Giovannucci et al., 2002; Etminan et al., 2004) and gastric cancer (Yang et al., 2013).

In the early 1980s, the scientific community started to focus on the exploration of microbial antioxidants. Since then, researchers have discovered and characterized a variety of antioxidant compounds from microorganisms in hope to be developed into novel therapeutic agents (Hall, 2001). Due to the pathophysiological complexity of the human diseases, the bioprospecting activities in search for more effective and specific antioxidants from natural resources is still required. In this context, *Streptomyces* bacteria emerges as one of the good sources of natural compounds since they are prolific producers of bioactive secondary metabolites. Furthermore, several studies have reported the detection of compounds with antioxidant property extracted from *Streptomyces* spp., for instance, thiazostatin A and thiazostatin B (Shindo et al., 1989), diphenazithionin (Hosoya et al., 1996), dihydroherbimycin A (Chang and Kim, 2007) as well as 5-(2,4-dimethylbenzyl)pyrrolidin-2-one (Saurav and Kannabiran, 2012).

The present study showed that MUSC 93J^T merits assignment to a novel species in the genus *Streptomyces* based on the polyphasic approach analyses. Since the strain MUSC 93J^T is a novel *Streptomyces* species, it would be interesting to investigate the antioxidant potential of this strain. Hence, the strain was further examined for its antioxidant potential using ABTS, metal chelating, and SOD activity assays. According to the results of antioxidant assays, it can be observed that MUSC 93J^T extract exhibited significant free radical scavenging activity (Table 3). In ABTS assay, ABTS radical cation was produced by the reaction between a strong oxidizing agent potassium persulfate with ABTS salt and the ability of antioxidant to scavenge the ABTS radical generated in the aqueous phase will be measured (Shalaby and Shanab, 2013). The results showed that MUSC 93J^T extract was capable of scavenging 11.80 ± 3.75% of ABTS radicals at the highest test concentration of 2 mg/mL. Besides, MUSC 93J^T extract exhibited metal chelating activity of 50.06 ± 1.95% at 2 mg/mL concentration. This indicated the antioxidative potential of MUSC 93J^T extract through prevention of transition metals from enhancing the production of ROS (Ser et al., 2016b). In addition, the SOD assay also confirmed the antioxidant potential of MUSC 93J^T extract. In SOD assay, the superoxide anion scavenging activity of this extract was determined by the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST) reduction method. The superoxide anion radical produced from hypoxanthine-xanthine oxidase reaction reduces WST-1 to produce yellow formazan (Dudonné et al., 2009; Tan et al., 2015). MUSC 93J^T extract exhibited superoxide dismutase (SOD)-like activity which may subsequently prevent the formation of yellow

TABLE 3 | Radical scavenging activity of MUSC 93J^T evaluated using ABTS, metal chelating, and SOD assays.

Antioxidants assays	Concentration of MUSC 93J ^T extract (mg/mL)	Mean ± standard error (%)
ABTS	0.25	5.65 ± 2.56
	0.50	1.35 ± 1.76
	1.00	4.60 ± 3.24
	2.00	11.80 ± 3.75
Metal chelating	0.25	7.86 ± 2.87
	0.50	18.10 ± 2.05
	1.00	33.02 ± 1.07
	2.00	50.06 ± 1.95
SOD	0.25	36.02 ± 3.89
	0.50	51.55 ± 3.54
	1.00	70.29 ± 2.76
	2.00	83.32 ± 2.62

WST-1 formazan. The SOD-like activity of this extract was 83.32 ± 2.62% at the highest tested concentration of 2 mg/mL. All of these assays revealed significant antioxidant potential of MUSC 93J^T extract and thus suggested the presence of antioxidant(s) in it.

Cytotoxic Activity of Strain MUSC 93J^T Extract

In present study, cytotoxic potential of MUSC 93J^T extract was examined on human colon cancer cell lines namely HCT-116, HT-29, Caco-2, and SW480 by using the MTT assay. MTT assay is a tetrazolium-based colorimetric assay which operates by measuring the mitochondrial activity in living cells only. The activity of mitochondrial dehydrogenase enzyme of viable cells will transform the MTT tetrazolium salt (yellow) to MTT formazan crystal (purple) (Gerlier and Thomasset, 1986; Ser et al., 2015a; Tan et al., 2015). The use of different type of human colon cancer cell lines with different molecular characteristics as the panels for this experimentation is to assess varying efficacy of cytotoxic activity toward different genetic makeup of cancer cell lines (Tan et al., 2015). The tested results of MUSC 93J^T extract against tested colon cancer cell lines were shown in **Figure 3**.

The results revealed that MUSC 93J^T extract showed varying levels of cytotoxicity against HCT-116, HT-29, Caco-2, and SW480. It was found that the extract exhibited highest cytotoxic effect on SW480 with cell viability recorded at 63.6 ± 3.0% when tested with highest concentration of 400 µg/mL, followed by HCT-116 with cell viability 84.3 ± 11.5% (at concentration 400 µg/mL), then Caco-2 with cell viability 87.5 ± 5.3% (at concentration 400 µg/mL), and lastly the lowest cytotoxic effect on HT-29 with cell viability 88.4 ± 4.4% (at concentration 400 µg/mL; **Figure 3**). Furthermore, it can be observed that there was a dose dependent effect when the extract was tested against SW480 cells. SW480 cells were significantly inhibited ($p < 0.05$)

by increased concentration of the extract. As for the human normal colon CCD-18Co cells, no significant cytotoxic effect was exerted by MUSC 93J^T extract against these cells (**Figure 3**). Overall, the results suggested that MUSC 93J^T extract is more cytotoxic toward the colon cancer cells lines than the normal colon cells with particularly stronger cytotoxic activity against colon cancer cell line SW480.

GC-MS Analysis of Strain MUSC 93J^T Extract

GC-MS analysis was conducted to aid in chemical profiling and to identify compounds that present in the extract. The results of GC-MS analysis revealed that strain MUSC 93J^T extract contains nine compounds (**Table 4**): 2(5H)-Furanone (1), 1-Nonanol (2), Phenol, 2,4-bis (1,1-dimethylethyl)- (3), Benzoic acid, 4-ethoxy-, ethyl ester (4), Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester (5), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (6), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (7), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (8), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (9) with chemical structures as shown in **Figure 4**. From this analysis, butenolides, fatty alcohol, phenolic, benzoic acid esters, hydrocarbon, pyrrolopyrazine, and dicarboxylic acid ester were the main classes of compounds present in strain MUSC 93J^T extract.

Pyrrolopyrazines are capable of exerting a variety of bioactivities such as antioxidant, antitumor, anti-angiogenesis, and antimicrobial (Ser et al., 2015a,b). According to the GC-MS analysis, three pyrrolopyrazine compounds were detected in the MUSC 93J^T extract, including Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (6), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (7), and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (8). In previous studies, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (6) was reported to be present in *Streptomyces mangrovisoli*, a novel *Streptomyces* species isolated from mangrove forest in Malaysia by Ser et al. (2015b) and it was suggested that this compound may be responsible for the antioxidant activity of this species. In addition, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (6) was also found in *Streptomyces pluripotens* and *Bacillus* sp. with the capability to reduce oxidative damages by free radicals (Gopi et al., 2014; Ser et al., 2015a). Moreover, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (7) was detected in *Streptomyces* sp. VITMK1 isolated from India mangrove soil by Manimaran et al. (2015). Mithun and Rao (2012) also reported the detection of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (7) in *Micrococcus luteus* with promising anticancer activity on HCT15. Also, all three pyrrolopyrazine compounds: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (6), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (7), and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (8) were detected in *Streptomyces* sp. MUM 256 isolated from mangrove forest in Malaysia by Tan et al. (2015) which exhibited antioxidant and anticancer activities that could be due to the presence of these pyrrolopyrazines.

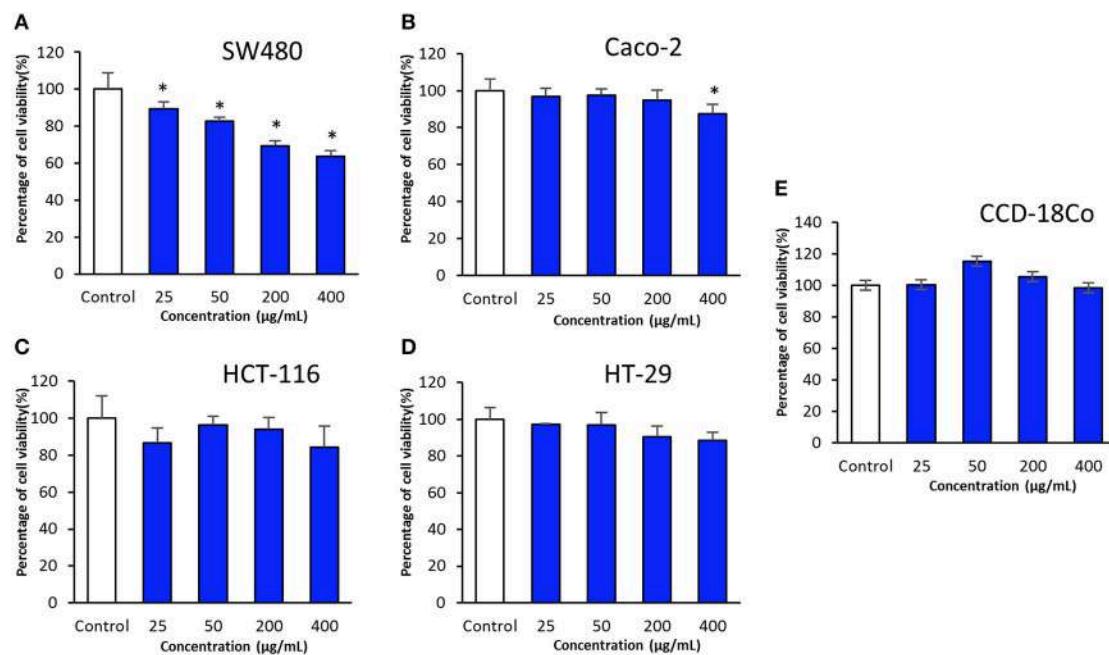


FIGURE 3 | Cytotoxic activity of MUSC 93J^T extract against human colon cancer and normal cell lines. The measurement of cell viability was done using MTT assay. The graphs show cytotoxicity effect of MUSC 93J^T extract against (A) SW480, (B) Caco-2, (C) HCT-116, (D) HT-29, and (E) CCD-18Co. All data are expressed as mean \pm standard deviation and significance level are set as 0.05. Symbol (*) indicates $p < 0.05$ significant difference between the cells treated with MUSC 93J^T extract and control (without MUSC 93J^T extract).

TABLE 4 | Compounds identified from MUSC 93J^T extract using GC-MS.

No.	Retention time (min)	Compound	Class	Molecular formula	Molecular weight (MW)	Quality (%)
1	13.787	2(5H)-Furanone	Butenolides	C ₄ H ₄ O ₂	84	83
2	27.325	1-Nonanol	Fatty alcohol	C ₉ H ₂₀ O	144	83
3	44.457	Phenol, 2,4-bis(1,1-dimethylethyl)-	Phenolic compound	C ₁₄ H ₂₂ O	206	93
4	44.892	Benzoic acid, 4-ethoxy-, ethyl ester	Benzoic acid esters	C ₁₁ H ₁₄ O ₃	194	91
5	47.758	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	Hydrocarbon	C ₁₆ H ₃₀ O ₄	286	78
6	53.165	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	Pyrrolopyrazine	C ₇ H ₁₀ N ₂ O ₂	154	96
7	59.076	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Pyrrolopyrazine	C ₁₁ H ₁₈ N ₂ O ₂	210	83
8	72.031	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Pyrrolopyrazine	C ₁₄ H ₁₆ N ₂ O ₂	244	80
9	76.883	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Dicarboxylic acid ester	C ₁₆ H ₂₂ O ₄	278	87

Hence, the antioxidant and cytotoxic activities exhibited by MUSC 93J^T extract could be mainly due to the presence of these pyrrolopyrazine compounds.

Besides, phenolic compounds have been regarded as important antioxidant agents responsible in scavenging ROS (Narendhran et al., 2014). Phenol, 2,4-bis(1,1-dimethylethyl)- (3) was the phenolic compound detected in strain MUSC 93J^T extract. Some of the recent studies have demonstrated the presence of phenol, 2,4-bis(1,1-dimethylethyl)- in *Streptomyces* bacteria through GC-MS analysis. For instance, Narendhran et al. (2014) successfully detected phenol, 2,4-bis(1,1-dimethylethyl)- in *Streptomyces cavouresis* KUV39 isolated from vermicompost samples collected in India. The study also presented that this

compound could be responsible for the antioxidant and cytotoxic properties of *Streptomyces cavouresis* KUV39. Besides, phenol, 2,4-bis(1,1-dimethylethyl)- was also detected in *Streptomyces* sp. MUM256 with potential antioxidant activity in the study conducted by Tan et al. (2015). A recently discovered novel *Streptomyces antoxidans* by Ser et al. (2016c) reported the detection of phenol, 2,4-bis(1,1-dimethylethyl)- was detected in the extract, which may have contributed to the strain's free radical scavenging activities.

The compound 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (9) detected in MUSC 93J^T extract has been previously reported to possess potential antibacterial, antifungal, and cytotoxic activities (Saxena et al., 2015; Tan et al., 2015).

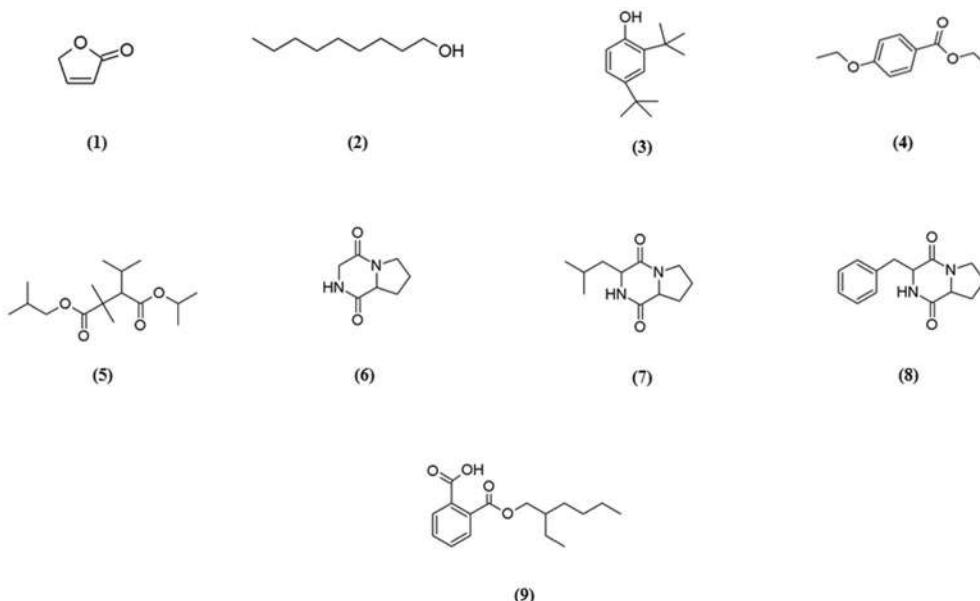


FIGURE 4 | Chemical structures of constituents detected in MUSC 93J^T extract.

This compound was also detected in other *Streptomyces* sp., with cytotoxic activity against several cancer cell lines such as liver cancer cell line HepG2 and breast cancer cell line MCF7 (Krishnan et al., 2014).

Lastly, other detected compounds in MUSC 93J^T including 2(5H)-Furanone (1), 1-Nonanol (2), Benzoic acid, 4-ethoxy-, ethyl ester (4), and Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester (5) were also previously found to be present in microbes. For instance, 2(5H)-Furanone was detected in *Lactobacillus helveticus* (Ndagijimana et al., 2006), 1-Nonanol was detected in *Streptomyces albidoeflavus* (Sunesson et al., 1997), Benzoic acid, 4-ethoxy-, ethyl ester was detected in *Bacillus* sp. (Mishra and Thakur, 2016), and Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester was detected in *Aspergillus carbonarius* ITEM 5010 (Sinha et al., 2015).

Overall, most of the chemical compounds identified by GC-MS are well-known for their antioxidant and anticancer activities. Therefore, we propose that these compounds, either alone or in combination, might be the main contributing factors for the antioxidant and cytotoxic properties present in MUSC 93J^T extract.

Description of *Streptomyces colonosanans* sp. nov.

Streptomyces colonosanans (co.lo.no.sa'nans. Gr. n. kolon, intestine, colon; L. part. adj. sanans, healing; N.L. part. adj. colonosanans, colon-healing).

Cells stain Gram-positive, forming light yellow aerial and vivid yellow substrate mycelium on ISP 2 agar. The colors of the aerial and substrate mycelium are media-dependent (Table S1). Cells grow well on ISP 2 agar, ISP 6 agar, and *Streptomyces*

agar after 7 days at 28°C, cells grow moderately on starch casein agar, and cells grow weakly on nutrient agar and did not grow on actinomycetes isolation agar, ISP 3, ISP 4, ISP 5, and ISP 7 agar. Cells grow at 0–4% NaCl tolerance (optimum 0–2%), 24–36°C (optimum 28–32°C), at pH 6.0–7.0 (optimum pH 6.0). Cells are positive for catalase and hemolytic activities. Hydrolysis of soluble starch, casein and tributyrin (lipase) are positive; but negative for hydrolysis of chitin carboxymethylcellulose and xylan.

The following compounds are utilized as sole carbon sources: α-D-glucose, α-hydroxy-butyric acid, β-hydroxyl-D,L-butyric acid, D-cellobiose, dextrin, D-fructose-6-phosphate, L-fucose, D-galactose, D-galacturonic acid, D-glucose-6-phosphate, D-gluconic acid, D-glucuronic acid, D-mannose, D-melibiose, D-raffinose, D-trehalose, gelatin, gentiobiose, glucuronamide, glycerol, L-galactonic acid lactone, L-lactic acid, p-hydroxyphenylacetic acid, Tween 40, γ-amino-butyric acid, acetoacetic acid, and α-keto-butyric acid. The following compounds are not utilized as sole carbon sources: acetic acid, α-D-lactose, α-keto-glutaric acid, β-methyl-D-glucoside, bromo-succinic acid, citric acid, D-arabitol, D-aspartic acid, D-fructose, D-fucose, D-lactic acid methyl ester, D-malic acid, D-maltose, D-mannitol, D-saccharic acid, D-salicin, D-serine, D-sorbitol, D-turanose, formic acid, glycyl-L-proline, inosine, L-malic acid, L-rhamnose, methyl pyruvate, mucic acid, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-neuraminic acid, pectin, propionic acid, quinic acid, stachyose, sucrose, myo-inositol, and 3-methyl glucose. The following compounds are not utilized as sole nitrogen sources: L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, and L-serine.

TABLE 5 | General features of *Streptomyces colonosanans* MUSC 93J^T genome.

Streptomyces colonosanans MUSC 93J ^T	
Genome size (bp)	7,015,076
Contigs	166
Contigs N ₅₀ (bp)	99,963
G + C content %	69.90
Protein coding genes	5,859
tRNA	66
rRNA	3 (5S), 1 (16S), 1 (23S)

The cell wall peptidoglycan contains LL-diaminopimelic acid. The predominant menaquinones were identified as MK-9(H₈) and MK-9(H₆). The whole cell sugars are glucose, mannose and ribose. The polar lipids consist of aminolipid, diphosphatidylglycerol, lipid, phospholipid, phosphatidylinositol, phosphatidylethanolamine, and phosphoglycolipid. The predominant cellular fatty acids (>10.0%) are anteiso-C_{15:0}, C_{16:0}, and iso-C_{16:0}.

The type strain is MUSC 93J^T (=DSM 102042^T =MCCC 1K02298^T), isolated from mangrove soil collected from mangrove forest located in Kuching, state of Sarawak, Malaysia. The 16S rRNA gene sequence of strain MUSC 93J^T has been deposited in GenBank/EMBL/DDBJ under the accession number KP998434. The genome of MUSC 93J^T consists of 7,015,076 bp with average coverage of 53.0-fold and the G + C content of the genomic DNA of the type strain is 69.90 mol%. The whole project of MUSC 93J^T has been deposited at DDBJ/EMBL/GenBank under accession number MLYP00000000. A total of 5,859 coding genes was predicted and assigned to 402 subsystems, along with 66 tRNA and 5 RNA genes (Table 5). Based on RAST annotation, most of the genes were involved in amino acids and derivatives metabolism (9.18%), followed by carbohydrates metabolism (6.21%) and protein metabolism subsystems (4.91%).

CONCLUSION

This study has revealed that strain MUSC 93J^T is a novel species of the genus *Streptomyces*, isolated from the soil of mangrove forest located in Kuching, Sarawak. The name *Streptomyces colonosanans* sp. nov. is proposed and the type

strain is MUSC 93J^T (=DSM 102042^T =MCCC 1K02298^T). This study revealed that the extract of strain MUSC 93J^T has significant antioxidant potential with radical scavenging activity up to 83.32 ± 2.62% via SOD assay. Additionally, this strain exhibited cytotoxic activity against several human colon cancer cell lines, with highest cytotoxic effect on SW480 with cell viability of 63.6 ± 3.0%. Chemical analysis via GC-MS further confirms that the strain is capable of producing chemo-preventive related metabolites. Hence, this study demonstrated the biopharmaceutical potential of novel strain *Streptomyces colonosanans* MUSC 93J^T with capability to produce bioactive compounds responsible for the antioxidant and cytotoxic activities. Strain MUSC 93J^T serves as a potentially high quality resource for drug discovery and further studies pertaining the development of chemo-preventive drugs from this strain are highly valuable.

AUTHOR CONTRIBUTIONS

The experiments, data analysis, and manuscript writing were performed by JL and HS, while AD, SS, SIB, TK, NM, K-GC, BG, and LL provided vital guidance, insight and technical support for the completion of the project. L-HL and BG founded the research project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00877/full#supplementary-material>

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Marine Rare Actinobacteria: Isolation, Characterization, and Strategies for Harnessing Bioactive Compounds

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Actinobacteria are prolific producers of thousands of biologically active natural compounds with diverse activities. More than half of these bioactive compounds have been isolated from members belonging to actinobacteria. Recently, rare actinobacteria existing at different environmental settings such as high altitudes, volcanic areas, and marine environment have attracted attention. It has been speculated that physiological or biochemical pressures under such harsh environmental conditions can lead to the production of diversified natural compounds. Hence, marine environment has been focused for the discovery of novel natural products with biological potency. Many novel and promising bioactive compounds with versatile medicinal, industrial, or agricultural uses have been isolated and characterized. The natural compounds cannot be directly used as drug or other purposes, so they are structurally modified and diversified to ameliorate their biological or chemical properties. Versatile synthetic biological tools, metabolic engineering techniques, and chemical synthesis platform can be used to assist such structural modification. This review summarizes the latest studies on marine rare actinobacteria and their natural products with focus on recent approaches for structural and functional diversification of such microbial chemicals for attaining better applications.

Keywords: marine rare actinobacteria, bacterial characterization, bioactive compounds, metagenomics, host engineering

INTRODUCTION

Actinobacteria are Gram-positive bacteria with high GC contents in DNA. They have characteristics presence of intracellular proteasomes, and spores if present are exospores (Cavalier-Smith, 2002). The order *Actinomycetales* under phylum *Actinobacteria* includes major producer strains of diverse bioactive compounds. *Actinomycetales* includes 11 suborders viz. *Actinomycineae*, *Actinopolysporineae*, *Catenulisporineae*, *Corynebacterineae*, *Glycomycineae*, *Jiangellineae*, *Micromonosporineae*, *Propionibacterineae*, *Pseudonocardineae*, *Streptomyxineae*, and *Streptosporangineae* (<http://www.bacterio.net/-classifphyla.html>). The genus *Streptomyces* under sub-order *Streptomyxineae* have been characterized as most important producer of bioactive microbial metabolites (Berdy, 2005). Recently, previously underexplored genera are reported as important resources of diverse bioactive metabolites (Tiwari and Gupta, 2013). These so called rare-actinobacteria are commonly categorized as strains other than *Streptomyces* (Berdy, 2005) or actinobacteria strains with less frequency of isolation under normal parameters (Lazzarini et al., 2001; Baltz, 2006).

The un-explored and under-explored habitats including marine ecosystems are believed to be rich sources of such rare actinobacteria, with tremendous potential to produce interestingly new compounds (Hong et al., 2009). These marine actinobacteria with potential of producing bioactive compounds have attracted major attention to search for unique compounds with pharmaceutical and biotechnological applications (Bull and Stach, 2007; Subramani and Aalbersberg, 2013; Azman et al., 2015). Recently, there are reports on the discovery of rare actinobacteria from wide range of terrestrial and aquatic locations, including deep seas (Goodfellow et al., 2012). Reports on the analysis of geographical origins of the marine rare actinobacteria, with special focus on the isolation of specific compounds, and precise bioactivities are predominant indications of increasing global interest on the natural compounds from marine rare actinobacteria (Blunt et al., 2007).

ISOLATION AND CHARACTERIZATION OF MARINE RARE ACTINOBACTERIA

Generally, for uncovering the marine rare actinobacteria, isolation efforts have been focused on rare locations as deep-sea sediments to obtain new marine diversities (Fenical and Jensen, 2006). The specialized sampling techniques using sophisticated equipment (Fenical and Jensen, 2006), remotely operated vehicles (Pathom-Aree et al., 2006) and even human (Bredholdt et al., 2007), have provided easy access to unprecedented microbial diversity. However, marine rare actinobacteria are usually difficult to culture compared to their terrestrial counterparts mostly due to their special growth requirements (Zotchev, 2012) or unknown culture conditions. It has been observed that hardly <2% of bacterial cells can form colonies by conventional plate cultivation. A large number of them belong to “viable but not culturable” (VBNC) strains (Bernard et al., 2000). Recently, strategies such as mimicking the natural environment in terms of pH, oxygen gradient, nutrient compositions, etc is employed. With these improvements, some previously VBNC species can now be grown with more efficiency (Kaeberlein et al., 2002; Zengler et al., 2002; Vartoukian et al., 2010; Stewart, 2012).

Moreover, the laborious microscopic techniques are being replaced with techniques utilizing recent advances in genomics, proteomics, and bioinformatics for identification and characterization of microbial diversity in robust manner (Rastogi and Sani, 2011). The genomic analysis by genetic fingerprinting (Nübel et al., 1999), DNA-DNA hybridization techniques (Pinhassi et al., 1997), and the construction of metagenomic library and sequencing (Kisand et al., 2012) have been employed for identifying and characterizing the diversity within marine samples. The development of next generation sequencing (NGS) (Webster et al., 2010) and nanopore sequencing (Deamer et al., 2016) has made the process robust and less time consuming. The analysis of RNA expression and regulation using metatranscriptomics (Ogura et al., 2011) or determination of protein profile by metaproteomics (Slattery et al., 2012) can be directly linked to available genome in the

database. The coupled metagenomics and metatranscriptomic analysis was successfully used for determining the microbial communities in deep sea water of the North Pacific Ocean (Wu J. et al., 2013). Thus, the combination of both culture dependent (grow and isolate) and culture independent (analysis of nucleic acids and proteins) approaches have revolutionized the characterization and isolation of diverse marine organisms including rare actinobacteria (Hirayama et al., 2007; Zeng et al., 2012).

DISCOVERY OF BIOACTIVE COMPOUNDS FROM MARINE RARE ACTINOBACTERIA

Actinobacteria including *Streptomyces* contribute for approximately half of the characterized bioactive compounds up to date (Berdy, 2005). However, the chances of discovery of novel bioactive molecules from *Streptomyces* has significantly declined (Fenical et al., 1999), presumably due to easy chances of genetic exchange between species during evolution (Freel et al., 2011). Therefore, special attention is given to isolation, screening, and culturing of rare actinobacteria from rare environmental locations as marine sources. The list below summarizes some of the representative compounds isolated from diverse marine rare actinobacteria during last 10 years (Table 1A).

REINVIGORATING NATURAL PRODUCT DISCOVERY FROM MARINE RARE ACTINOBACTERIA

Though isolation and cultivation of marine rare actinobacteria is difficult, the development of novel and facile bacterial cultivation platforms such as hollow-fiber membrane chamber (HFMC) and iChip for *in situ* cultivation of previously uncultivable microbial species have expanded the scope of natural product discovery (Aoi et al., 2009; Nichols et al., 2010). By utilizing rationally designed iChip platform, Ling et al. (2015) has successfully isolated previously uncultivable soil bacteria *Eleftheria terrae* and characterized its bioactive molecule (Ling et al., 2015).

It is assumed that strain divergence (phylogenetic or ecological) can have great impact on metabolism and biosynthetic pathway and result in novel chemistry and bioactivities, so research is focused on previously unexplored strains (Monciardini et al., 2014). However, it is unrealistic to assume that every unexplored strain can provide bioactive compounds (Donadio et al., 2010). Hence, systematic approaches need to be employed for utilizing the true potential of natural products from marine rare actinobacteria. Some of the key foundations can be categorized as:

1. Identification of target strains/molecules,
 2. Systematic enrichment of production,
 3. Explicit modification for functional/structural diversity.
1. Identification of target strains/molecules
- The accessible diversity of useful microbial molecules have almost been exhausted by traditional approaches, hence

TABLE 1 | Overview of achievements in study of bioactive molecules derived from marine rare actinobacteria.**A. Examples of bioactive compounds isolated from various marine rare actinobacteria**

Compound name	Isolation source	Bacterial source	Biological activities	References
INDEPENDENT ISOLATES				
Pseudonocardians	Deep-sea sediment of South China Sea	<i>Pseudonocardia</i> sp. SC510 01299	Antibacterial and cytotoxic	Li et al., 2011
Caerulomycins	Marine sediments from the seashore of Weihai, China	<i>Actinoalloteichus cyanogriseus</i> WH1-2216-6	Cytotoxic, antibacterial	Fu et al., 2011
Marinacarbolines,	Marine sediment sample from South China Sea	<i>Marinactinospora thermotolerans</i> SC510 00652	Antimalarial	Huang et al., 2011
Salinosporamides (Commercial name <i>Marizomib</i>)	Deep sea-water of Bahamas Islands, Bahamas	<i>Salinispora tropica</i> (strain CNB-392)	Cytotoxic	Feling et al., 2003; Williams et al., 2005
Abyssomicins	Sediment sample from the Sea of Japan, Japan	<i>Verrucosispora</i> sp. AB-18-032	Antibacterial	Bister et al., 2004; Riedlinger et al., 2004
Marinomycins	Sediment sample offshore of La Jolla, USA	<i>Marinispora</i> strain CNQ-140	Cytotoxic	Kwon et al., 2006
Levantilides	Deep-sea sediment Eastern Mediterranean Sea	<i>Micromonospora</i> M71-A77	Cytotoxic	Gärtner et al., 2011
Salinoquinones	Deep sea-water of Bahamas Islands, Bahamas	<i>Salinispora arenicola</i> CNS-325.	Cytotoxic	Murphy et al., 2010
Neomaclafungin	Marine sediment from Usa bay, Kochi Prefecture, Japan.	<i>Actinoalloteichus</i> sp. NPS702	Antifungal	Sato et al., 2012
Marthiapeptide A	Deep-sea sediment of the South China Sea	<i>Marinactinospora thermotolerans</i> SC510 00652	Antibacterial, Cytotoxic	Zhou et al., 2012
Lucentamycins	Sediment sample from Bahamas island, Bahamas	<i>Nocardiopsis lucentensis</i> (strain CNR-712)	Cytotoxic	Cho et al., 2007
Juvenimycin C	Sediment collected off the coast of Palau, USA	<i>Micromonospora</i> sp (CNJ-878)	Cancer chemo preventive	Carlson et al., 2013
Levantilide C	Shallow coastal waters near the island of Chiloe, Chile.	<i>Micromonospora</i> strain FIM07-0019	Antiproliferative	Fei et al., 2013
Nocapyrone	Sediment sample, Ulleung Basin, Eastern sea, Korea	<i>Nocardiopsis</i> sp.	Reduced the pro-inflammatory factor	Kim et al., 2013
Nocardiamides	Sediment sample from La Jolla Canyon, San Diego, California, USA.	<i>Nocardiopsis</i> sp. CNX037	Low antibacterial activity	Wu Z. C. et al., 2013
Cyanogramides	Marine sediments from the seashore of Weihai, China	<i>Actinoalloteichus cyanogriseus</i> WH1-2216-6	Multidrug-resistance (MDR) reversing activity	Fu et al., 2014
Taromycin	Marine sediment sample from La Jolla Submarine Canyon, San Diego, California, USA.	<i>Saccharomonospora</i> sp. CNQ-490	Antibacterial	Yamanaka et al., 2014
Lodopyridone	Marine sediment sample from La Jolla Submarine Canyon, San Diego, California, USA.	<i>Saccharomonospora</i> CNQ490	Modest cytotoxic activity	Maloney et al., 2009
Lynamicins	Marine sediment off the coast of San Diego, California, USA	<i>Marinispora</i> NPS12745	Antibacterial	McArthur et al., 2008
Saccharothrixones	Sediment sample from Heishijiao Bay, Dalian, China	<i>Saccharothrix</i> sp. 10-10	Cytotoxic	Gan et al., 2015
Saliniketals	Sediment sample from Island of Guam, USA	<i>Salinispora arenicola</i> CNR-005	Prevention of carcinogenesis	Williams et al., 2007a
Arenicolides	Sediment sample from Island of Guam, USA	<i>Salinispora arenicola</i> CNR-005	Moderate cytotoxicity	Williams et al., 2007b
Lagumycin B, Dehydrorabelomycin, Phenanthroviridone, WS-5995 A	Sediment sample from Cát Bà Peninsula, East Sea Vietnam	<i>Micromonospora</i> sp.	Cytotoxic	Mullowney et al., 2015
Dermacozines, Phenazine derivatives	Sediment sample from Mariana Trench	<i>Dermacoccus abyssi</i> sp. nov., strains MT1.1 and MT1.2	Cytotoxic and anti-oxidant	Abdel-Mageed et al., 2010

(Continued)

TABLE 1 | Continued

Compound Name	Isolation Source	Bacterial Source	Biological Activities	References
Fijiolides	Sediment sample from the Beqa Lagoon, Fiji	<i>Nocardiopsis</i> CNS-653	Inhibitor of TNF- α -induced NF κ B activation	Nam et al., 2010
Fluostatin	Sediment sample from South China Sea	<i>Micromonospora rosaria</i> SCSIO N160	Antimicrobial	Zhang et al., 2012
Retimycin	Deep sea-water of Bahamas Islands, Bahamas	<i>S. arenicola</i> strain CNT-005.	Cytotoxic	Duncan et al., 2015
Sioxanthin	Deep sea-water of Bahamas Islands, Bahamas	<i>Salinispora tropica</i> CNB-440	Siderophore	Richter et al., 2015
Lobosamides	Sediment sample from Point Lobos, Monterey Bay, California, USA.	<i>Micromonospora</i> sp. RL09-050-HVF-A	Antitryposomal	Schulze et al., 2015a
Salinipostins	Sediment sample from Keawekahaka Bay, Hawaii, USA	<i>Salinispora</i> sp. RL08-036-SPS-B	Antimalarial	Schulze et al., 2015b
Isomethoxyneihumicin	Sediment sample at Chichijima, Ogasawara, Japan	<i>Nocardiopsis alba</i> KM6-1	Cytotoxic	Fukuda et al., 2016
Nocarimidazoles	Sediment sample off the coast of southern California, USA	<i>Nocardiopsis</i> sp. CNQ115	Weak antibacterila	Leutou et al., 2015
Cyclomarine	Marine sediment from Palau, Republic of Palau	<i>S. arenicola</i> CNS-205	Anti-inflammatory	Schultz et al., 2008
Cyclomarazine				
ISOLATES IN SYMBIOTIC ASSOCIATION				
JBIR-65	Symbiont to unidentified marine sponge from Ishigaki Island, Okinawa Prefecture, Japan	<i>Actinomadura</i> sp. SpB081030SC-15	Anti-oxidant	Takagi et al., 2010
Nocapyrone	Symbiont to sponge <i>Halichondria panacea</i> from Baltic Sea, Germany	<i>Nocardiopsis</i> sp. HB383	Weak cytotoxic	Schneemann et al., 2010
Arenjimycin	Symbiont to ascidian <i>Ecteinascidia Turbinata</i> from Sweetings Cay, Grand Bahama Island, USA	<i>Salinispora arenicola</i>	Antimicrobial and ytotoxic	Asolkar et al., 2010
Bendigoles	Symbiont to sponge <i>Suberites japonicas</i> from unspecified source	<i>Actinomadura</i> sp. SBMs009	Antimicrobial and cytotoxic	Simmons et al., 2011
Thiocoraline	Symbiont to sponge <i>Chondrilla caribensis</i> from Florida Keys, USA	<i>Verrucosispora</i> sp.	Cytotoxic	Wyche et al., 2011
Peptidolipins	Symbiont to ascidian <i>Trididemnum orbiculatum</i> from Florida Keys, USA	<i>Nocardia</i> sp.	Antibacterial	Wyche et al., 2012
Anthracyclinones	Symbiont to tunicate <i>Eudistoma vannamei</i> from Taiba Beach, Ceará, Brazil	<i>Micromonospora</i> sp.	Cytotoxic	Sousa et al., 2012
Halomadurone	Symbiont to ascidian <i>Ecteinascidia turbinata</i> , from Florida Keys, USA	<i>Actinomadura</i> sp.	Active against neurodegenerative diseases	Wyche et al., 2013
Solwaric acids	Symbiont to ascidian, <i>Trididemnum orbiculatum</i> from Florida Keys, USA	<i>Solwaraspora</i> sp.	Antibacterial	Ellis et al., 2014
Forazoline A	Symbiont to ascidian, <i>Ecteinascidia turbinata</i> from Florida Keys	<i>Actinomadura</i> sp. WMMB-499	Antifungal	Wyche et al., 2014
Rifamycins	Symbiont to sponge, <i>Pseudoceratina clavata</i> . From Great Barrier Reef, Australia	<i>Salinispora</i> sp. strain M403	Antibacterial	Kim et al., 2006
Saccharothrixmicines	Symbiont to marine mollusk <i>Anadara broughtoni</i> from Sea of Japan	<i>Saccharothrix espanaeensis</i> An 113	Antibacterial, Antifungal	Kalinovskaya et al., 2010

B. Approaches used for production and structural/functional diversification of bioactive compounds derived from marine rare actinobacteria

Compound name	Genus	Particulars	Biological activity	References
Retimycin	<i>Salinospora</i>	MS/MS spectrum pattern based genome mining	Cytotoxic, Antibacterial	Duncan et al., 2015
Thiolactomycin	<i>Salinospora</i>	Antibiotic resistance gene based genome mining, heterologous expression	Bacterial fatty acid synthase inhibitor	Tang et al., 2015

(Continued)

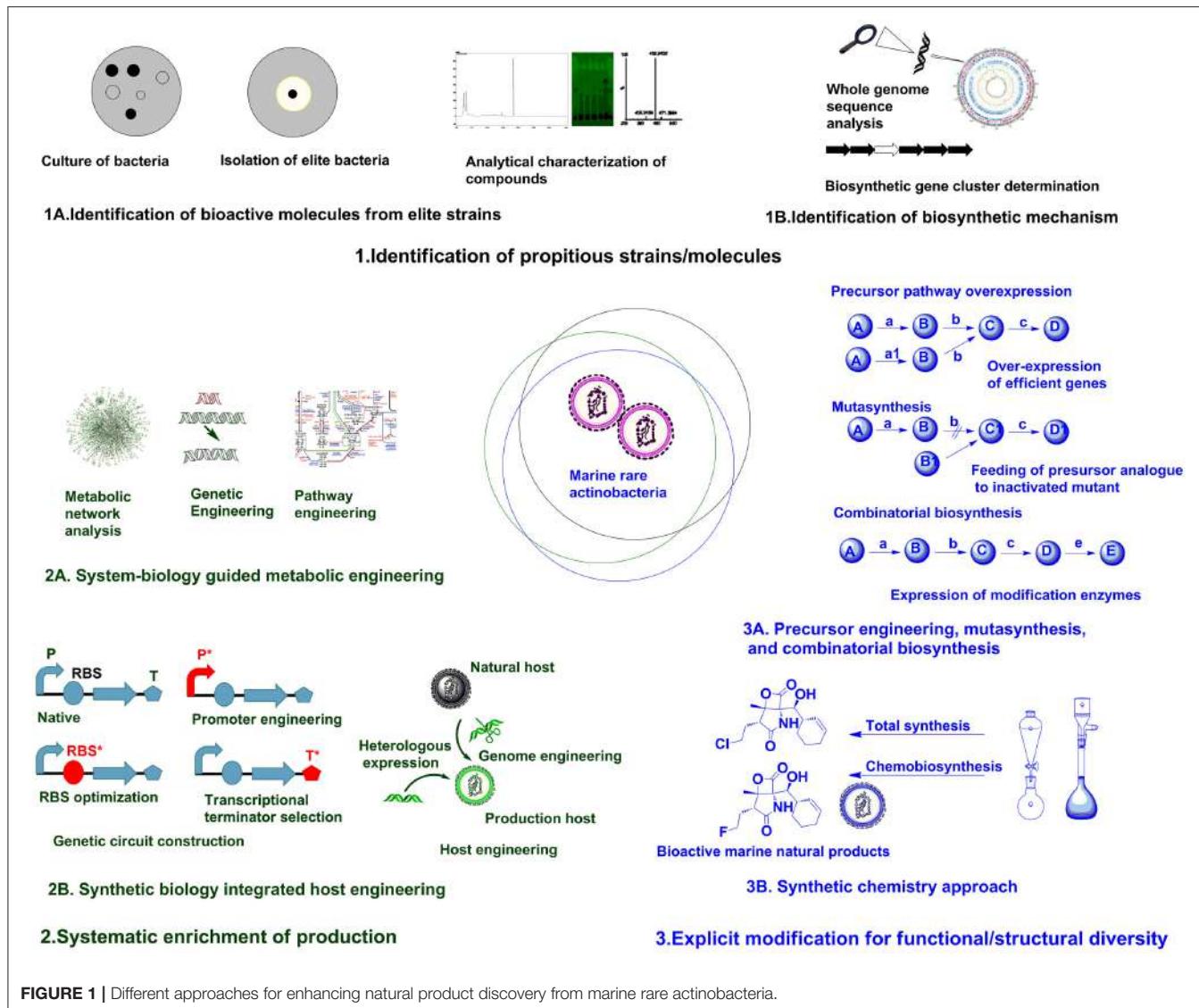
TABLE 1 | Continued

Compound name	Genus	Particulars	Biological activity	References
Lomaiviticin	<i>Salinospora</i>	Bioactivity guided genome mining	Cytotoxic	Kersten et al., 2013
Salinosporamide K	<i>Salinospora</i>	Genome mining, metabolomics and transcriptomics	Cytotoxic	Eustáquio et al., 2011
Taromycin	<i>Saccharomonospora</i>	BCG Genome mining, heterologous expression	Antibacterial	Yamanaka et al., 2014
Enterocin	<i>Salinospora</i>	BCG Genome mining, heterologous expression	Antibacterial	Bonet et al., 2014
Fluostatins	<i>Micromonospora</i>	Heterologous expression	Antibacterial	Yang et al., 2015
Thiocoraline	<i>Micromonospora</i>	Heterologous expression	Cytotoxic	Lombó et al., 2006
Bromosalinosporamide	<i>Salinospora</i>	Precursor directed biosynthesis	Cytotoxic	Lam et al., 2007
Salinosporamide A	<i>Salinospora</i>	Precursor pathway modulation	Cytotoxic	Lechner et al., 2011
Salinosporamide X1, Salinosporamide X2	<i>Salinospora</i>	Combinatorial biosynthesis	Cytotoxic	McGlinchey et al., 2008
Salinosporamide X3	<i>Salinospora</i>	Mutasynthesis	Cytotoxic	Nett et al., 2009
Salinosporamide X4				
Salinosporamide X5				
Salinosporamide X6				
Salinosporamide X7				
Fluorosalinosporamide	<i>Salinospora</i>	Mutasynthesis	Cytotoxic	Eustáquio and Moore, 2008
Salinosporamides analogs	<i>Salinospora</i>	Chemobiosynthesis	Cytotoxic	Liu et al., 2009
Salinosporamide A	<i>Salinospora</i>	Total chemical synthesis	Cytotoxic	Reddy et al., 2004; Endo and Danishefsky, 2005; Kaiya et al., 2011; Logan et al., 2014
Homosalinosporamide	<i>Salinospora</i>	Total chemical synthesis	Cytotoxic	Nguyen et al., 2010
Salinosporamides analogs	<i>Salinospora</i>	Chemobiosynthesis	Cytotoxic	Liu et al., 2009
Salinosporamide E	<i>Salinospora</i>	Semi-synthesis	Cytotoxic	Macherla et al., 2005
Bromosalinosporamide				
Iodosalinosporamide, Azidosalinosporamide, Hydroxysalinosporamide				
Methylsalinosporamide	<i>Salinospora</i>	Semi-synthesis	Cytotoxic	Manam et al., 2008
Tosylsalinosporamide				
Dansylsalinosporamide				
Hydroxysalinosporamide				
Flurosalinosporamide				

it is speculated that unstudied marine rare actinobacteria can provide reservoir of new microbial molecules (Schorn et al., 2016). Recently, direct connection of genomic information to biomolecule can be attained in culture independent approach as introducing environment (eDNA) into a suitable expression host (metagenomic libraries) (Handelsman, 2004). But, compound rediscovery due to similar strain replications is a major limitation of this approach. To maximize the capacity to mine metagenomes for attaining biomolecules with novel activities, there is requisite for parallel developments in techniques for bioactivity screening, isolation and separation methods, and analytical chemistry (Trindade et al., 2015). Robust techniques for analytical characterization of compounds (**Figure 1A**) based on UV absorbance, high pressure liquid chromatography (HPLC), mass spectrometry, and nuclear magnetic resonance (NMR) analysis can be used to scrutinize the discovery of

new compounds (Liu et al., 2012). The techniques utilizing coupling of biochemical analytical methods with genome information such as, in glycogenomics (Kersten et al., 2013), peptidogenomics (Medema et al., 2014), and metabolomics (Maansson et al., 2016) are recent advances facilitating easy access to diverse biomolecules. The results of such analytical analysis can be subsequently compared against databases repositories, such as MarinLit, ChemSpider, Pubchem, etc., to avoid already known compounds (Forner et al., 2013). Hence, robust analytical facilities and comparison with reference databases can assist on characterization of diverse chemical structures.

The prime focus in drug discovery is identification of new bioactive chemical or discovery of previously unreported biological activity with known chemical structure. High throughput screening (HTS) can provide easy means for evaluating desired bioactivities against an array natural



products (Monciardini et al., 2014). The robust screening strategies ranging from the classic whole cell assays to more sophisticated antisense based assay have been reviewed elsewhere (Silver and Bostian, 1990; Singh et al., 2011; Farha and Brown, 2016). Recently, the integrative approach of metabolite profiling, bioactivity studies and taxonomic studies have been utilized for characterizing different marine actinobacteria and biological properties of metabolites produced by them (Betancur et al., 2017). Such integrative approaches can be fascinating tool for directly assessing bioactivities at preliminary stages of study.

The next focus in drug discovery is understanding the biogenesis of bioactive molecule in producer strains. The rapid development of genome sequencing methods has revolutionized such studies by unveiling information about the whole genome architecture (Figure 1B). The challenge now is mining the data and connect the predicted

biosynthetic gene clusters (BGC) to bioactive molecules. A plethora of *in silico* tools are available for determining the nature of gene clusters (Weber and Kim, 2016). The classic genome mining approach (focusing on unique biosynthetic enzyme) has transitioned to the concept of comparative genome mining (complete BGC to next BGC comparison) and culture independent-metagenome mining (Ziemert et al., 2016). Due to its efficacy in studying BGCs, the genome mining concept has been expanded to different marine rare actinobacteria for getting insight on biosynthesis mechanisms of different secondary metabolites. The analysis of genome sequence of *Micromonospora* sp. RV43, *Rubrobacter* sp. RV113, and *Nocardiopsis* sp. RV163 isolated from Mediterranean sponges revealed presence of numerous gene clusters of different secondary metabolites (Horn et al., 2015). The 5.2 Mb genome of marine rare actinobacteria, *Salinispora tropica* CNB-440 (Udwary et al.,

2007) was interpreted using bioinformatics revealing at least 19 novel secondary metabolite BCGs. Later, diverse compounds have been characterized from *S. tropica*, including anticancer agent salinosporamide A, lymphocyte kinase inhibitor lymphostatin, DNA-cleaving agent calicheamicin, novel lysin-primed polyene macrolactam polyketide, and various siderophores (Kersten et al., 2013). Biosynthetic analysis of the draft genome of *Saccharomonospora* sp. CNQ490 has revealed 19 conspicuous BGC, indicating diverse secondary metabolic capacity (Yamanaka et al., 2014). Using precise bioinformatics tools, 75 genomes from closely related *Salinispora* species were compared and 124 distinct prominent BCGs were predicted which are far greater than known compound classes from these bacteria (Ziemert et al., 2014). Duncan et al. (2015) has simultaneously compared a large number of complex microbial extract in a large number of *Salinispora* species. This molecular networking was coupled with genome sequence data for comparative analysis of metabolite profile and BCG to develop pattern-based genome mining (PBMG) approach. Concurrently, a novel non-ribosomal peptide, retimycin A was isolated and characterized based on genome and metabolome analysis (Duncan et al., 2015). Therefore, genome mining approach has provided new avenues on discoursing novel natural products from marine rare actinobacteria.

2. Systematic enrichment of production

Generally, genome information is the starting point for pathway discovery. Various “omics” based tools have been employed for engineering pathways for secondary metabolite production in various actinobacteria (Chaudhary et al., 2013; Hwang et al., 2014). But the lack of full understanding of physiological transition stage for secondary metabolite production is a major consideration during manipulation of cellular processes using metabolic engineering (Licona-Cassani et al., 2015). Engineering primary metabolism for enhancing the pools of building blocks without compromising the growth is a major constraint in most metabolic engineering approaches (Olano et al., 2008). System biology protocols have been successfully used to study physiological parameters, leading to the discovery of the activation of NPs biosynthesis and manipulation of pathways (Licona-Cassani et al., 2015). Genome scale metabolic models are valuable for predicting organisms' phenotypes from genotypes basically by providing simulated mathematical prediction of cellular behavior under different genetic and physiological conditions (Henry et al., 2010; Ates et al., 2011). Community system biology approaches provide understanding about the complex relationship of individual members in a community and the modes of interactions they are engaged (Zengler and Palsson, 2012). The systematic application of systems biological approaches as metabolic network analysis coupled with pathway engineering or genetic engineering (**Figure 2A**) from a single strain to the larger community level can provide breakthrough in rational metabolic engineering approaches.

Synthetic biology is particularly focused on precise design and construction of new biological systems (metabolic pathways or genetic circuits) that are not prevalent in nature (Andrianantoandro et al., 2006). Previously, efforts in

synthetic biology have been largely focused on creating and perfecting genetic devices. But the current focus is directed to customizable larger scale system engineering by assembling devices or modular organizations (Purnick and Weiss, 2009). Most often, biologically valuable natural products are produced in lower titer or are cryptic under normal laboratory conditions, whereas many rare actinobacteria are not amenable to genetic manipulation. Hence, in such cases transferring natural products biosynthesis into well-developed heterologous host is a logical approach for producing parent NPs or generating novel analogs through biosynthetic engineering (Wenzel and Müller, 2005). Direct cloning and refactoring of previously silent lipopeptide gene cluster of *Saccharomonospora* sp. CNQ490 have been achieved by heterologous expression in *Streptomyces coelicolor* to yield taromycin A by Transformation Assisted Recombination (TAR)-based genetic platform (Yamanaka et al., 2014). Besides, tuning of metabolic pathway by altering promoters (Siegl et al., 2013; Wang et al., 2013), terminators (Pulido and Jimenez, 1987), and RBS (Bai et al., 2015) and/or host manipulation by genome engineering (Siegl and Luzhetsky, 2012; Tong et al., 2015) are providing new avenues for systemic level metabolic engineering of actinobacteria. Promoter exchange (Horbal et al., 2012) and the use of exogenous principal sigma factor (σ HrdB) (Wang et al., 2014) have been utilized for increasing teicoplanin in an industrial strain of *Actinoplanes teichomyceticus*. Approach for constructing genetic circuit or holistic host engineering (**Figure 2B**) can be an effective approach for designing and synthesizing unnatural but effective molecules from marine rare actinobacteria.

3. Explicit modification for functional/structural diversity

Fundamentally, engineering or modulating the precursor pathways can lead to enhancement or diversification of natural products (Dhakal et al., 2016). Combinatorial biosynthesis exploits the shuffling of anabolic pathways by precursor directed biosynthesis, enzyme level modulations, and pathway level recombination, leading to novel natural products (Sun et al., 2015; Winn et al., 2016). The precursor-directed in-situ synthesis (PDSS) has been successfully employed for generating new congeners of saccharothriolides from *Saccharothrix* sp. A1506 (Lu et al., 2016). Such type of precursor modulations can be manifested chemically or biologically to generate structural diversity in compounds from marine rare actinobacteria. Mutasynthesis is another variant of modulation of anabolic pathway by generating mutant strain deficient in key aspects of biosynthetic pathway and substituting natural precursor with analog of precursor to produce new natural products (Kennedy, 2008). Mutasynthesis couples the power of chemical synthesis with molecular biology to create diverse derivatives of medicinally valuable natural products (Weissman, 2007). One such example is the production of fluorinated analog fluorosalinosporamide. It has better proteasome inhibition and cytotoxic activity than naturally produced salinosporamides isolated from various *Salinispora* species (Feling et al., 2003). The halogenase gene *sall* in *Salinispora tropica* has been inactivated and

5'-fluoro-5'-deoxyadenosine, a fluorinated analog of its natural precursor 5'-chloro-5'-deoxyadenosine, has been used to generate fluorosalinosporamide by chemistry mediated mutasynthesis (Eustáquio and Moore, 2008). In another approach, *sall* was replaced by fluorinase gene *fIA* from *Streptomyces catteleya*. The mutant strain *sall*⁻/*fIA*⁺ produced fluorosalinosporamide in the presence of inorganic fluoride (Eustáquio et al., 2010). Moreover, combinatorial biosynthetic approach by feeding L-3-cyclohex-2'-enylalanine (CHA) residue in SalX disruption mutant of *S. tropica* enabled the generation of other unnatural salinosporamide derivatives such as salinosporamide X1 and salinosporamide X2, with lower activity (McGlinchey et al., 2008). But in another approach utilizing mutasynthetic approach with fine-tuned feeding of readily available amino acid precursors to SalX disruption mutant of *S. tropica* led to generation of many salinosporamide derivatives. Among them salinosporamide X7 exhibited equal to slightly improved cytotoxic potential than the natural counterpart (Nett et al., 2009). Hence, such approaches of precursor engineering, mutasynthesis, and combinatorial biosynthesis (**Figure 3A, Table 1B**) can be rationally utilized to diversify structure and perform structure-activity relationship studies of versatile molecules from various marine rare actinobacteria.

The advent of combinatorial synthetic chemistry has created huge excitement in the pharmaceutical industry by generating libraries of millions of compounds which could be screened by HTS (Butler, 2004). The total synthesis of complex natural products offers greater potential for direct access to bioactive molecule from marine sources. However, large scale production of complex natural product remains elusive due to low yields and high cost (Yeung and Paterson, 2005). Recent achievement as total synthesis of natural products in absence of protecting groups can lead to development of superior molecules with greater flexibility (Young and Baran, 2009). The generation of microbial chemicals by total enzymatic synthesis has been used as alternative to total chemical synthesis (Cheng et al., 2007). There have been ample of examples illustrating improvement in physical and biological properties of natural products (including many marine natural products) by chemical modifications, semisynthesis, mutasynthesis, and chemobiosynthesis (Hamann, 2003; Kennedy, 2008) mediated by biological and chemical techniques. Bioinspired total synthesis of salinosporamides and structurally related derivatives have provided access to novel functionalities of tremendously effective molecule (Nguyen et al., 2010; Chen et al., 2012). Suitable integration of synthetic chemistry (**Figure 3B, Table 1B**) with biological production system can be utilized for generating structurally and functionally

diverse analogs/derivatives of target molecule. One of the successful example illustrating application of synthetic chemistry in marine natural products is rationalized for structural/functional diversification of salinosporamides (Baran et al., 2007; Potts and Lam, 2010). The synergy between genome sequencing, mass spectroscopy based analysis and bio-inspired synthesis have been utilized for studying biosynthetic mechanism and structural diversification of nocardioazine B from *Nocardiopsis* sp. CMB-M0232 (Alqahtani et al., 2015). Hence, it is no doubt that rational integration of biological processes and chemical techniques (Dhakal and Sohng, 2015, 2017) can provide new foundations for drug discoveries from marine rare actinobacteria.

FUTURE OUTLOOK

As evident from examples above, the innovative methods for procurement of bioactive molecules from potent strains, efficient production and/or modifications by biological and chemical methods can assist in harnessing the full potential of biomolecules derived from marine rare actinobacteria. Further, tuning of structural and functional properties based on structure activity relationship studies can lead to development of superior analogs. But the prime focus should be on application of cutting edge translational research, such as transferring the achievements of discovery or synthesis of such biomolecule to the industrial bench-tops and clinics. The successful collaboration between biologists/chemists in academics and/or pharmaceutical companies can open new avenues for development of highly effective drugs. Salinosporamide A (*Marizomib*) has been a significant representation of compound derived from marine rare actinobacteria leading to phase trials. It is no doubt that exploration of new candidate strains with sophisticated techniques will certainly unravel tremendous opportunities to identify novel natural products and improve their applicability by structural/functional diversifications.

AUTHOR CONTRIBUTIONS

DD, ARP, BS, and JS made substantial, direct, and intellectual contribution to the work, and approved it for publication with full consent.

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***Streptomyces antioxidantans* sp. nov., a Novel Mangrove Soil Actinobacterium with Antioxidative and Neuroprotective Potentials**

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A novel strain, *Streptomyces antioxidantans* MUSC 164^T was recovered from mangrove forest soil located at Tanjung Lumpur, Malaysia. The Gram-positive bacterium forms yellowish-white aerial and brilliant greenish yellow substrate mycelium on ISP 2 agar. A polyphasic approach was used to determine the taxonomy status of strain MUSC 164^T. The strain showed a spectrum of phylogenetic and chemotaxonomic properties consistent with those of the members of the genus *Streptomyces*. The cell wall peptidoglycan was determined to contain LL-diaminopimelic acid. The predominant menaquinones were identified as MK-9(H₆) and MK-9(H₈), while the identified polar lipids consisted of aminolipid, diphosphatidylglycerol, glycolipid, hydroxyphosphatidylethanolamine, phospholipid, phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol and lipid. The cell wall sugars consist of galactose, glucose and ribose. The predominant cellular fatty acids (>10.0%) were identified as iso-C_{15:0} (34.8%) and anteiso-C_{15:0}(14.0%). Phylogenetic analysis identified that closely related strains for MUSC 164^T as *Streptomyces javensis* NBRC 100777^T (99.6% sequence similarity), *Streptomyces yogyakartensis* NBRC 100779^T (99.6%) and *Streptomyces violaceusniger* NBRC 13459^T (99.6%). The DNA-DNA relatedness values between MUSC 164^T and closely related type strains ranged from 23.8 ± 0.3% to 53.1 ± 4.3%. BOX-PCR fingerprints comparison showed that MUSC 164^T exhibits a unique DNA profile, with DNA G + C content determined to be 71.6 mol%. Based on the polyphasic study of MUSC 164^T, it is concluded that this strain represents a novel species, for which the name *Streptomyces antioxidantans* sp. nov. is proposed. The type strain is MUSC 164^T (=DSM 101523^T = MCCC 1K01590^T). The extract of MUSC 164^T showed potent antioxidative and neuroprotective activities against hydrogen peroxide. The chemical analysis of the extract revealed that the strain produces pyrazines and phenolic-related compounds that could explain for the observed bioactivities.

Keywords: *Streptomyces antioxidantans*, actinobacteria, mangrove, neuroprotective, antioxidative

INTRODUCTION

Many therapeutic agents, such as antibiotics, anti-inflammatory and antioxidant compounds have been isolated from microorganisms (Bérdy, 2005; Williams, 2009). Discovery of these bacterial-derived bioactive compounds has a major impact on human health, helping people to live longer and reducing the mortality rate due to infectious and/or chronic diseases. In recent years, the accumulation of free radicals or oxidative stress has been identified as one of the major contribution to neuronal loss and occur early in all major neurodegenerative diseases (Lin and Beal, 2006; Fischer and Maier, 2015; Leszek et al., 2016). By reducing the presence of free radicals, increased intake of antioxidants is known to prevent and decrease the risk of these chronic diseases (Devasagayam et al., 2004; Bonda et al., 2010). Thus, continuous efforts have been directed toward searching for potent, natural antioxidants to prevent the deleterious effects of free radicals.

Over the years, exploring new taxa remains as one of the successful strategies which lead to discovery of therapeutic agents (Williams, 2009). As the most prolific producer of bioactive compounds, *Streptomyces* genus was initially proposed by Waksman and Henrici (1943) and metabolites isolated from these organisms have been shown to possess pharmaceutically relevant activities such as anti-inflammatory, antimicrobial, antioxidant activities (Bérdy, 2005; Wang et al., 2013; Kumar et al., 2014; Ser et al., 2015a, 2016a; Tan et al., 2016). Moreover, the metabolites derived from *Streptomyces* are described as potent protective agents in neuronal cells against oxidative stress induced damage. In fact, a recent study by Leiros et al. (2013) has identified seven bioactive compounds produced by *Streptomyces* sp. which protects against hydrogen peroxide (H_2O_2) challenge in primary cortical neurons. Unfortunately, many previous drug screening program focused on novel actinomycetes from terrestrial source, which in turn resulted in inefficient rediscovery of known bioactive compounds. Thus, researchers began to divert their attention to new or underexplored habitats, in hope to find new species that may yield promising bioactive compounds.

As one of the world's most dynamic environments, the mangrove ecosystem yields commercial forest products, supports coastal fisheries and protects coastlines (Alongi, 2008). Recently, there has been a renewed interest in the mangrove microorganisms' resources, considering that the changes in salinity and tidal gradient in the mangrove can trigger metabolic adaptations that could result in valuable metabolites production (Hong et al., 2009; Lee et al., 2014a; Azman et al., 2015). Several studies have discovered novel actinobacteria from the poorly explored mangrove environments, demonstrated by the isolation of *Streptomyces xiamenensis* (Xu et al., 2009), *Streptomyces sanyensis* (Sui et al., 2011), *Streptomyces qinglanensis* (Hu et al., 2012), *Streptomyces pluripotens* (Lee et al., 2014b), *Streptomyces gilvigriseus* (Ser et al., 2015b), and *Streptomyces mangrovisoli* (Ser et al., 2015a). Some of these novel strains are known to be bioactive strains as they were found to produce potent compounds with antibacterial, antifibrotic and antioxidant activities. Overall, these findings emphasized that these mangrove-derived Gram-positive filamentous bacteria could

be potentially useful for discovery of new drugs or drug leads for neurodegenerative diseases which role of oxidative stress has been implicated, including Parkinson's diseases, Alzheimer's disease and multiple sclerosis.

In this study, a novel strain, MUSC 164^T was discovered from a mangrove soil located in east coast of Peninsular Malaysia. A polyphasic approach determined that MUSC 164^T represents a novel species of the *Streptomyces* genus, for which the name *Streptomyces antioxidantans* sp. nov. is proposed. As a means to explore the bioactivities possessed by the strain, the extract of MUSC 164^T was subjected to several antioxidant assays prior to *in vitro* neuroprotective screening against hydrogen peroxide (H_2O_2). Gas chromatography-mass spectrometry (GC-MS) was used to perform chemical analysis for MUSC 164^T extract in order to reveal the chemical constituents present in the extract. Taken altogether, this study has implicated the potential of the mangrove-derived strain *Streptomyces antioxidantans* sp. nov. in producing bioactive compounds, specifically with antioxidative and neuroprotective activities.

MATERIALS AND METHODS

Isolation and Maintenance of Isolate

Strain MUSC 164^T was recovered from a soil sample collected at site MUSC-TLS4 (3° 48' 21.3" N 103° 20' 3.3" E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular Malaysia in December 2012. Topsoil samples of the upper 20 cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored at –20°C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50°C; Takahashi et al., 1996). Five grams of the pretreated air-dried soil was mixed with 45 mL sterilized water and mill ground, spread onto the isolation medium ISP 2 (Shirling and Gottlieb, 1966) supplemented with cycloheximide (25 µg/mL) and nystatin (10 µg/mL), and incubated at 28°C for 14 days. Pure cultures of strain MUSC 164^T were isolated and maintained on slants of ISP 2 agar and in glycerol suspensions (20% v/v).

Genomic and Phylogenetic Analyses

Extraction of DNA was performed as previously described (Hong et al., 2009), followed by 16S rRNA gene amplification carried out as stated by Lee et al. (2014b). Using CLUSTAL-X software, the 16S rRNA gene sequence of strain MUSC 164^T was aligned with representative sequences of related type strains of the genus *Streptomyces* retrieved from the GenBank/EMBL/DDBJ databases (Thompson et al., 1997). Subsequently, the alignment was verified manually and adjusted before constructing the phylogenetic trees with the neighbor-joining (Saitou and Nei, 1987; **Figure 1**) and maximum-likelihood algorithms (Felsenstein, 1981; Figure S1), using the MEGA version 6.0 (Tamura et al., 2013). Evolutionary distances for the neighbor-joining algorithm were computed using Kimura's two-parameter model (Kimura, 1980). Calculations of sequence similarity was performed using EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>) (Kim et al., 2012). The stability of the resultant trees

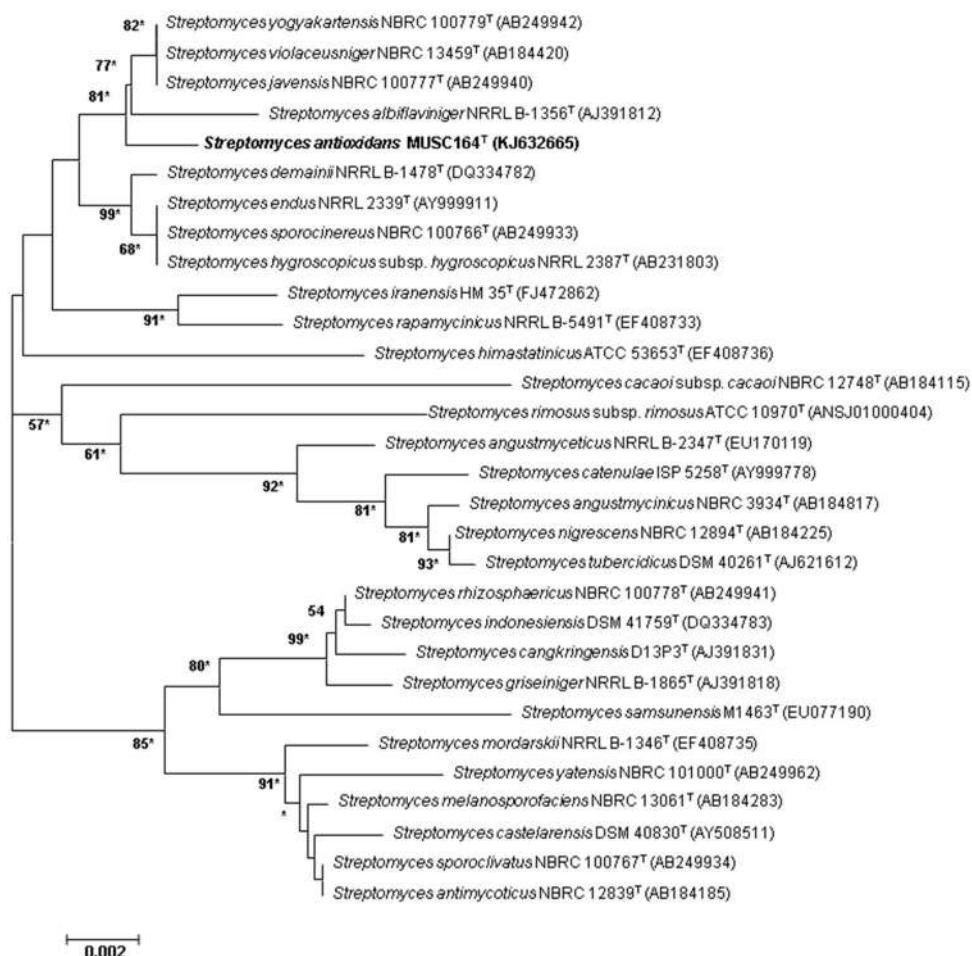


FIGURE 1 | Neighbor-joining phylogenetic tree based on almost complete 16S rRNA sequences (1491 nucleotides) showing the relationship between strain MUSC 164^T and representatives of some other related taxa. Numbers at nodes indicate percentages of 1000 bootstrap re-samplings, only values above 50% are shown. Bar, 0.002 substitutions per site. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm.

topologies were evaluated by using the bootstrap resampling method of Felsenstein (1985).

BOX-PCR fingerprint analysis was carried out to characterize strain MUSC 164^T and the closely related strains using the primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3'; Versalovic et al., 1991; Lee et al., 2014c). The PCR condition for BOX-PCR was performed as described by Lee et al. (2014d) and the PCR products were visualized by 2% agarose gel electrophoresis.

Genomic DNA extractions for DNA-DNA hybridization of strain MUSC 164^T, *Streptomyces javensis* NBRC 100777^T, *Streptomyces violaceusniger* NBRC 100779^T and *Streptomyces yogyakartensis* NBRC 13459^T were performed by the Identification Service of the DSMZ, Braunschweig, Germany following the protocol of Cashion et al. (1977). DNA-DNA hybridization was conducted as described by De Ley et al. (1970) with slight modifications described by Huss et al. (1983). The G + C content of strain MUSC 164^T was determined by HPLC (Mesbah et al., 1989).

Chemotaxonomic Characteristics

The analyses of peptidoglycan amino acid composition and sugars of strain MUSC 164^T were conducted by the Identification Service of the DSMZ using published protocols (Schumann, 2011). Analysis of respiratory quinones, polar lipids (Kates, 1986) and fatty acids (Sasser, 1990) were carried out by the Identification Service of the DSMZ. Major diagnostic cell wall sugars of strain MUSC 164^T were obtained as described by Whiton et al. (1985) and analyzed by TLC on cellulose plates (Staneck and Roberts, 1974).

Phenotypic Characteristics

The cultural characteristics of strain MUSC 164^T were determined following growth on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, and ISP 7 agar (Shirling and Gottlieb, 1966), actinomycetes isolation agar (AIA; Atlas, 1993), *Streptomyces* agar (SA; Atlas, 1993), starch casein agar (SCA; Küster and Williams, 1964) and nutrient agar (Macfaddin, 2000) for 14 days at 28°C. The colony color was examined by using the ISCC-NBS color charts (Kelly,

1964). Light microscopy (80i, Nikon) and scanning electron microscopy (JEOL-JSM 6400) were utilized to evaluate the morphology of the strain after incubation on ISP 2 medium at 28°C for 7–14 days (**Figure 2**). Gram staining was performed and confirmed by using KOH lysis (Cerny, 1978). The growth temperature range was tested at 12–48°C (at intervals of 4°C) on ISP 2 agar, while the pH range for growth was tested in tryptic soy broth (TSB) between pH 4.0–10.0 (at intervals of 1.0 pH unit). Tolerance of NaCl was tested in TSB with concentrations ranging from 0 to 18% (w/v) at intervals of 2%. The responses to temperature, pH and NaCl were observed for 14 days. Catalase activity and production of melanoid pigments were determined following protocols described by Lee et al. (2014e). Hemolytic activity was examined after incubation at 32°C for 7–14 days using blood agar medium containing 5% (w/v) peptone, 3% (w/v) yeast extract, 5% (w/v) NaCl, and 5% (v/v) horse blood (Carrillo et al., 1996). Amylolytic, cellulase, chitinase, lipase, protease, and xylanase activities were determined by growing cells on ISP 2 medium as described by Meena et al. (2013). The carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates (Biolog, USA) according to the manufacturer's instructions.

All of the phenotypic assays mentioned were performed concurrently for strain MUSC 164^T, *Streptomyces javensis* NBRC 100777^T, *Streptomyces violaceusniger* NBRC 100779^T and *Streptomyces yogyakartensis* NBRC 13459^T.

Extract Preparation of MUSC 164^T

Seed medium was prepared by cultivating strain MUSC 164^T in TSB for 14 days prior to fermentation process. Fermentation was conducted in 500 mL Erlenmeyer flask containing 200 mL of sterile FM 3 medium, shaking at 200 rpm for 7–10 days at 28°C (Hong et al., 2009; Lee et al., 2012). The cell mass was separated by centrifugation at 12000 × g for 15 min and the supernatant was filtered and freeze-dried. The freeze-dried sample was extracted repeatedly with methanol. Subsequently, the extracting solvent

was removed and concentrated by rotary vacuum evaporator at 40°C. The extract of MUSC 164^T was retrieved and suspended in dimethyl sulphoxide (DMSO) as vehicle reagent prior to bioactivity screening assays.

Determination of Antioxidant Activity of MUSC 164^T Extract

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity by MUSC 164^T extract was determined using previous protocol with minor modification (Ser et al., 2015a). The reduction in radical is measured as decrease in the absorbance of 515 nm. Volume of 195 µL of 0.016% DPPH ethanolic solution was added to 5 µL of extract solution to make up final volume of 200 µL. Gallic acid was included as positive control. Reactions were carried out at room temperature in dark for 20 min before measurement with spectrophotometer at 515 nm. DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

SOD activity was determined using SOD assay Kit – WST (Sigma-Aldrich) following manufacturer's instructions (Tan et al., 2015). Twenty microliter of sample solution was added to sample and blank 2 wells, while 20 µL of ddH₂O was added to blank 1 and blank 3 wells. Subsequently, WST working solution (20 µL) was then added to each well followed by 20 µL of enzyme working solution to the sample and blank 1 wells. The resultant mixtures were mixed thoroughly and incubated at 37°C for 20 min. The absorbance was read at 450 nm and superoxide anion scavenging activity was calculated as follows:

$$\text{SOD activity} = \frac{((\text{Absorbance of blank 1} - \text{Absorbance of blank 3}) - (\text{Absorbance of sample} - \text{Absorbance of blank 2}))}{(\text{Absorbance of blank 1} - \text{Absorbance of blank 3})} \times 100\%$$

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was performed as previously described in published literature with some modifications (Miser-Salihoglu et al., 2013; Ser et al., 2016b). ABTS radical cation (ABTS•) was generated by reacting ABTS stock solution (7 mM) and potassium persulphate (2.45 mM) for 24 h prior to assay. The change in radical amount was indicated by decrease in absorbance at 743 nm. Metal-chelating activity was measured as described by Manivasagan et al. (2013) with slight modification. 2 mM of FeSO₄ was added to extract and the reaction was initiated by adding 5 mM of ferrozine before measuring at 562 nm using spectrophotometer.

Cell Lines Maintenance and Growth Condition

Neuronal SH-SY5Y cells were maintained in DMEM media (supplemented with 10% FBS) in humidified incubator (5% CO₂ in air at 37°C) as described by Wong et al. (2012) with minor modification.

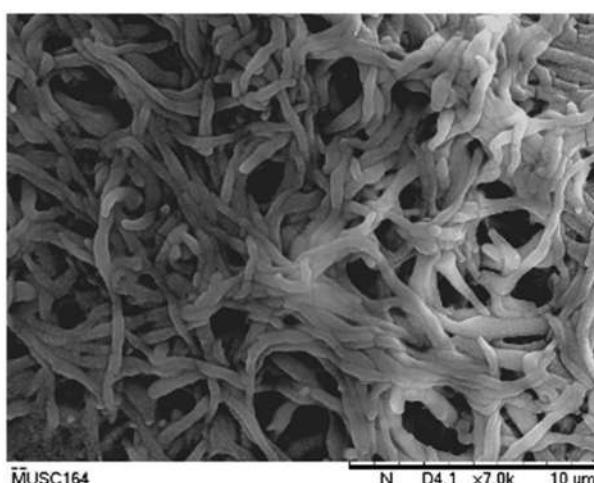


FIGURE 2 | Scanning electron microscope of *Streptomyces antioxidantans* MUSC 164^T.

Assessment of Toxicity and Neuroprotective Activity of MUSC 164^T Extract

Confluence cells were harvested and seeded at a density of 3×10^4 cells/well into a sterile flat bottom 96-well plate. The seeded cells were allowed to adhere for 48 h before the experiment. Firstly, toxicity of the extract was investigated by treating the cells MUSC 164^T extract (0–400 µg/mL) for 24 h prior to measurement of cell viability using MTT assay (Chan et al., 2012). On the other hand, for evaluation of neuroprotective activity, cells were pretreated with MUSC 164^T (0–400 µg/mL) extract for 2 h prior to treatment with H₂O₂ (Wong et al., 2012). For these screenings, media with DMSO was included as negative control along with a non-inoculated extract. Subsequently, MTT assay was performed 24 h after H₂O₂ treatment. Tetrazolium salt solution was added into each well and incubated further for 4 h. Plates were analyzed in microplate reader at 570 nm (with a reference wavelength of 650 nm). The percentage of cell viability was calculated as follows:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\%$$

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed in accordance with our previous developed method with slight modification (Supriady et al., 2015; Ser et al., 2015a). The machine used was Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m × 250 × 0.25 µm and used helium as carrier gas at 1 mL/min. The column temperature was programmed initially at 40°C for 10 min, followed by an increase of 3°C/min to 250°C and was kept isothermally for 5 min. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those from NIST 05 Spectral Library.

Statistical Analysis

Experiments to evaluate bioactivities were performed in quadruplicate and all results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with SPSS statistical analysis software. A difference was considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Biologically active compounds isolated from microorganisms remain to be vital for development of new drugs, particularly pharmaceutical and agricultural industry (Bérdy, 2005; Jensen et al., 2005). Oxidative stress has been implicated in pathogenesis of various chronic human disease, particularly neurodegenerative diseases (Radi et al., 2014). An imbalance in free radicals production and antioxidant mechanisms causes

modifications and damages on biological macromolecules including protein, lipid and DNA. These detrimental effects of free radicals eventually lead to neuronal cell loss—a scenario which is commonly seen in neurodegenerative diseases (Uttara et al., 2009). Antioxidants are responsible of removing or reducing amount of these harmful radicals; high intake of antioxidants have been associated with reduced risk of developing neurodegenerative diseases (Bonda et al., 2010; Lassmann and van Horssen, 2015). The need to search for novel, potent antioxidative agents to combat against these diseases has called upon researchers to venture into new or underexplored habitats for the discovery of bioactive strains that could produce potent antioxidant(s) (Harvey, 2000; Penesyan et al., 2010). In current study, the mangrove forest soil-derived MUSC 164^T strain shows abundant growth on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7 agar, actinomycetes isolation agar, nutrient agar and starch casein agar after 7–14 days at 28°C. The strain grows moderately on *Streptomyces* agar, and does not grow on ISP 4 agar. The colors of the aerial and substrate mycelium were media-dependent as indicated by Table S1. Both aerial and vegetative hyphae were abundant, well developed and not fragmented as observed from 14-day-old culture grown on ISP 2 agar. These morphological features are consistent with assignment of the strain to the genus *Streptomyces* (Williams et al., 1989). Growth was found to occur at 26–36°C (optimum 28–32°C), with 0–6% NaCl tolerance (optimum 0–2%) and at pH 6.0–8.0 (optimum pH 7.0). Cells were found to be positive for catalase but lack of hemolytic activity and melanoid pigment production. Furthermore, cells were capable of hydrolyzing soluble starch and carboxymethylcellulose, but unable to hydrolyze casein, chitin, tributyrin (lipase) and xylan. Using a range of phenotypic properties, strain MUSC 164^T can be differentiated from closely related members of the genus *Streptomyces* (Table 1). Furthermore, chemical sensitivity assays showed that the strain was resistant to aztreonam, fusidic acid, guanine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin RV, sodium bromate, sodium butyrate, 1% sodium lactate, tetrazolium blue, tetrazolium violet, troleandomycin, and vancomycin.

The nearly complete 16S rRNA gene sequence was obtained for strain MUSC 164^T (1491 bp; GenBank/EMBL/DDBJ accession number KJ632665) and phylogenetic trees were reconstructed to determine the phylogenetic position of this strain (Figure 1, Figure S1). Phylogenetic analysis exhibited that closely related strains include *Streptomyces javensis* NBRC 100777^T, *Streptomyces yogyakartensis* NBRC 100779^T and *Streptomyces violaceusniger* NBRC 13459^T, as they formed a distinct clade at high bootstrap value of 81% (Figure 1). The analysis of 16S rRNA gene sequence for strain MUSC 164^T exhibited highest similarity to strain *Streptomyces javensis* NBRC 100777^T (99.6% sequence similarity), *Streptomyces yogyakartensis* NBRC 100779^T (99.6%) and *Streptomyces violaceusniger* NBRC 13459^T (99.6%); while the type strains of other species of the genus *Streptomyces* showed sequences similarities below 99.3%. The DNA-DNA relatedness values between strain MUSC 164^T and *Streptomyces javensis* NBRC

TABLE 1 | Differentiation characteristics of strain MUSC 164^T and type strains of phylogenetically closely related species of the genus *Streptomyces*.

Characteristic	1	2	3	4
MORPHOLOGY (ON ISP 2):				
Color of aerial mycelium	Yellowish white	Yellowish white	Pale orange yellow	Vivid greenish yellow
Color of substrate mycelium	Brilliant greenish yellow	Light yellow	Brilliant yellow	Brilliant Greenish yellow
GROWTH AT:				
26°C	+	+	+	(+)
36°C	(+)	+	+	-
pH 8	(+)	-	-	+
4% NaCl	(+)	+	+	-
Catalase	+	+	+	+
Hemolytic	-	-	-	-
HYDROLYSIS OF:				
Casein (protease)	-	+	-	+
Tributyrin (lipase)	-	+	+	+
Starch (amylolytic)	+	+	+	+
Carboxymethylcellulose (cellulase)	+	+	+	+
Xylan (xylanase)	-	+	+	+
CARBON SOURCE UTILIZATION:				
Dextrin	-	+	+	+
Gentiobiose	-	-	+	+
Sucrose	+	-	-	-
D-turanose	+	-	-	-
Stachyose	+	-	-	-
α-D-lactose	-	+	+	+
D-melibiose	-	-	+	+
N-acetyl-neuraminic acid	+	-	-	-
D-mannose	-	-	+	+
D-fructose	-	-	+	+
D-galactose	-	-	+	-
L-fucose	-	+	+	+
Inosine	-	-	+	+
D-mannitol	-	+	+	+
p-hydroxy-phenylacetic acid	+	-	-	-
acetoacetic acid	+	-	-	-
CHEMICAL SENSITIVITY ASSAYS:				
Troleandomycin	+	-	-	-
Vancomycin	+	-	-	-
Fusidic acid	+	-	-	-
Lincomycin	+	-	-	-
Niaproof 4	+	-	-	-
Lithium chloride	+	-	-	-
Guanidine HCl	+	-	-	-

Strains: 1, *Streptomyces antioxidantans* sp. nov. MUSC 164^T; 2, *Streptomyces javensis* NBRC 100777^T; 3, *Streptomyces yogyakartensis* NBRC 100779^T; 4, *Streptomyces violaceusniger* NBRC 13459^T. All data were obtained concurrently in this study. +, Positive; -, negative; (+), weak.

All strains are positive for utilization of D-cellulose, D-raffinose, N-acetyl-D-glucosamine, α-D-glucose, gelatin, glucuronamide, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, and α-keto-butyric acid. All strains are negative for assimilation of D-maltose, β -methyl-D-glucoside, D-salicin, N-acetyl-β -D-mannosamine, N-acetyl-D-galactosamine, 3-methyl glucose, D-fucose, D-sorbitol, D-glucose-6-PO₄, D-serine and citric acid.

100777^T (23.8 ± 0.2%), *Streptomyces yogyakartensis* NBRC 100779^T (30.2 ± 2.7%) and *Streptomyces violaceusniger* NBRC 13459^T (53.1 ± 4.4%) were significantly below 70% which was reported as the threshold value for the delineation of bacterial species (Wayne et al., 1987). The BOX-PCR analysis revealed a unique fingerprint pattern by strain MUSC 164^T as compared

with its closely related type strains (Figure S2). These results further supported the results of DNA-DNA hybridizations and phylogenetic analysis, which indicated the novel status of strain MUSC 164^T in the genus *Streptomyces*.

The major cellular fatty acids in MUSC 164^T were identified as iso-C_{15:0} (34.8%) and anteiso-C_{15:0} (14.0%) (Table 2). The

fatty acids profile of MUSC 164^T displayed some levels of similarities with those of closely related phylogenetic neighbors such as *Streptomyces javensis* NBRC 100777^T, *Streptomyces yogyakartensis* NBRC 100779^T and *Streptomyces violaceusniger* NBRC 13459^T, as they contain iso-C_{15:0} (24.1–36.3%) as their predominant fatty acids. Nonetheless, the fatty acid profile of MUSC 164^T was quantitatively different from those of these type strains; for instance, iso-C_{15:0}(34.8%) was found to be predominant in strain MUSC 164^T (Table 2), but the amount of the same fatty acid was much lesser in *Streptomyces yogyakartensis* NBRC 100779^T (24.1%). The polar lipids of MUSC 164^T were aminolipid, diphosphatidylglycerol, glycolipid, hydroxyphosphatidylethanolamine, phospholipid, phosphatidyl inositol, phosphatidylethanolamine, phosphatidylglycerol and lipid. The differences in polar lipid profiles indicated that MUSC 164^T differs from related type strains; for example, strain MUSC 164^T contain aminolipid and glycolipid (Figure S3A) that were not detected in *Streptomyces javensis* NBRC 100777^T (Figure S3B).

Strain MUSC 164^T presented a type I cell-wall as it contains LL-diaminopimelic acid (Lechevalier and Lechevalier, 1970), an amino acid which has been found in many species of the genus *Streptomyces* (Lee et al., 2005, 2014b; Xu et al., 2009; Hu et al., 2012; Ser et al., 2015a, Ser et al., 2015b). The predominant menaquinones of strain MUSC 164^T were detected as MK-9(H₆) (51%) and MK-9(H₈) (39%). These findings parallel those reported by Kim et al. (2003). The cell wall sugars detected were galactose, glucose and ribose. The G + C content of strain MUSC 164^T was 71.6 mol% which falls within the range of 67.0–78.0 mol% described for species of the genus *Streptomyces* (Kim et al., 2003).

Based on the results of phylogenetic analysis, DNA-DNA hybridization, chemotaxonomic and phenotypic analysis, it is evident that strain MUSC 164^T is different from all other species in the genus *Streptomyces*; the strain represents a novel species within the genus *Streptomyces*, for which the name *Streptomyces antioxidantans* sp. nov. is proposed. As an attempt to explore bioactivities possessed by the strain, MUSC 164^T extract was subjected to several antioxidant assays. DPPH assay revealed the ability of MUSC 164^T to produce free radical scavenging compound(s) as 2 mg/mL of extract showed significant activity at 18.31 ± 2.03% (Table 3). Similarly, the extract was able to reduce ABTS radical and chelate ferrous ion significantly with highest activity recorded to be 30.38 ± 2.27 and 43.66 ± 0.98%, respectively. Additionally, SOD assay suggested antioxidant(s) in MUSC 164^T extract with ranging antioxidant activity of 53.09–79.84%, depending on extract concentration. In summary, all the antioxidant assays suggested the potential of MUSC 164^T to produce potent antioxidant(s) that could scavenge free radicals and may reduce occurrence of oxidative stress.

As antioxidant assays revealed presence of antioxidant(s) in MUSC 164^T extract, *in vitro* cellular screening assay was then performed. The aim of this screening is to investigate if the extract is capable of protecting neuronal cells against the oxidative stress cellular damage elicited by an oxidative stress inducer, H₂O₂. Even though with short half-life, H₂O₂

TABLE 2 | Cellular fatty acid composition of strain MUSC 164^T and its closely related *Streptomyces* species.

Fatty acid	1	2	3	4
iso-C _{11:0}	—	0.1	—	—
C _{12:0}	0.1	0.1	—	—
iso-C _{13:0}	1.5	1.0	0.5	0.5
anteiso-C _{13:0}	0.2	0.1	—	0.1
C _{13:0}	0.1	—	—	—
iso-C _{14:0}	2.5	1.7	3.1	3.8
C _{14:0}	0.6	0.4	0.3	0.3
iso-C _{15:0}	34.8	36.3	24.1	32.5
anteiso-C _{15:0}	14.0	10.1	5.5	6.9
C _{15:1B}	1.2	0.1	0.6	0.2
C _{15:0}	3.7	0.7	1.4	0.7
iso-C _{16:1 H}	0.9	1.1	3.5	2.2
iso-C _{16:0}	7.0	9.1	21.3	15.1
C _{16:1 Cis 9}	6.4	3.7	4.3	3.8
anteiso-C _{15:0 2OH}	0.3	—	—	—
C _{16:0}	5.9	5.4	4.4	4.4
iso-C _{15:0 3OH}	0.1	—	—	—
C _{16:0 9Methyl}	5.3	9.1	10.4	10.0
anteiso-C _{17:1 C}	1.1	1.2	1.2	1.1
iso-C _{17:0}	7.6	13.9	10.1	13.8
anteiso-C _{17:0}	3.9	4.8	3.1	3.3
C _{17:1 Cis 9}	1.1	0.3	1.4	0.2
C _{17:0 Cyclo}	0.7	0.2	1.1	0.4
C _{17:0}	0.8	0.3	0.5	0.1
C _{17:0 10Methyl}	—	—	1.0	0.1
iso-C _{18:1 H}	—	0.1	1.3	0.2
iso-C _{18:0}	—	0.1	0.3	0.2
iso-C _{17:0 2OH}	—	—	0.1	—
C _{18:0}	—	0.1	0.1	0.2
iso-C _{19:0}	—	—	—	0.1

Strains: 1, *Streptomyces antioxidantans* sp. nov. MUSC 164^T; 2, *Streptomyces javensis* NBRC 100777^T; 3, *Streptomyces yogyakartensis* NBRC 100779^T; 4, *Streptomyces violaceusniger* NBRC 13459^T. —, <0.1% or not detected. All data are obtained concurrently from this study.

is highly soluble in water and dissociates to hydroxyl and superoxide ions, which leads to oxidative damage to important macromolecules and ultimately cell death (Triana-Vidal and Carvajal-Varona, 2013). Thus, this molecule has been used widely in oxidative studies (Whittemore et al., 1995; Suematsu et al., 2011; Wong et al., 2012; Triana-Vidal and Carvajal-Varona, 2013). Initially, the viability of SH-SY5Y cells was examined following exposure to the extract up to 400 µg/mL and no significant toxic effect was observed in SH-SY5Y (i.e., cell viability remains >98%). In subsequent neuroprotective experiment, the obtained results have demonstrated MUSC 164^T extract confers a significant level of protection on neuronal cells when challenged with H₂O₂. Highest cell viability was recorded at 80.62 ± 2.75% when neuronal cells were pre-treated with 400 µg/mL of extract (Figure 3) prior to induction by H₂O₂. As antioxidant assays suggested presence of antioxidant(s) in the extract, these compounds can

TABLE 3 | Radical scavenging activity of MUSC 164^T evaluated using different antioxidant assays.

Antioxidant assays	Concentration of MUSC 164 ^T extract (mg/mL)	Mean ± standard deviation (%)
DPPH	0.125	ND
	0.25	ND
	0.5	2.42 ± 1.58*
	1.0	6.66 ± 1.91*
	2.0	18.31 ± 2.03*
ABTS	0.125	5.14 ± 0.94*
	0.25	8.27 ± 1.63*
	0.5	10.63 ± 0.54*
	1.0	18.14 ± 1.72*
	2.0	30.38 ± 2.27*
Superoxide dismutase-like	0.09375	53.09 ± 2.53*
	0.1875	56.92 ± 1.71*
	0.375	69.46 ± 0.64*
	0.75	69.94 ± 1.51*
	1.5	79.84 ± 1.16*
Metal-chelating	0.125	NA
	0.25	14.41 ± 2.08*
	0.5	20.48 ± 1.58*
	1.0	29.29 ± 0.66*
	2.0	43.66 ± 0.98*

Symbol (*) indicates $p < 0.05$ significant difference between MUSC 164^T extract and controls (without MUSC 164^T extract).

*NA, not available, ND, not detected.

possibly quench H₂O₂, preventing oxidative damage against the neuronal cells. Previous study has showed that compounds produced by *Streptomyces* sp. protect primary cortical neurons against oxidative stress, accompanied by alteration in the expression of apoptotic genes and transcription factor for antioxidant pathways (Leiros et al., 2013). Thus, further study on underlying neuroprotective mechanisms induced by MUSC 164^T would be helpful for the development of new drugs in preventing the occurrence of neurodegenerative disorders.

Following the evaluation of bioactivities possessed by MUSC 164^T extract, GC-MS was performed to assist with the chemical profiling and to identify compounds present in the extract. This technique enables identification of compounds present in *Streptomyces* extract as GC separates the compounds and MS generates characteristic mass profile for each of the compounds present (Pollak and Berger, 1996; Karanja et al., 2010; Jog et al., 2014). Our results revealed 24 compounds present in MUSC 164^T extract (Table 4) and their chemical structures (Figure 4, Figure S4) as Pyrazine, 2,5-dimethyl- (1), Pyrazine, 2,3-dimethyl- (2), Dimethyl trisulfide (3), Pyrazine, 2-ethyl-5-methyl- (4), Pyrazine, trimethyl- (5), Pyrazine, 3-ethyl-2,5-dimethyl- (6), 4-Pyridinamine, N,N,2-trimethyl- (7), 2,3-Dimethyl-5-ethylpyrazine (8), Benzoic acid, methyl ester

(9), Pyrazine, 2-methyl-5-(1-propenyl)-, (E)- (10), Pyrazine, 3,5-diethyl-2-methyl- (11), 2-Piperidinone (12), Pyrazine, 2,5-dimethyl-3-(2-methylpropyl)- (13), Indolizine (14), Pyrazine, 2,5-dimethyl-3-(3-methylbutyl)- (15), Pyrazine, 3,5-dimethyl-2-propyl- (16), 2,3,5-Trimethyl-6-ethylpyrazine (17), Phenol, 2,4-bis(1,1-dimethylethyl)- (18), 1,2,3,4-Tetrahydrocyclopenta[b]indole (19), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (20), Phenol, 3,5-dimethoxy- (21), Hexadecanoic acid, methyl ester (22), Pentadecanoic acid, 14-methyl, methyl ester (23) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-phenylmethyl)- (24).

Through GC-MS, most of the compounds were identified to be heterocyclic organic compounds. Naturally occurring phenolic compounds have been widely accepted as potent antioxidants and these compounds are believed to play an important role in the prevention of chronic diseases resulting from oxidative stress (Soobrattee et al., 2005; Gülcin and Beydemir, 2013). The phenolic compound 18 has been detected in previously isolated *Streptomyces* sp. from vermicompost and mangrove forest (Narendhran et al., 2014; Tan et al., 2015; Ser et al., 2015a,c). This compound has been shown to exhibit antioxidant activity, probably owing to its hydrogen-donating ability (Brewer, 2011; Narendhran et al., 2014). On the other hand, pyrazines are heterocyclic compounds that contain two nitrogen atoms in their aromatic ring; some of these compounds are known to exhibit various bioactivities including antimicrobial, anticancer, antioxidant, neuroprotection against ischemia/reperfusion injuries and hypoxia (Premkumar and Govindarajan, 2005; Jia et al., 2009; Baldwin et al., 2013; Tan et al., 2015; Ser et al., 2015a,c). Previous studies have demonstrated that microorganisms are capable of producing compounds 1, 2, 5, 6, 15, and 17 with antioxidant activity (Sun et al., 2013; Citron et al., 2015; Pongsetkul et al., 2015). *Bacillus methylo trophicus* KOSM11 was used in fermentation industry for traditional soybean paste and found to produce compound 1 and 5; the presence of these pyrazines and their related compounds in various food and plants have been linked with antioxidant activities (Liu et al., 2012; Sun et al., 2013; Xu et al., 2015). Furthermore, two pyrrolopyrazines were observed in MUSC 164^T extract, which are compound 20 and 24. These compounds were previously detected in several *Streptomyces* sp. and they have been associated with antioxidant activity exhibited by these strains (Gopi et al., 2014; Tan et al., 2015; Ser et al., 2015a,c). Gopi et al. (2014) has also reported that these compounds were highly capable of scavenging or reducing amount of free radicals when assessed with reducing power assay. Thus, the detection of these heterocyclic compounds present in MUSC 164^T could account for the antioxidant activity and protect SH-SY5Y cells against H₂O₂ insults.

The current study described a novel streptomycete designated as MUSC 164^T that produces a mixture of compounds, with some of them could be responsible for the free radical scavenging activities detected via several antioxidant assays. Furthermore, the extract has demonstrated its potential in conferring neuroprotection against oxidative insults, possibly by preventing oxidative stress and activating of antioxidant defense systems that are crucial for survival

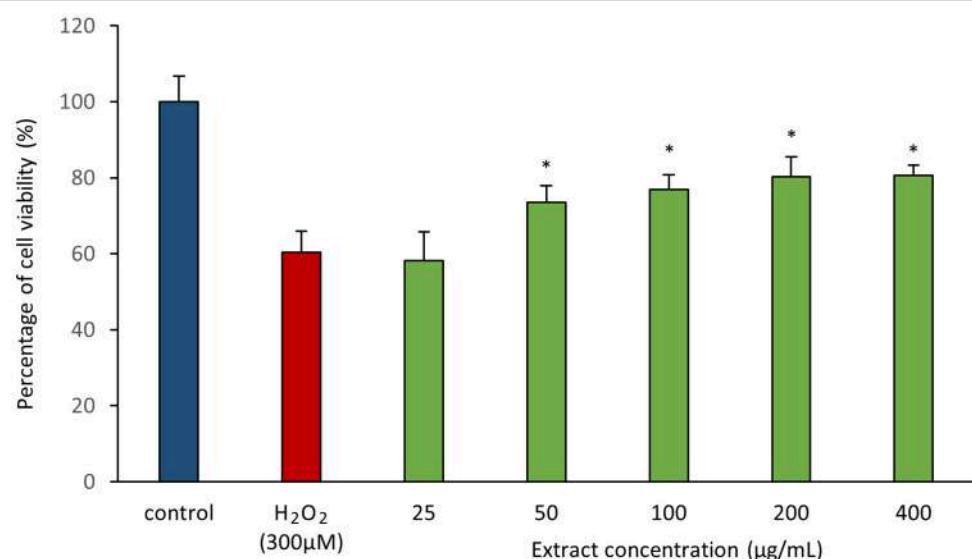


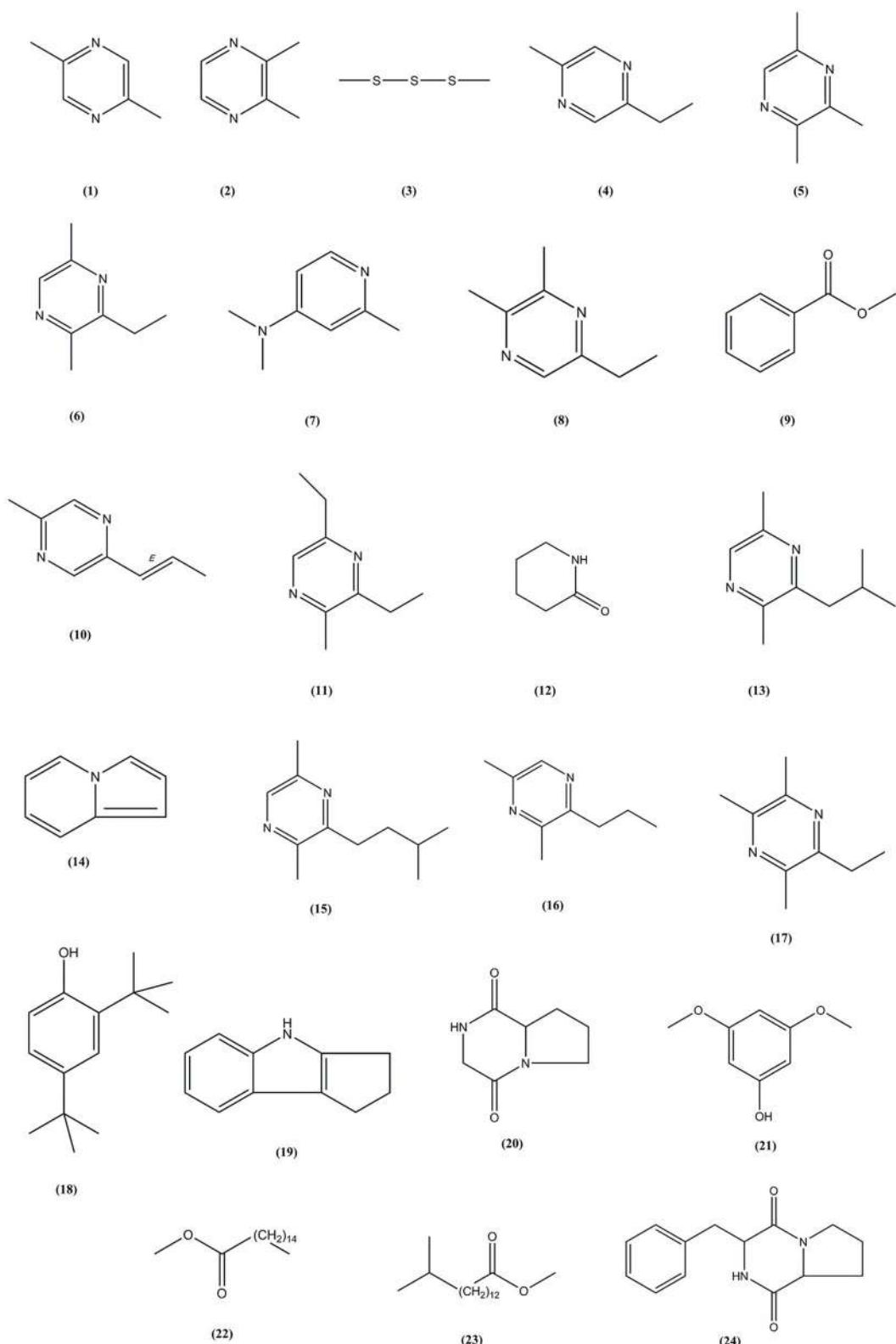
FIGURE 3 | Neuroprotective activity of MUSC 164^T extract against hydrogen peroxide (H₂O₂) in SH-SY5Y cells. The measurement of cell viability was done using MTT assay. Media containing 0.5% DMSO was used as control. All data are expressed as mean \pm standard deviation. Symbol (*) indicates $p < 0.05$ significant difference between the inducer and cells treated with MUSC 164^T extract.

TABLE 4 | Compounds identified from MUSC 164^T extract using GC-MS.

No	Retention time (min)	Compound	Formula	Molecular weight (MW)	Quality (%)
1	13.484	Pyrazine, 2,5-dimethyl-	C ₆ H ₈ N ₂	108.14	90
2	14.022	Pyrazine, 2,3-dimethyl-	C ₆ H ₈ N ₂	108.14	80
3	17.111	Dimethyl trisulfide	C ₂ H ₆ S ₃	126.26	78
4	19.526	Pyrazine, 2-ethyl-5-methyl-	C ₇ H ₁₀ N ₂	122.17	90
5	19.595	Pyrazine, trimethyl-	C ₇ H ₁₀ N ₂	122.17	78
6	24.218	Pyrazine, 3-ethyl-2,5-dimethyl-	C ₈ H ₁₂ N ₂	136.19	94
7	24.544	4-Pyridinamine, N,N,2-trimethyl-	C ₈ H ₁₂ N ₂	136.19	53
8	24.630	2,3-Dimethyl-5-ethylpyrazine	C ₈ H ₁₂ N ₂	136.19	91
9	24.985	Benzoic acid, methyl ester	C ₈ H ₈ O ₂	136.15	90
10	25.265	Pyrazine, 2-methyl-5-(1-propenyl)-, (E)-	C ₈ H ₁₀ N ₂	134.18	59
11	28.464	Pyrazine, 3,5-diethyl-2-methyl-	C ₉ H ₁₄ N ₂	150.22	72
12	29.540	2-Piperidinone	C ₅ H ₉ NO	99.13	59
13	30.701	Pyrazine, 2,5-dimethyl-3-(2-methylpropyl)-	C ₁₀ H ₁₆ N ₂	164.25	80
14	34.970	Indolizine	C ₈ H ₇ N	117.15	64
15	36.057	Pyrazine, 2,5-dimethyl-3-(3-methylbutyl)-	C ₁₁ H ₁₈ N ₂	178.27	83
16	37.024	Pyrazine, 3,5-dimethyl-2-propyl-	C ₉ H ₁₄ N ₂	150.22	64
17	44.142	2,3,5-Trimethyl-6-ethylpyrazine	C ₉ H ₁₄ N ₂	150.22	68
18	44.474	Phenol, 2,4-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O	206.32	95
19	46.969	1,2,3,4-Tetrahydro-cyclopenta[b]indole	C ₁₁ H ₁₁ N	157.21	87
20	53.251	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C ₇ H ₁₀ N ₂ O ₂	154.17	97
21	56.158	Phenol, 3,5-dimethoxy-	C ₈ H ₁₀ O ₃	154.16	58
22	58.041	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	94
23	59.242	Pentadecanoic acid, 14-methyl, methyl ester	C ₁₇ H ₃₄ O ₂	270.45	90
24	72.065	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-phenylmethyl)-	C ₁₄ H ₁₆ N ₂ O ₂	244.29	97

of SH-SY5Y cells. In conclusion, these preliminary studies have revealed the antioxidative and *in vitro* neuroprotective properties of MUSC 164^T which merit for further investigations

focusing on the isolation and characterization of chemical compounds using bioassay-guided purification. The identified bioactive principles could essentially be important for the

FIGURE 4 | Chemical structures of constituents detected in *MUSC 164^T* extract.

development of pharmacological agents for neurodegenerative diseases.

DESCRIPTION OF *Streptomyces antioxidantans* sp. nov.

Streptomyces antioxidantans sp. nov. (an.ti.o'xi.dans. Gr. pref. anti, against; N.L. v. oxidare, to oxidize; N.L. part. adj. antioxidantans, non-oxidizing, referring to the antioxidant properties of this strain).

Gram-positive actinobacteria that forms yellowish-white aerial and brilliant greenish yellow substrate mycelium on ISP 2 agar. The colors of the aerial and substrate mycelium are media-dependent (Table S1).

Abundant growth was observed on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7, actinomycetes isolation agar, starch casein agar and nutrient agar after 7–14 days at 28°C; cells grow moderately on *Streptomyces* agar, and does not grow on ISP 4 agar. Cells grow at 26–36°C (optimum 28–32°C), pH 6.0–8.0 (optimum pH 7.0), with 0–6% NaCl tolerance (optimum 0–2%). Cells are positive for catalase but negative for hemolytic activity and melanoid pigment production. Soluble starch and carboxymethylcellulose are hydrolyzed but casein, chitin, xylan, and tributyrin (lipase) are not. The following compounds are utilized as sole carbon sources: acetic acid, acetoacetic acid, α-D-glucose, α-hydroxy-butyric acid, α-keto-butyric acid, α-keto-glutaric acid, β-hydroxyl-D,L-butyric acid, bromo-succinic acid, D-cellulose, D-fructose-6-phosphate, D-galactose, D-galacturonic acid, D-glucuronic acid, D-lactic acid methyl ester, D-malic acid, D-raffinose, D-saccharic acid, D-trehalose, D-turanose, formic acid, gelatin, glucuronamide, L-galactonic acid lactone, L-lactic acid, L-rhamnose, N-acetyl-D-glucosamine, N-acetyl-neuraminic acid, pectin, p-hydroxyl-phenylacetic acid, quinic acid, stachyose, sucrose, Tween 40, γ-amino-butyric acid, and myo-inositol. The following compounds are not utilized as sole carbon sources: α-D-lactose, β-methyl-D-glucoside, citric acid, D-arabitol, D-aspartic acid, Dextrin, D-fructose, D-fucose, D-glucose-6-phosphate, D-gluconic acid, D-maltose, D-mannitol, D-mannose, D-melibiose, D-salicin, D-serine, D-sorbitol, gentiobiose, glycerol, glycyl-L-proline, inosine, L-fucose, L-malic acid, methyl pyruvate, mucic acid, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, propionic acid, and 3-methyl glucose. The following compounds are not utilized as sole carbon sources: L-alanine, L-arginine, L-aspartic acid,

L-histidine, L-pyroglutamic acid; while L-glutamic acid and L-serine are utilized as sole nitrogen sources.

The cell wall peptidoglycan contains LL-diaminopimelic acid. The predominant menaquinones are MK-9(H₆) and MK-9(H₈). The polar lipids consist of aminolipid, diphosphatidylglycerol, glycolipid, hydroxyphosphatidylethanolamine, phospholipid, phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol, and lipid. The cell wall sugars are galactose, glucose and ribose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}.

The type strain, MUSC 164^T (=DSM 101523^T = MCCC 1K01590^T) was isolated from mangrove soil collected from the Tanjung Lumpur mangrove forest (state of Pahang, Peninsular Malaysia). The 16S rRNA gene sequence of strain MUSC 164^T has been deposited in GenBank/EMBL/DDBJ under the accession number KJ632665. The G + C content of the genomic DNA of the type strain is 71.6 mol%.

AUTHOR CONTRIBUTIONS

The experiments, data analysis and manuscript writing were performed by H-LS, LT-HT, B-HG, and L-HL, UDP, SNAM, W-FY, and K-GC provided vital guidance and technical support. L-HL and B-HG founded the research project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00899>

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Diverse Cone-Snail Species Harbor Closely Related *Streptomyces* Species with Conserved Chemical and Genetic Profiles, Including Polycyclic Tetramic Acid Macrolactams

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Streptomyces are Gram-positive bacteria that occupy diverse ecological niches including host-associations with animals and plants. Members of this genus are known for their overwhelming repertoire of natural products, which has been exploited for almost a century as a source of medicines and agrochemicals. Notwithstanding intense scientific and commercial interest in *Streptomyces* natural products, surprisingly little is known of the intra- and/or inter-species ecological roles played by these metabolites. In this report we describe the chemical structures, biological properties, and biosynthetic relationships between natural products produced by *Streptomyces* isolated from internal tissues of predatory *Conus* snails, collected from the Great Barrier Reef, Australia. Using chromatographic, spectroscopic and bioassays methodology, we demonstrate that *Streptomyces* isolated from five different *Conus* species produce identical chemical and antifungal profiles – comprising a suite of polycyclic tetramic acid macrolactams (PTMs). To investigate possible ecological (and evolutionary) relationships we used genome analyses to reveal a close taxonomic relationship with other sponge-derived and free-living PTM producing *Streptomyces* (i.e., *Streptomyces albus*). In-depth phylogenomic analysis of PTM biosynthetic gene clusters indicated PTM structure diversity was governed by a small repertoire of genetic elements, including discrete gene acquisition events involving dehydrogenases. Overall, our study shows a *Streptomyces-Conus* ecological relationship that is concomitant with specific PTM chemical profiles. We provide an evolutionary framework to explain this relationship, driven by anti-fungal properties that protect *Conus* snails from fungal pathogens.

Keywords: *Streptomyces*, cone snails, natural product, phylogenomics, polycyclic tetramic acid macrolactams (PTMs)

INTRODUCTION

Polycyclic tetramic acid macrolactams (PTMs) are widespread natural products produced by members of the phyla Actinobacteria and Proteobacteria (class gammaproteobacteria). PTM-producing bacteria have been isolated from complex biological systems, such as sponges, plants and insects (Shigemori et al., 1992; Jakobi and Winkelmann, 1996; Nakayama et al., 1999; Yu et al., 2007; Blodgett et al., 2010). Examples of PTMs produced by symbiotic bacteria include alteramide A produced by *Alteromonas* sp., isolated from the marine sponge *Halichondria okadai* (Shigemori et al., 1992), maltophilin produced by *Stenotrophomonas maltophilia*, isolated from the rhizosphere of rape plants (Jakobi and Winkelmann, 1996), xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88, isolated from the root of sugar beet (Nakayama et al., 1999), and frontaliamides produced by *Streptomyces* sp. SPB78, isolated from the southern pine beetle (Blodgett et al., 2010). These observations suggest that PTMs play important biological and ecological roles yet-to-be described.

Structurally, PTMs are composed of a polycyclic carbocycle (5-5, 5-5-6 or 5-6-5 ring system), a macrolactam core, and a tetramic acid moiety (Zhang et al., 2016). Functional analysis using the host *Escherichia coli* for heterologous expression, and *in vitro* reconstitution of individual functional polyketide (PKS) and non-ribosomal synthetase (NRPS) enzymes, have determined that the tetramate polyene precursor is produced via a hybrid PKS/NRPS, whereas the PKS domain is responsible for the PTM backbone assembly (Li et al., 2014) and the NRPS module incorporates L-ornithine (Blodgett et al., 2010). In addition, the biosynthetic pathways of several PTMs (i.e., frontaliamides, ikarugamycin, and dihydromaltophilin – also known as heat-stable antifungal factor HSFAF) have been elucidated using different *in vivo* and *in vitro* approaches (Yu et al., 2007; Halo et al., 2008; Blodgett et al., 2010; Lou et al., 2011; Antosch et al., 2014; Li et al., 2014; Zhang et al., 2014; Greunke et al., 2017). For instance, the six-gene (*ftdA-F*) frontaliamide biosynthetic gene cluster (BGC), identified by gene knock out of the *ftd* biosynthetic cluster in *Streptomyces* sp. SPB78, incorporates a sterol desaturase-like enzyme, iterative hybrid PKS/NRPS, two desaturase-like enzymes, a zinc dependent alcohol desaturase and a cytochrome P450 enzyme (Blodgett et al., 2010). The biosynthesis of ikarugamycin from *Streptomyces* sp. was elaborated through heterologous expression of three main biosynthetic components, the iterative hybrid PKS/NRPS enzyme, phytoene desaturase-like enzyme and alcohol dehydrogenase encoded by *ikaA*, *ikaB*, and *ikaC* genes, respectively (Antosch et al., 2014; Zhang et al., 2014; Greunke et al., 2017). Finally, dihydromaltophilin biosynthesis was described in *Lysobacter enzymogenes* strains a decade ago through gene disruption, *in vitro* biochemical assays and heterologous expression experiments (Yu et al., 2007; Lou et al., 2011; Xie et al., 2012). The dihydromaltophilin gene cluster is formed by an iterative hybrid PKS/NRPS, sterol desaturase-like enzyme, ferredoxin reductase, alcohol dehydrogenase and three FAD-dependent oxidoreductase like enzymes.

Cone snails are marine molluscs that comprise the large genus *Conus*, with currently over 800 species identified (Costello et al., 2013). Queensland, Australia, represents a biodiversity hotspot for these molluscs with 133 of 166 Australian species. Cone snails prey on fish, worms or other snails using venoms composed mainly of small peptides (<5 kDa) known as conotoxins or conopeptides. Conotoxins are chemically stable ribosomally synthesized and post-translationally modified peptides that possess high specificity against diverse neuronal targets, many of which are implicated in human diseases (Cruz et al., 1985; Vetter and Lewis, 2012; Inserra et al., 2013). Given the wide range of potential applications of conotoxins as pharmaceuticals, cone snails have proved to be an excellent source of molecules for drug discovery and development (Adams et al., 1999; Halai and Craik, 2009). An example of this is the conopeptide marketed under the name Prialt, used clinically as a pain therapeutic (Miljanich, 2004). Previous studies on cone snail-associated bacteria as producers of bioactive metabolites reported neuroactive metabolites (Lin et al., 2011, 2013).

In the current study, we explored the biosynthetic repertoire of five cone snail-derived *Streptomyces* isolated from cone snail specimens collected from the Lady Musgrave Island, Great Barrier Reef, Australia. The observation that several *Streptomyces* isolates displayed common phenotypic colony morphology when grown under specific culture conditions, suggested a taxonomic bias, and perhaps a common (ecological-symbiotic) relationship. This prompted us to conduct a chemical and phylogenomic investigation, in which we confirmed that all isolates were taxonomically related, and shared similar chemical and biological (i.e., antifungal) profiles. A phylogenomic approach was used to establish direct correlations between sequence phylogeny, the presence/absence of enzymes within the PTM BGCs, and the associated natural products. Overall, this report demonstrates a specific association between a discrete lineage of *Streptomyces* and cone snails, and provides an evolutionary framework for further investigations into the biosynthesis of PTMs.

MATERIALS AND METHODS

Cone Snail Collection, Dissection and Microbial Isolation

Cone snails and microorganisms were manipulated under sterile conditions provided by a LabTech class II biological safety cabinet and incubated in either a MMM FrioCell incubators (Lomb Scientific, NSW, Australia) or an Innova 42R incubator shaker (John Morris, NSW, Australia) with temperature set at 26.5°C. Cone snail-derived *Streptomyces* were isolated from the stomach and hepatopancreas of five *Conus* species collected in 2011 from the Lady Musgrave Island at the Great Barrier Reef. Freshly collected cone snail specimens were transported in local seawater to the laboratory. The cone snail taxonomy was determined based on an informed observation of the physical characteristics including color of the body, cyphon and outer shell (Röckel et al., 1995) and according to the taxonomic keys available at the *Conus*

biodiversity website¹. Specimens were dissected and tissue was homogenized using a mortar and pestle, homogenate was serially diluted and applied to ISP-4 agar plates (Bacto DIFCO, Cat No. 277210), sealed with parafilm and incubated for 3–4 weeks.

Taxonomy of *Streptomyces* Isolates

A pure culture of *Streptomyces* isolates obtained by single colony serial transfer on agar plates was cryopreserved at –80°C in 20% aqueous glycerol. Five *Streptomyces* isolates: CMB-CS038, CMB-CS145, CMB-CS143, CMB-CS143, CMB-CS132, CMB-CS138 were obtained from five distinct species of cone snails, namely *Conus miles*, *C. ebraeus*, *C. flavidus*, *C. coronatus*, and *C. emaciatus*, respectively. The isolates initially formed a white colony, with gray spores after 10 days of incubation at 26.5°C. Genomic DNA from all the isolates was extracted from liquid cultures using the DNAeasy Plant Mini Kit (QIAGEN) as per the manufacturer's protocol. Bacterial taxonomic identification was performed by rRNA amplification. The rRNA genes were amplified by PCR using the universal primers 27F (5'-AGAGTTGATCMTGGCTCAG-3') and 1498R (5'-TACGGYTACCTGTTACGACTT-3') purchased from Sigma-Aldrich. The PCR mixture (50 µL) contained genomic DNA (1 µL, 20–40 ng), four deoxynucleoside triphosphates (dNTP, 200 µM each), MgCl₂ (1.5 mM), primer (0.3 µM each), 1 U of *Taq* DNA polymerase (Fisher Biotech) and PCR buffer (5 µL, 10×). PCR was performed as follows: initial denaturation at 95°C for 3 min, 30 cycles in series of 94°C for 30 s (denaturation), 55°C for 60 s (annealing) and 72°C for 60 s (extension), followed by one cycle at 72°C for 6 min. The PCR products were purified with PCR purification kit (QIAGEN) and sequenced. 16S DNA sequence (~1200 bp) was used as a query to BLAST against NCBI GenBank available database.

General Experimental Procedures

Analytical grade solvents were used for both liquid and solid phase extractions (SPE). Spectrophotometric-grade solvents were used for UV and chiroptical measurements. Deuterated solvents were purchased from Cambridge Isotopes (Andover, MA, United States). Specific optical rotations ([α]_D) were measured on a JASCO P-1010 polarimeter in a 100 mm × 2 mm cell at room temperature. CD spectra were recorded at 22°C on a JASCO J-810 spectropolarimeter. Liquid chromatography-diode array-mass spectrometry (HPLC-DAD-MS) data were acquired on an Agilent 1100 series separation module equipped with an Agilent 1100 series HPLC/MSD mass detector and diode array multiple wavelength detector. Semi-preparative and preparative HPLCs were performed using Agilent 1100 series HPLC instruments with corresponding detectors, fraction collectors and software inclusively. Pure compounds or fractions eluting from semi-preparative and preparative HPLCs were dried on a Christ freeze dryer. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance 600 MHz spectrometer with either a 5 mm PASEL 1H/D-13C Z-Gradient probe or 5 mm CPTCI 1H/19F-13C/15N/DZ-Gradient cryoprobe, controlled by TopSpin 2.1 software. In all cases spectra were acquired at 25°C (unless otherwise specified) in solvents as specified above, with

reference to residual ¹H or ¹³C signals in the deuterated solvents. Electrospray ionization mass spectrometry (ESIMS) experiments were carried out on an Agilent 1100 series LC/MSD (quadrupole) instrument in both positive and negative modes. High-resolution ESIMS spectra were obtained on a Bruker microTOF mass spectrometer either by direct injection in MeCN at 3 µL/min using sodium formate clusters as an internal calibrant.

Cultivation of Microbes and Chemical Analysis

Streptomyces sp. CMB-CS038, CMB-CS145, CMB-CS143, CMB-CS132, CMB-CS138 were cultivated for approximately 15 days in a petri dish (10 cm) containing ISP-4 agar (Burlington, NC, United States). The ISP-4 agar was extracted with 3:1 EtOAc:MeOH (30 mL) and the organic phase concentrated *in vacuo* to yield approximately 10 mg of crude extract. A solution of crude extract prepared in MeOH (1 mg/mL) was subjected to HPLC-DAD-ESI(±)MS analysis (Zorbax SB-C₈ column, 150 mm × 4.6 mm column, 5 µm, 1 mL/min gradient elution from 90% H₂O/MeCN to 100% MeCN over 15 min, with constant 0.05% formic acid modifier). *Streptomyces* sp. CMB-CS038 was also cultivated in other media: Marine agar (Becton Dickinson, Franklin Lakes, NJ, United States), Nutrient Agar (Becton Dickinson, Franklin Lakes, NJ, United States) and R2A agar (Becton Dickinson, Franklin Lakes, NJ, United States) to explore the secondary metabolite production capability of this strain.

Purification and Identification of PTMs from CMB-CS038

A seed culture of *Streptomyces* sp. CMB-CS038 was used to inoculate ISP-4 agar (400 plates), which were incubated at 26.5°C for 10 days, after which the agar was diced and extracted EtOH:MeOH 3:1 (2 L × 1.5 L). The solvent was concentrated *in vacuo* at 40°C and dried under N₂ at 40°C to yield a crude extract (820 mg). The crude extract was triturated into hexane (150 mg) and MeOH (650 mg) solubles, with the latter subjected to sequential fractionations by C₈ SPE (30% H₂O/MeCN to 100% MeCN) and semi-preparative HPLC purification (Zorbax-SB C₁₈ column 250 µm × 9.4, 5 µm, 3 mL/min, gradient elution from 10% H₂O/MeCN to 100% MeCN, with constant 0.01% TFA modifier, over 40 min) to afford dihydromaltophilin.

Antibiotic Assays

Activities were measured against Gram-positive bacterium *Staphylococcus aureus* ATCC 25923, Gram-negative bacteria *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and a fungus *Candida albicans* ATCC 90028 by the broth micro-dilution method. The test was performed (in triplicate) in 96-well microtiter plates by serial dilution in tryptic soy broth for bacteria and Sabouraud broth for fungi, respectively. Test compounds were prepared and serially (10-fold) diluted in 10% DMSO. An aliquot (20 µL) of each dilution was transferred to a 96-well microtiter plate, followed by freshly prepared microbial broth (180 µL, 10⁴–10⁵ cfu/mL cell density) to give a final test compound concentration ranging from 32 to 0.125 µg/mL.

¹<http://biology.burke.washington.edu/conus/catalogue/index.php>

For crude extracts, an aliquot was prepared from dried extract at a concentration of 1 mg/mL (2020 μ L). Assay plates were incubated at 37°C for 24 h for bacteria and at 26.5°C for 48 h for yeast. The optical density of each well was measured at 600 nm using a microtitre plate spectrophotometer (POLARstar Omega plate, BMG LABTECH, Offenburg, Germany). The minimum inhibitory concentration (MIC_{50}) was determined as the lowest concentration of a test compound that inhibits 50% of microorganism growth. Broth medium with and without microbial inoculation were used as negative controls. The MIC_{50} (μ M) for positive control tetracycline against *Staphylococcus aureus* ATCC 25923 was 0.26 μ M and for Gram-negative bacteria *E. coli* ATCC 25922 was 0.12 μ M and *Pseudomonas aeruginosa* ATCC 27853 was 0.26 μ M. The MIC_{50} for positive control ketoconazole against *Candida albicans* ATCC 90028 was 0.22 μ M.

Cytotoxicity Assays

Activity was measured using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay modified from that previously described (Carmichael et al., 1987) using adherent NCI-H460 (human lung carcinoma), SW-620 (human colorectal adenocarcinoma) cells. Briefly, cells were harvested with trypsin and dispensed into 96-well microtitre assay plates at 2,000 cells/well and incubated for 18 h at 37°C with 5% CO₂ (to allow cells to attach). Test compounds were dissolved in 5% DMSO in PBS (v/v) and aliquots (20 μ L) were tested over a series of final concentrations ranging from 10 nM to 30 μ M. Control wells were treated with 5% aqueous DMSO. After 68 h incubation at 37°C with 5% CO₂, an aliquot (20 μ L) of MTT in PBS (4 mg/mL) was added to each well (final concentration of 0.4 mg/mL), and the microtitre plates incubated for a further 4 h at 37°C with 5% CO₂. After this final incubation the medium was aspirated and precipitated formazan crystals dissolved in DMSO (100 μ L/well). The absorbance of each well was measured at OD_{580 nm} at r.t. on a POLARstar Omega microtitre plate reader. IC₅₀ values were calculated using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, United States), as the concentration of analyte required for 50% inhibition of cancer cell growth (compared to negative controls). All experiments were performed in duplicate. Vinblastin was used as a positive control for the MTT assay showing an IC₅₀ value of 0.05 μ M for NCI-H460 lung cancer cell line and 0.01 μ M for SW-620 colon cancer cells.

Genome Sequencing, Annotation and Mining

The genomic DNA of the isolated strains was sequenced using the MiSeq Illumina platform in the 250 bases paired-end format at the Ramaciotti Centre (Sydney, NSW, Australia). The reads obtained were trimmed using Trimmomatic v0.32 (Bolger et al., 2014) and assembled using Velvet v1.2.10 (Zerbino and Birney, 2008), K-mers ranging from 31 to 171 (increasing 10 units per iteration) were tested and the largest assemblies in the lowest number of contigs were selected. RAST (Aziz et al., 2008) and antiSMASH version 3 (Weber et al., 2015) were used for genome annotation.

Phylogenomic Analysis of PTMs Biosynthesis

For the phylogenomic analysis of PTMs, their BGCs were mined from a genomes database including the genomes of our isolates, actinobacterial genomes selected for their taxonomic distribution and genomes from non-actinobacteria species reported to produce PTMs (Zhang et al., 2016). The sequences were retrieved from GenBank. PTM BGCs were analyzed using the CORASON-BGC pipeline (CORe Analysis of Synthenic Orthologs of Natural products BGCs²) and the ikarugamycin BGC as deposited in MiBiG database (accession number: BGC0001435) (Medema et al., 2015) as query. First, we searched for IkaB homologs using BlastP (*e*-value 0.000001, bitscore 1000). Second, neighborhoods of 15 genes upstream and downstream *ikaB* were obtained from the genomes previously annotated using RAST (Aziz et al., 2008). Finally, the selected gene neighborhoods were analyzed using bidirectional best-hits against the ikarugamycin BGC to identify further syntenic homologs. The gene neighborhoods sharing at least three syntenic homologs with *E*-values of less than 1E-6 were considered orthologous PTM BGCs. The amino acid sequences of the homologs of the NRPS-PKS (IkaA) and the beta subunit of the RNA polymerase (rpoB) from selected genomes were identified with BlastP (*e*-value 0.00000001 for both, bitscore 4000 for *ikaA* and 200 for *rpoB*) extracted, aligned, trimmed and the resulting matrix was used for phylogenetic reconstruction with MrBayes v.3.2.6 (Huelsenbeck et al., 2001) using a mixed substitution model for a million generations. This phylogenetic tree was used to order the representation of the BGCs analyzed and obtained with CORASON-BGC.

RESULTS

Isolation of *Streptomyces* spp. from Marine Cone Snails

Five venomous cone snail specimens were collected and transported from the Great Barrier Reef to the microbiology laboratory, in local ocean water at room temperature (~26°C). Detailed morphological examinations indicated that the collected cone snail specimens belonged to three different taxonomic groups, each one belonging to a different species, namely, *C. emaciatus* and *C. flavidus* (*Virgiconus* group), *C. miles* (*Rhizoconus* group) and *C. coronatus* and *C. ebraeus* (*Virroconus* group). A total of 150 microbial strains were isolated from dissected cone snail tissues (venom duct, stomach foot and hepatopancreas) using ISP-4 solid media. Environmental sampling (rock surface areas and organisms found near the cone snails such as sea stars and sea gherkins) during cone snail collection ruled out a plausible environmental contamination given the lack of growth of *Streptomyces*-like organisms. We also found that isolation of PTM producing strains was tissue independent, as all strains were obtained from different organs of the snail (Supplementary Table S1). Most of the microbial isolates (70%) were non-sporulating unicellular bacteria while the rest

²<https://github.com/nselem/EvoDivMet/tree/master/CORASON>

were spore-forming bacteria, and only one fungus. Based on microscopic and macroscopic phenotypes (e.g., hyphae forming, pigmentation, formation of aerial mycelium and sporulation) (**Figure 1**), we selected a total of five *Streptomyces* isolates to explore their metabolic potential, taking into consideration that all the selected strains were derived from different cone snails. For simplicity, we assigned the following isolation codes: CMB-CS038 (*C. miles*), CMB-CS145 (*C. flavidus*), CMB-CS132 (*C. emaciatus*), CMB-CS143 (*C. coronatus*), and CMB-CS138 (*C. ebraeus*).

Initial Metabolic Profiling Shows That All Cone Snail-Associated *Streptomyces* spp. Produce a Similar Subset of PTMs

Streptomyces strains were first cultured on ISP-4 solid media. After 240 h, extracellular metabolites were extracted using 3:1 MeOH:EtOAc, then dried *in vacuo*, resuspended in MeOH, and analyzed by HPLC-DAD-MS. Initial HPLC-DAD-MS chromatograms showed identical chemical profiles for all five different strains over retention time (t_R) 8.0 to 13.0 min, inclusive of common UV-vis chromophores (λ_{max} 220 and 323) and ESI-MS m/z ions (m/z 511 [M-H] $^-$, m/z 509 [M-H] $^-$, m/z 493[M-H] $^-$ and m/z 491 [M-H] $^-$) (**Figure 2**). Additionally, the same solvent extracts also exhibited antifungal and cytotoxic properties when tested against *Candida albicans* and two human cancer cell lines (SW-620 and NCI-H460) (data not shown).

Identification and Biological Activity of PTMs Produced by Cone Snail-Associated *Streptomyces* Isolates

We evaluated *Streptomyces* CMB-CS038 on four different media (ISP-4 agar, marine agar, nutrient agar and R2A agar) and detected significant production of PTMs only on ISP-4 agar. In order to elucidate the chemical structures of the target PTMs, we scaled up the cultivation (400 \times ISP-4 petri plates). The resulting extract was fractionated by semi-preparative HPLC to afford PTM 1. Comparison of the NMR (Supplementary Table S2 and Figures S2–S4) and ECD data (Supplementary Figure S5) for **1** with the literature data confirm it to be identical to dihydromaltophilin (Graupner et al., 1997), the absolute configuration of which was solved in 2015 (Xu et al., 2015). The yield of three minor PTMs **2–4** co-metabolites (**Figure 2**) was not sufficient for NMR spectroscopic analysis; however, HPLC-DAD-MS analysis using single ion extraction (SIE) and HPLC co-injection with authentic standards confirmed the presence of xanthobaccin C (**3**) (Hashidoko et al., 2000) and the frontalamide precursor FI-3 (**4**) (Blodgett et al., 2010) (**Figure 3**). We sourced authentic standards xanthobaccin C and frontalamide intermediate FI-3 and confirmed their structures by *de novo* spectroscopic analysis, and comparison with literature data (Blodgett et al., 2010). We determined that **2** is new to the literature, and based on the molecular formula and biosynthetic grounds, we tentatively assign the structure as Δ^{30} -dihydromaltophilin. We attribute common absolute configurations to co-metabolites (**1–4**). Due

to limited material, biological assays were only performed for dihydromaltophilin (**1**), which showed antifungal activity against *Candida albicans* (IC_{50} 3 μM) and cytotoxicity against human colon (SW-620, IC_{50} 3.0 μM) and lung (NCI-H460, IC_{50} 5 μM) carcinoma cells (Supplementary Figures S7, S8). See the section “Discussion” (below) for commentary on ambiguities, redundancies and limitations in PTM trivial nomenclature and structure assignments.

Cone Snail-Associated *Streptomyces* Are Taxonomically Related, Have Reduced Genomes and Conserved PTM BGCs

The observation that different cone snail-associated *Streptomyces*, isolated from different cone snails, produce the same subset of antifungal PTMs is intriguing, and led us to speculate an ecological and evolutionary relationship between *Conus* and their *Streptomyces*. To gain insights into this we sequenced the genomes of five cone snail-associated *Streptomyces* isolates, and used a phylogenomic approach to establish taxonomic relationships, with a view to reconstruct the evolution of the PTM biosynthetic pathway.

Preliminary analysis of the 16s rRNA sequence amplified from our isolates indicated a close genetic association with *Streptomyces albus* J1074, a terrestrial strain with a long-standing laboratory history, which is known to produce PTMs and has been exploited as a heterologous host due to its limited biosynthetic repertoire and small genome size (Olano et al., 2014). The taxonomic relationships of our strains were further investigated with an emphasis on PTM producer strains whose genomes were available, using the *rpoB* molecular marker, which provides better resolution for the genus *Streptomyces* than the 16s rRNA (**Figure 4**). Our phylogenetic analysis strongly suggests that all cone snail-associated *Streptomyces*, irrespective of the *Conus* species, are closely related and cluster in the same clade with the previously reported sponge-associated strains GVA94-10 and PVA 94-07 (Ian et al., 2014) and with *Streptomyces albus* J1074 (Zaburannyi et al., 2014).

Interestingly, all these *Streptomyces* are known to produce PTMs and have reduced genomes compared with the average *Streptomyces* genome length (8.3 Mbp), with a standard deviation of 1.1 Mbp as calculated from 395 publicly available *Streptomyces* genomes. Consistently, the genomes of our five *Streptomyces* isolates include identical PTM BGCs and reduced genomes with an average genome length of 7 Mbp (**Figure 4**). Overall, these observations suggest a taxonomic specificity, genome reduction and a conserved PTM BGCs, as common traits in marine host-associated *Streptomyces* (**Figure 4**).

Evolutionary Reconstruction of the PTM Biosynthetic Pathway

Given the widespread presence of the PTM BGCs in host-associated *Streptomyces*, we decided to study BGC evolution in more detail. Using a phylogenomic approach, we identified 134 PTM BGCs (Supplementary Figure S6), from which a subset of 31

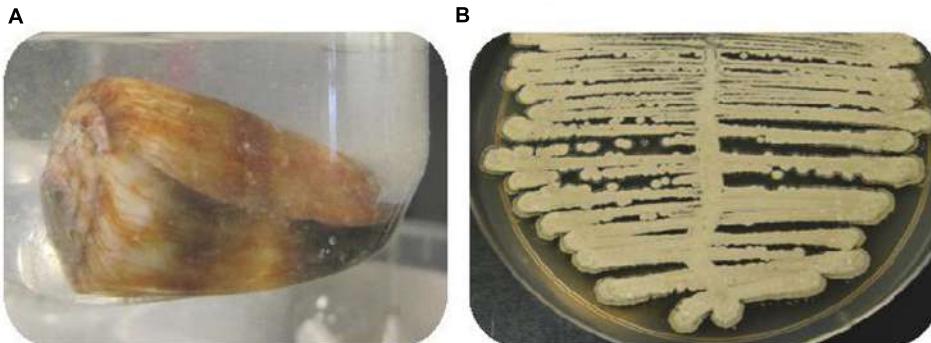


FIGURE 1 | (A) Image of cone snail *C. miles* **(B)** ISP-4 agar plate cultivation displaying the common phenotype of *Streptomyces* sp. after 10 days incubation at 26.5°C.

BGCs was selected for having known products and representing diverse taxonomic groups. Our analyses show that the PTM BGC is conserved and widespread within Actinobacteria, and that the three members of the Gammaproteobacteria class included in the matrix, namely, *Lysobacter gummosus*, *L. enzymogenes*, and *Saccharophagus degradans*, seem to have acquired the biosynthetic capacity to produce PTMs (maltophilin-related), most likely through horizontal gene transfer from Actinobacteria. Moreover, assuming that all PTM chemical variants are the result of sequence divergence within their BGCs, we established direct correlations between sequence phylogeny, the presence/absence of enzymes within the BGC, and the chemical structures of PTMs produced by these biosynthetic systems. Our analyses included both the data obtained in this study for the cone snail-associated strains, and that available in the literature (Zhang et al., 2016; Saha et al., 2017).

We found that the PTM BGCs are highly conserved, and given the current dataset, the enzymatic repertoire of the family includes only 7 enzymes: a PKS-NRPS hybrid system, a sterol desaturase (SD), a cytochrome P450 (CYP450), an oxidoreductase (OxR) and 3 dehydrogenases, named dehydrogenase 1 (DH1), 2 (DH2), and 3 (DH3), for simplicity (Figure 5A). We also found that the hybrid PKS/NRPS and DH1 are the only genes that are universally conserved. To a lesser extent, the OxR is highly conserved, as the only BGCs lacking this gene are those from *Saccharothrix syringae*, *Saccharopolyspora spinosa*, *Saccharopolyspora jianaxiensis*, plus three members of the *Streptomyces* genus (*Streptomyces* sp. NRRL F5135, *Streptomyces* sp. Mg1 and *Streptomyces scopuliridis*) (Figure 5A). OxR has been linked to the formation of the inner-membered ring, either in PTMs with three or two rings. A total of eleven BCGs analyzed lack the SD coding gene. SD is responsible for the addition of a hydroxyl group at the C-4 (see Figure 3 for the numbering system) in the latest stage of the pathway. The cytochrome P450 coding gene is also a well-conserved gene, only missing in *Salinispora arenicola*, *Actinokineospora* sp., *Saccharothrix syringae* and two members of the *Streptomyces* family (*Streptomyces* sp. NRRL WC 3742 and *S. aureofaciens*) (Figure 5A). Within this context, it is interesting to note that the

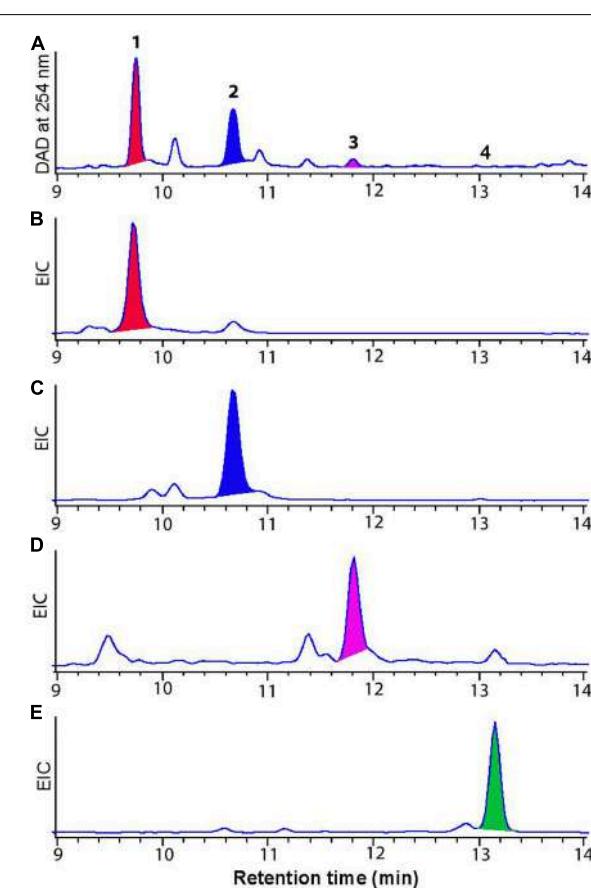


FIGURE 2 | (A) HPLC-DAD-MS (254 nm) chromatogram of cone snail-associated *Streptomyces* sp. extracts cultivated on ISP-4 agar **(B)** Single ion extraction (SIE) at m/z 511 [M-H]⁻ corresponding to dehydromaltophilin **(1)** **(C)** at m/z 509 [M-H]⁻ corresponding to **2**, **(D)** at m/z 493 [M-H]⁻ corresponding to xanthobaccin **(3)**, and **(E)** at m/z 491 [M-H]⁻ corresponding to Fl-3 **(4)**.

strains isolated from cone snails show the most conserved and widespread PTM BGC, encoding for the hybrid PKS/NRPS, sterol desaturase, DH 1 and DH 2, oxidoreductase and cytochrome

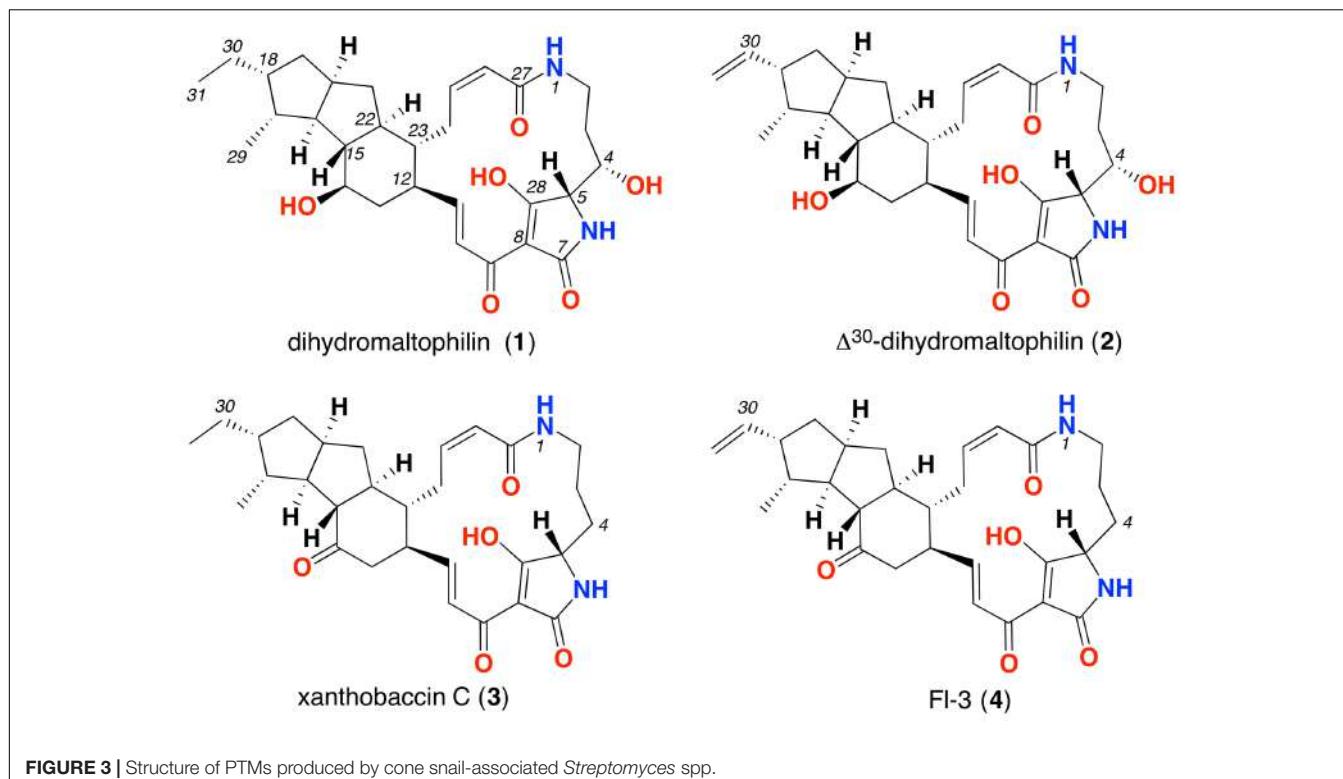


FIGURE 3 | Structure of PTMs produced by cone snail-associated *Streptomyces* spp.

P450 genes (Figure 5A). The reconstruction of the evolutionary events that lead to the PTM chemical diversity is shown in Figure 6.

DISCUSSION

Prior to addressing our experimental data, it is first necessary to comment on PTM scientific literature, and in particular ambiguities and redundancies associated with the assignment of chemical structures, trivial nomenclature and scaffold numbering schemes. The structure elucidation of any natural product is critically dependent on the isolation of a pure sample of sufficient quality and size to enable the acquisition and analysis of spectroscopic data (i.e., NMR, MS, $[\alpha]_D$, CD, UV-vis). Such an analysis must unambiguously assign the planar structure, enabled by the use of a consistent and informative carbon skeleton (scaffold) numbering scheme. With a definitive planar structure in hand, structure elucidation can advance to assigning relative and then absolute configurations. New natural products are typically allocated a unique trivial name, while re-isolated known natural products retain the prior-published trivial nomenclature. Where a class of natural products (i.e., PTMs) is isolated by multiple researchers, from varying sources, at different times, it is best practice to use of a consistent (common) scaffold numbering scheme and related trivial nomenclature. Unfortunately, the published history of bacterial PTMs diverges from best practice, with competing (even redundant) trivial nomenclatures and numbering schemes, and on occasion inadequate levels of spectroscopic

characterisation. Consider the historical summary outlined below.

Maltophilins (Xanthobaccins, HSAFs, Lysobacteramide B, Frontalamides, FIs, Pactamides)

A planar structure for maltophilin was first reported in 1996 from *Stenotrophomonas maltophilia* R3089 (Jakobi and Winkelmann, 1996). In 1999 maltophilin was re-isolated from *Stenotrophomonas* sp. strain SB-K88, at which time it was assigned a partial relative configuration, and designated with the additional trivial name xanthobaccin A (Hashidoko et al., 1999). Although these same authors went on to acknowledge co-metabolites xanthobaccins B and C (Nakayama et al., 1999; Hashidoko et al., 2000), these structure assignments were not validated by spectroscopic characterization. Dihydromaltophilin was first reported in 1997 from a *Streptomyces* sp., as a co-metabolite with maltophilin (Graupner et al., 1997). Although both co-metabolites were assigned partial relative configurations consistent with earlier reports, they were also designated the additional trivial names A90931a and A90931b. To add to the confusion, in 1999 dihydromaltophilin was re-isolated from *Xanthomonas* sp KB-K88 (Nakayama et al., 1999), and designated the trivial name xanthobaccin B, and in 2007 was re-isolated from *L. enzymogenes* strain C3 (Yu et al., 2007), and designated the trivial name heat-stable antifungal factor (HSAF). This confusion was compounded by inconsistent use of carbon (scaffold) numbering, and its incorporation into subsequent trivial nomenclature. For example, in 2012

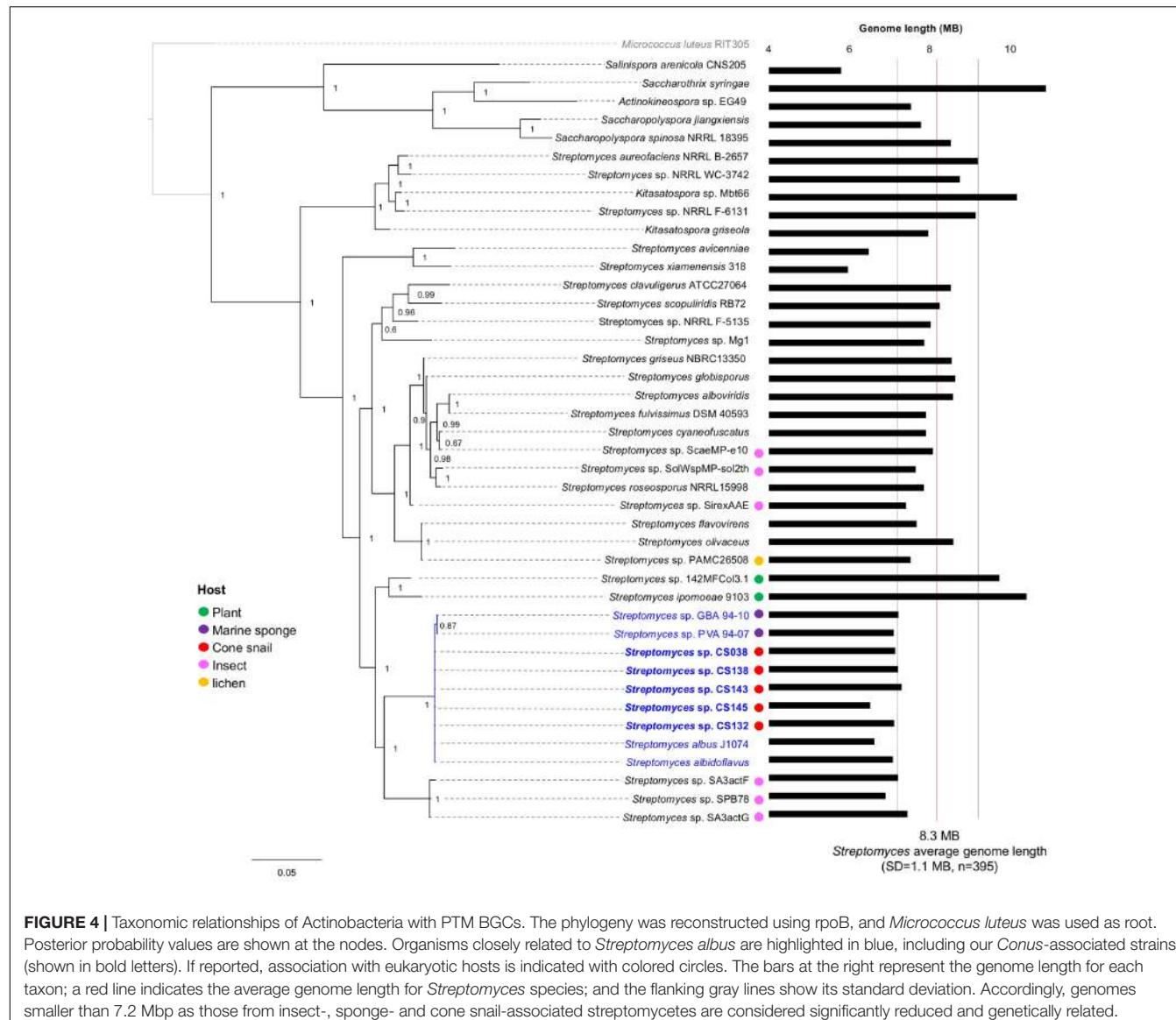


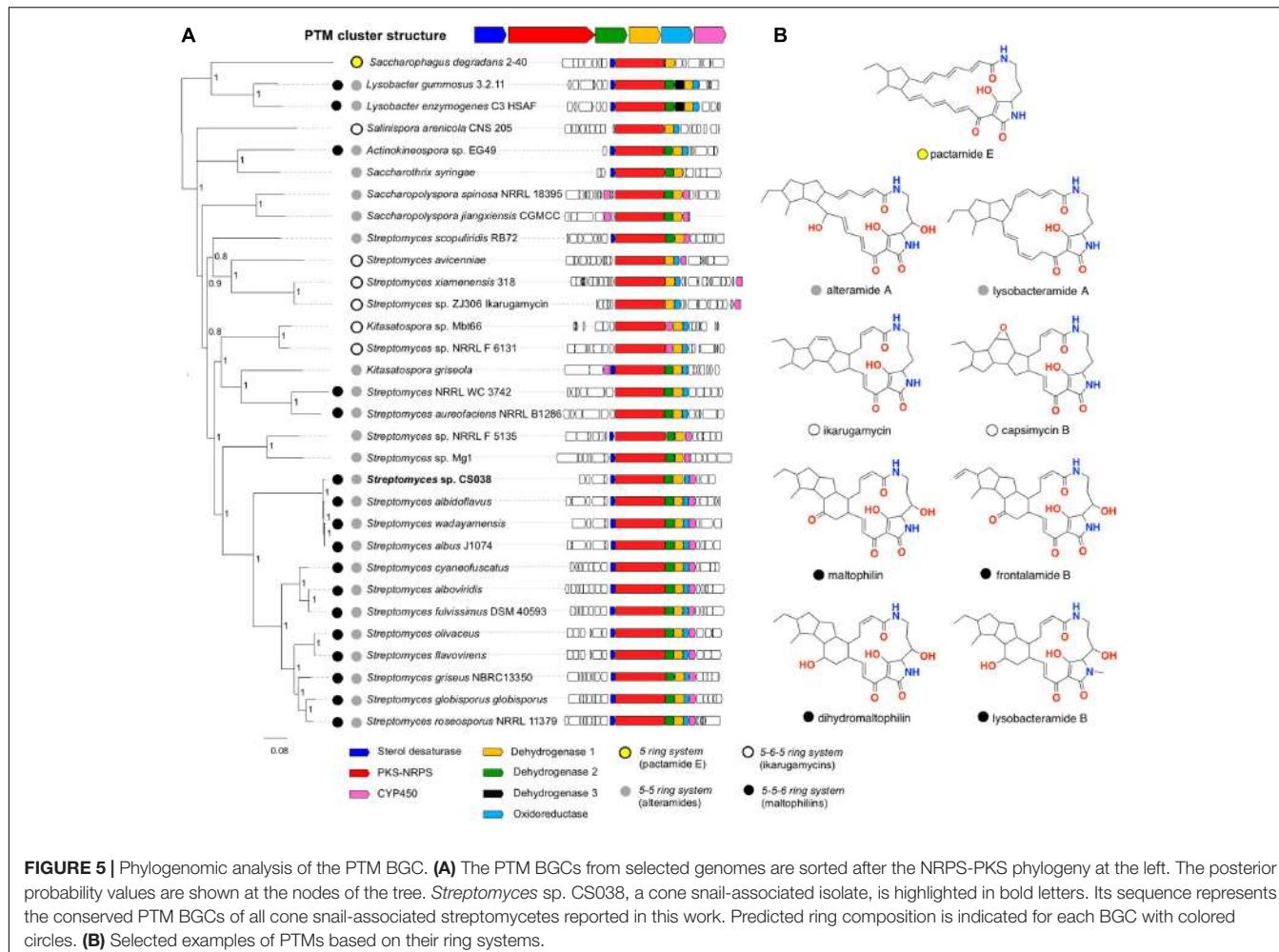
FIGURE 4 | Taxonomic relationships of Actinobacteria with PTM BGCs. The phylogeny was reconstructed using *rpoB*, and *Micrococcus luteus* was used as root. Posterior probability values are shown at the nodes. Organisms closely related to *Streptomyces albus* are highlighted in blue, including our *Conus*-associated strains (shown in bold letters). If reported, association with eukaryotic hosts is indicated with colored circles. The bars at the right represent the genome length for each taxon; a red line indicates the average genome length for *Streptomyces* species; and the flanking gray lines show its standard deviation. Accordingly, genomes smaller than 7.2 Mbp as those from insect-, sponge- and cone snail-associated streptomycetes are considered significantly reduced and genetically related.

and 2015 follow-up studies, *L. enzymogenes* strain C3 was also reported to yield 3-deOH-HSAF (Li et al., 2012), and lysobacteramides A and B (Xu et al., 2015), respectively. Of note this latter report successfully assigned an absolute configuration to dihydromaltophilin. In 2010 the biosynthetically related frontalamides A and B, and three biosynthetic intermediates FI-1, FI-2 and FI-3 were isolated from *Streptomyces* sp. SPB78 (Blodgett et al., 2010), however, it is important to note that none of these structure assignments were supported by an adequate level of spectroscopic characterization or data analysis. In 2017 pactamides A-F were reported from the marine-derived *Streptomyces pactum* SCSIO 02999 (Saha et al., 2017). The proliferation of trivial nomenclature and numbering systems, and inadequately characterized and documented PTM structures is unfortunate. This report acknowledges the published trivial nomenclature (except where it is redundant), and employs the numbering scheme of Graupner et al. (1997) (see

Figure 3). We also group the maltophilins, xanthobaccins, HSAFs, lysobacteramide B, frontalamides, FIs, and pactamides A-B, D and F in a common 5-5-6 ring system category (collectively known as maltophilins) (Figure 5B), based on the fact they share a common carbocyclic ring system embedded within the PTM scaffold.

Alteramide A (Aburatubolactam A, Lysobacteramide A, Pactamide C)

Alteramide was first reported in 1992 from a marine sponge-associated *Alteromonas* sp. (Shigemori et al., 1992), while the closely related aburatubolactam A was reported in 1996 from the marine mollusk-associated *Streptomyces* sp. SCRC-A20 (Bae et al., 1996). We group alteramide A, aburatubolactam A, lysobacteramide A, and pactamide C in a common 5-5 ring system category (collectively known as alteramides) (Figure 5B).



Ikarugamycins (Butremycin, Capsimycins)

The planar structure for ikarugamycin was first reported in 1977 from *Streptomyces phaeochromogenes* var. *ikaruganensis* Sakai (Ito and Hirata, 1977), and its absolute configuration confirmed by total synthesis (Pacquette and Macdonald, 1989). Butremycin, a hydroxylated ikarugamycin, was reported in 2014 from a mangrove river sediment-derived *Micromonospora* sp. K310 (Kyeremeh et al., 2014), while isoikarugamycin, 28-N-methylikarugamycin and 30-oxo-28-N-methylikarugamycin were reported in 2015 as co-metabolites with ikarugamycin from a marine sediment-derived *Streptomyces zhaozhouensis* CA-185989 (Lacret et al., 2015). In 2003 ikarugamycin epoxide was reported as a co-metabolite with ikarugamycin (and ripromycin) from *Streptomyces* sp. Tü 6239 (Bertasso et al., 2003). Capsimycin was first reported in 1979 from *Streptomyces* sp. C 49-87 (Aizawa et al., 1979), and later in 2017 as a co-metabolite with capsimycins B-G from a mangrove-derived *Streptomyces xiamenensis* 318 (Yu et al., 2017), with this latter report renaming ikarugamycin epoxide as capsimycin B. We group ikarugamycins, butremycin, and capsimycins in a common

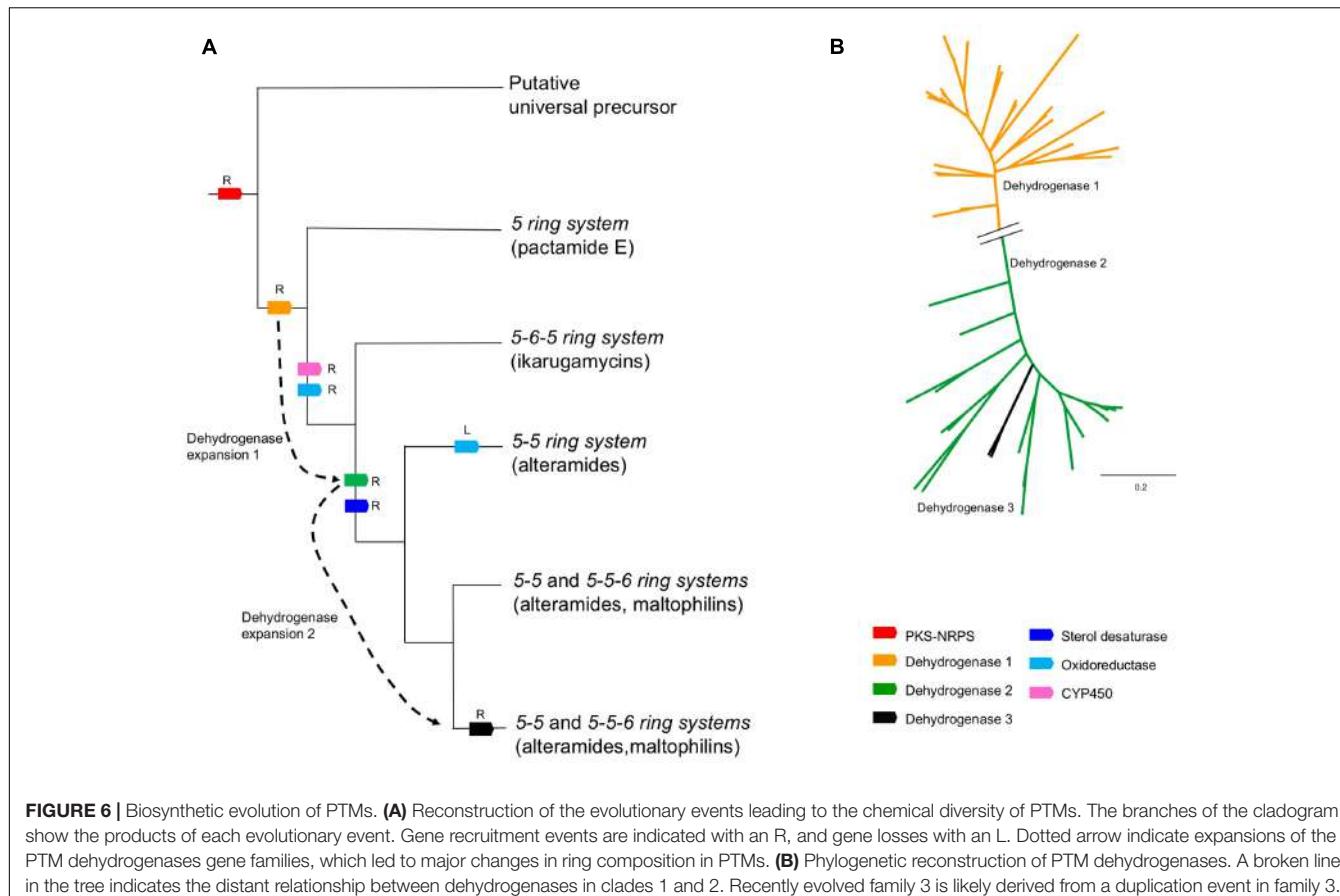
5-6-5 ring system category (collectively known as ikarugamycins) (Figure 5B).

Pactamide E

In 2017 pactamides A-F were reported from the marine-derived *Streptomyces pactum* SCSIO 02999 (Saha et al., 2017). Building on our categories outlined above, we acknowledge pactamide E as the sole known exemplar of a 5-ring system category of PTM (Figure 5B).

The PTM biosynthetic potential of *Streptomyces* sp. CMB-CS038 was studied by cultivation in different culture conditions and media, generating profiles comparable to other host-associated *Streptomyces* and with a generally very low level of production under standard culture conditions. We determined that ISP-4 agar (inorganic salt starch media) was optimal for the production of PTMs, with slower growth and a lack of PTMs production occurring in low salt media (Supplementary Figure S1). This is consistent with previous studies of marine adaptation in *Streptomyces albus* and related strains (Ian et al., 2014).

The genomes of cone snail-derived *Streptomyces* were sequenced and revealed a reduction in genome size, and



share a common clade taxonomically with other host-associated *Streptomyces*. This observation is in agreement with the fact that symbiotic (or associated) microorganisms display reduced genomes (McCutcheon and Moran, 2012). For example, two *Streptomyces* (S. sp. PVA 94-07 and S. sp. GBA 94-10) associated with sponges, which have been identified as closely related species to *Streptomyces albus* J1074, have a minimized genome of 6.8 Mb (Ian et al., 2014).

In addition to taxonomic specificity, we speculated that PTMs may deliver an ecological advantage in providing anti-infective protection against fungal pathogens. There are several observations that support this view. Firstly, despite routinely isolating marine-derived fungi from all marine substrates examined in our laboratory (e.g., sand, molluscs, fish, and algae), the cone snail samples examined during this study were remarkably deficient in fungal species. Secondly, literature accounts note the PTM alteramide very likely plays a key role in keeping coral reefs free of fungal pathogens (Moree et al., 2014). Finally, our biological activity assays correlate with previous observations of dihydromaltophilin (Graupner et al., 1997), and confirm significant antifungal activity.

We based the evolutionary reconstruction of PTM biosynthetic pathway on a relatively straightforward concept: all PTM BGC structural variations must be encoded in the

genome, thus, a deep look into the phylogeny based in the key conserved enzymes will directly highlight the genomic traits for a given chemical structure variation. For instance, we found that the hybrid PKS/NRPS and DH 1 constitute the core of the PTM BGC and potentially the minimal biosynthetic unit to form PTMs. This is supported by previous studies showing that DH 1 is essential for the formation of the *5-ring system* (Saha et al., 2017), which prompts us to predict involvement of the cryptic BGC found in *Saccharophagus degradans* 2–40.

In addition, the analysis shown indicates that the presence/absence of the dehydrogenase homologs determines the PTM ring composition (Figure 6A). This assumption was supported upon a dehydrogenase-based phylogenetic analysis that shows a remote expansion event leading to the evolution of DH1 and DH2, and a recent duplication event (DH3) (Figure 6B). Therefore, BGCs with the combo DH1/oxidoreductase will produce an ikarugamycins *5-6-5 ring system* (Figure 6A). The presence of the DH 1 and DH 2 correlates with the alteramides *5-5 ring system* (Figure 6A). Finally, DH 1, DH 2 and the oxidoreductase will assemble a maltophilins *5-5-6 ring system* (Figure 6A). While the lack of an oxidoreductase homolog is exclusive for the formation of a *5-5 ring system*, phylogenomics strongly suggests that bacteria producing the *5-5-6 ring system* can additionally

produce a *5-5 ring system*. For example, the *epi*-alteramide BGC produced by *Streptomyces albus* J1074 harbors DH 1, DH 2 and an oxidoreductase (Olano et al., 2014). On a chemical level, both lysobacteramide A (*5-5 ring system*) and lysobacteramide B (*5-5-6 ring system*) have been isolated from *L. enzymogenes* (Xu et al., 2015), which is consistent with our hypothesis. Consistent with this analysis, we identified the production of four PTMs (**1–4**) from *Streptomyces* sp. CMB-CS038, all displaying a *5-5-6 ring system* (Figure 3).

Our phylogenetic analysis also suggests that *Lysobacter* sp. acquired, most likely from an actinobacterium, the maltophilins BGC via horizontal gene transfer – capable of producing both maltophilin and dihydromaltophilin. *Lysobacter* PTM BGCs are unique as they include an extra dehydrogenase homolog (DH 3), which to the best of our knowledge, has not been linked to a particular function (Figure 6A). *Lysobacter* PTM BGCs also need to accommodate lysobacteramide B which features an *N*-methylated tetramic acid moiety. Regarding the sterol desaturase, our analysis was consistent with previous experimental evidence showing that the presence of this enzyme correlates with C-4 hydroxylation (see Figure 3 for the numbering system). In addition, our study shows that ikarugamycins lack C-4 hydroxylation step, and the related BGCs lack a sterol desaturase. Finally, activity of the cytochrome P450 homolog (CYP450) has been recently demonstrated through gene knock-out and complementation experiments in a capsimycin producing *Streptomyces* sp. Cytochrome P450 activity is responsible of an epoxide ring formation in the capsimycin and capsimycin B, and further side chain hydroxylation in capsimycin G (Yu et al., 2017).

Altogether, our combined chemical, bioactivity and phylogenomic analysis shows that, (i) there is a taxonomic bias of isolated cone snail-associated *Streptomyces* toward other host-associated *Streptomyces* species; (ii) this bias is reflected at a genomic level (i.e., common reduced genome), and biosynthetic level (i.e., common PTM BGCs), and (iii) there is an underlying antifungal property for PTMs that offers an ecological (survival) advantage. Our investigation also highlights the importance of combining natural products and analytical chemistry, next generation sequencing, and phylogenomic analysis, to achieve a better understanding of natural product biosynthesis, ecology and evolution. Specifically, our work emphasizes how metabolic pathway evolution within its ecological and taxonomic context can provide interesting

mechanistic hypotheses pinpointing specific genetic targets responsible of structural modifications.

AUTHOR CONTRIBUTIONS

MQ designed experiments, performed microbiological isolations, performed and analyzed spectroscopic and analytical experiments and wrote the manuscript. CL-C performed genomic analysis, phylogenomic analysis and wrote the manuscript. PC-M performed and analyzed phylogenomic analyses and genome mining and wrote the manuscript. AS performed analytical experiments, analyzed spectroscopic data and wrote the manuscript. EM analyzed genomic data and wrote the manuscript. FB-G analyzed genomic and phylogenomic data and wrote the manuscript. RC designed the analytical experiments, analyzed the analytical data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.02305/full#supplementary-material>

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New Dimensions of Research on Actinomycetes: Quest for Next Generation Antibiotics

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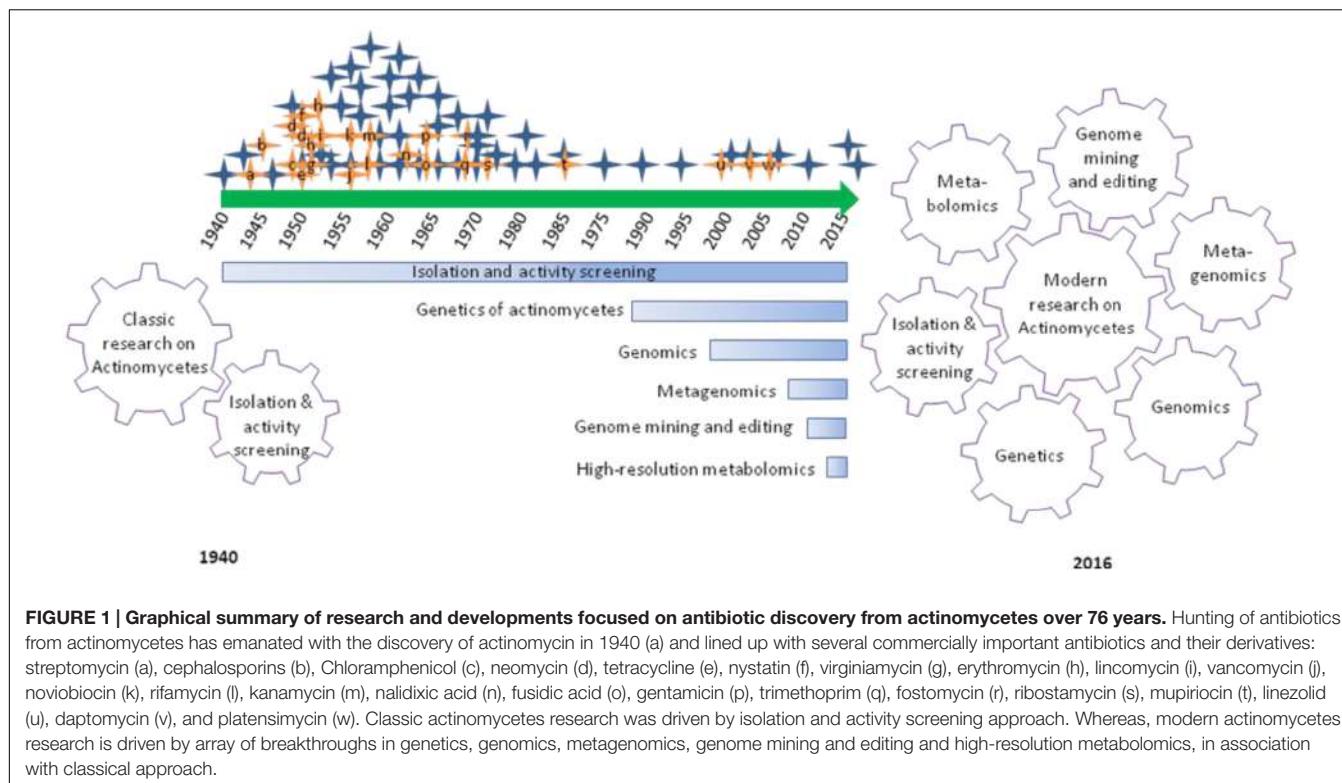
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Starting with the discovery of streptomycin, the promise of natural products research on actinomycetes has been captivating researchers and offered an array of life-saving antibiotics. However, most of the actinomycetes have received a little attention of researchers beyond isolation and activity screening. Noticeable gaps in genomic information and associated biosynthetic potential of actinomycetes are mainly the reasons for this situation, which has led to a decline in the discovery rate of novel antibiotics. Recent insights gained from genome mining have revealed a massive existence of previously unrecognized biosynthetic potential in actinomycetes. Successive developments in next-generation sequencing, genome editing, analytical separation and high-resolution spectroscopic methods have reinvigorated interest on such actinomycetes and opened new avenues for the discovery of natural and natural-inspired antibiotics. This article describes the new dimensions that have driven the ongoing resurgence of research on actinomycetes with historical background since the commencement in 1940, for the attention of worldwide researchers. Coupled with increasing advancement in molecular and analytical tools and techniques, the discovery of next-generation antibiotics could be possible by revisiting the untapped potential of actinomycetes from different natural sources.

Keywords: actinomycetes, natural products, antibiotics, drug discovery, genomics, metabolomics

INTRODUCTION

Actinomycetes are ubiquitous Gram-positive bacteria that constitute one of the largest bacterial phyla with characteristic filamentous morphology and high G+C DNA. The actinomycetes have been recognized as premier source and inspiration for a substantial fraction of antibiotics that play an important role in human health. The most striking fact is that these filamentous bacteria have



evolved with the wealth of biosynthetic gene clusters and thereby show an unprecedented potential in production biologically active natural product scaffolds. However, last two decades has seen a move by pharmaceutical giants away from microbial natural product discovery efforts, and such efforts continue to flourish in research institutes with promising results. The continued research efforts of academic research institutes, with post-genomic technological innovations, rejuvenate natural product research and compose a clarion call to worldwide researchers for tuning into microbial natural products research.

THE CLASSIC ACTINOMYCETES RESEARCH

If we look back to about 76 years of actinomycetes research that focused on hunting bioactive metabolites of public welfare, over 5000 compounds have been reported and contributed to the development of 90% of commercial antibiotics being used for either clinical or research needs. In this long course, actinomycetes research evolved several aspects from isolation and activity screening to modern post-genomic secondary metabolites research (Figure 1). The first report of streptomycin by Selman Waksman and associates in the 1940s and subsequent development as drug encouraged pharmaceutical companies and researchers to put their large scale efforts on microbial natural products research (Demain and Sanchez, 2009). The efforts were largely depending on the recovery of microorganisms from diverse environmental samples, and screening for the desired bioactivity. The approach brought the

golden era (1950–1970) of antibiotic discovery evidenced by the commercialization of several life-saving antibiotics including streptomycin, vancomycin, rifamycin, and so on (Mahajan and Balachandran, 2012). In subsequent decades, the rediscovery of known compounds and technical challenges associated with purification and structure elucidation of new compounds largely declined the classic efforts (Bérdy, 2012). Despite the evidence of a decline in microbial natural products research, continued innovations in sampling and acquisition of potential actinomycetes from previously unexplored sources are being continued by several academic research groups and mitigate risks of the rediscovery of known compounds and augmented availability of diverse actinomycetes that are fundamental matters to the long term actinomycetes research.

IN PROGRESS

Progress is crucial in several aspects of actinomycetes research that includes (1) isolation and dereplication of actinomycete isolates, (2) prediction and identification novel compounds, (3) enhancing production titers of potential compounds, (4) uncovering genome information and associated biosynthetic potential, (5) collection and processing of genomic data, (6) mining, editing and heterologous expression of cryptic gene clusters, and (7) comprehensive metabolic profiling, under a broad spectrum of main areas such as genetics, genomics and metabolomics.

Establishing actinomycete resources is one of the basic requirements for culture-dependent natural products research.

To address this, researchers are learning how to cultivate the unexplored actinomycete biodiversity in diverse environments and such efforts have led to cultivation of numerous novel actinomycetes from marine sediments (Becerril-Espinosa et al., 2013), hydrothermal vents (Thornburg et al., 2010), solar salterns (Jose and Jebakumar, 2013), desert soils (Mohammadipanah and Wink, 2016), red soils (Guo et al., 2015), sponges (Sun et al., 2015), insects (Matsui et al., 2012; Kurtbøke et al., 2015), and plants (Masand et al., 2015). On the other hand, dereplication of isolated strains has attained a new pitch with gene specific as well as metabolic fingerprinting approaches (Hou et al., 2012; Forner et al., 2013). Collectively, the united success in isolation and dereplication facilitates the prioritization of the isolates which could be cellular factories with the innate biosynthetic capability to produce novel compounds. One such approach has been practiced to isolate 64 distinctive actinomycetes from 12 different marine sponge species, and to prioritize two unique strains that showed anti-trypanosomal activity as well as uniqueness in metabolomic profile and richness of unidentified natural products (Cheng et al., 2015).

Prediction and identification of novel compounds from actinomycetes including those with low production titers have become relatively straight forward through the advent of high-resolution liquid chromatography-mass spectrometry (HR-LC-MS) and allied database search (Tawfik et al., 2013; Doroghazi et al., 2014; Wu et al., 2016). Recently, Wu et al. (2016) were able to demonstrate the employability of NMR-based metabolic profiling method to streamline microbial biotransformation and to determine the best harvesting time of actinomycetes for antibiotic production. Technical breakthroughs also in gene level understanding and recombineering of producer strains provide an attractive choice to improve the production titers of structurally complex natural products by microbial fermentation (Zhang et al., 2016).

Exploring the biology of secondary metabolites production in actinomycetes through genetics has provided a foremost share to our current knowledge. Dramatic and sustained increase in understanding the genetics and enzymology of secondary metabolites biosynthesis in actinomycetes, especially *Streptomyces* throughout the 1990s have also facilitated endurance of natural product search in this admirable bacterial group. As a noteworthy foundation, *S. coelicolor* A3(2) has genetically been recognized as a model for the actinomycetes, and the whole genome was announced with versatile *in vivo* and *in vitro* genetics (Bentley et al., 2002). The genome analysis of *S. coelicolor* A3(2) has revealed the abundance of previously uncharacterized gene clusters, metabolic enzymes, particularly those likely to be involved in the production of natural products. As a latest accomplishment, the marine actinomycete genus *Salinispora* has been established as a robust model organism for natural product research (Jensen et al., 2015). It has remarkable biosynthetic capacities with 17 diverse biosynthetic pathways of which only four had been linked to their respective products.

The genome information of cultured and uncultured actinomycetes is being promptly updated. Over 1304 actinomycetes genome have been reported as on March 2016 and with the advent of molecular genetics and next-generation

genome analysis rapid submissions are expected in near future. Analyses of genomes of actinomycetes have revealed that numerous 'cryptic' or 'orphan' biosynthetic gene clusters with the potential to direct the production of an ample number of novel, structurally diverse natural products (Challis, 2014; Gomez-Escribano et al., 2016). Subsequently, mining of actinomycetes genome has sketched new directions into the ongoing drug discovery efforts. One such approach has been to mine a collection of 10,000 actinomycetes for novel phosphonic acids, and have laid an intriguing foundation for rapid, large-scale discovery of other classes of natural products (Ju et al., 2015).

Improvements made in bioinformatics methods, particularly specific for natural product gene cluster identification and functional prediction aids in the processing of bulk genomic data of actinomycetes (Alam et al., 2011; Doroghazi et al., 2014; Abdelmohsen et al., 2015). However, sufficient insights into the biology and ecology of antibiotic production are needed to understand the precise triggers and cues required to activate silent gene clusters (Abdelmohsen et al., 2015; Kolter and van Wezel, 2016).

As a great breakthrough, the advent of RNA-guided DNA editing technology Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)/Cas9 substantially promises for application to genome modification in biosynthetic gene clusters of actinomycetes (Huang et al., 2015). Obviously, this molecular tool can be used in the engineering of non-model native hosts to heterologous production hosts for the biosynthesis of desired natural products. Continued technological and conceptual advances in engineering microbial hosts will open up opportunities to fully explore and harness Nature's immensely diverse chemical repertoire (Zhang et al., 2016).

FUTURE PERSPECTIVES

Actinomycetes have been recognized as a premier source of biopharmaceuticals especially antibiotics over several decades. Our universe is rich of diverse unexplored and underexplored environments that could be considered for isolation of novel members of actinomycetes. This could amend our actinomycetes repository with a continuous supply of novel biosynthetic gene clusters and natural product scaffolds on which current research reorient on. Continued advances in genomics and metabolomics reserve a next-generation natural products research and unwrap the wider opportunities on the exploitation of actinomycetes that represent an important asset for the discovery of pharmaceutically valuable compounds. The technological and conceptual advances will drive a transition of "searching for desired natural products" to "designing for desired products" from actinomycetes. Through this article, it is evinced that despite an interim decline in actinomycetes research, new avenues are open now and seek the active attention of researchers throughout the world. Those countries well endowed with the natural resources may deem to fund microbial natural products research especially actinomycetes research for extending the inventions of novel antibiotics of industrial

significance to triumph the escalating microbial resistance and infectious diseases.

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Reveromycins A and B from *Streptomyces* sp. 3–10: Antifungal Activity against Plant Pathogenic Fungi *In vitro* and in a Strawberry Food Model System

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This study was conducted to determine the antifungal activity of the metabolites from *Streptomyces* sp. 3–10, and to purify and identify the metabolites. Meanwhile, the taxonomic status of strain 3–10 was re-evaluated. The cultural filtrates of strain 3–10 in potato dextrose broth were extracted with ethyl acetate. The resulting crude extract at 1 and 5 µg/ml inhibited growth of 22 species in 18 genera of plant pathogenic fungi and Oomycetes, accounting for 92% of the total 24 tested species, suggesting that it has a wide antifungal spectrum. Two compounds were purified from the crude extract and were identified as reveromycins A and B, which demonstrated high antifungal activity against *Botrytis cinerea*, *Mucor hiemalis*, *Rhizopus stolonifer*, and *Sclerotinia sclerotiorum* under acidic pH conditions. Both the crude extract and reveromycin A from strain 3–10 at 10, 50, and 100 µg/ml showed high efficacy in suppression of strawberry fruit rot caused by the above-mentioned four pathogens. The efficacy was comparable to that of corresponding commercial fungicides (pyrimethanil, captan, dimetachlone) used in management of these pathogens. Morphological, physiological, and phylogenetic characterization showed that strain 3–10 is closely related to *Streptomyces yanglinensis* 1307^T, representing a novel phylotype in that species. This study reported a new strain with reveromycins-producing capability. The finding is important for further exploitation of reveromycins for agricultural use.

Keywords: *Streptomyces* sp. 3–10, reveromycins, plant pathogenic fungi, strawberry, antifungal activity, biological control

INTRODUCTION

Streptomyces species (Order: *Streptomycetales*, family: *Streptomycetaceae*) are Gram-positive, filamentous, and spore-producing actinobacteria with high G + C content in their genomes. They are generally regarded as pharmaceutically important biological resources, because many species of *Streptomyces* can produce various physiologically active metabolites, including antibiotics,

antimicrobial enzymes, antioxidants, as well as anti-inflammatory and anti-tumor compounds. It is estimated that up to 80% bioactive metabolites are produced by species of *Streptomyces* in Streptomycetales (Bérdy, 2012).

Streptomyces as well as other actinobacteria widely live or dwell in various terrestrial environments, including soil, plant, and air dust (Kettleson et al., 2013; Guo et al., 2015; Supong et al., 2016). While a few species of *Streptomyces* are plant pathogens (e.g., *S. scabies* caused potato scabies), majority of *Streptomyces* species live as saprophytes in soil or live as endophytes in plant tissues. The non-pathogenic *Streptomyces* usually produce beneficial effects on plant growth either through providing plants with nutrients from degradation of complex biological polymers like cellulose and chitin in soil (Brzezinska et al., 2013), or through secretion of plant hormones like indole-3-acetic acid, gibberellic acid, and zeatine (Solans et al., 2011). Meanwhile, many species of *Streptomyces* have been found to be capable of suppressing growth, development, and/or survival of plant pathogens (fungi, bacteria, nematodes, viruses) through diverse mechanisms, including production of antibiotics and antimicrobial enzymes (Anitha and Rabeeth, 2010; Zacky and Ting, 2013; Mander et al., 2016), hyperparasitism (Chen et al., 2016), and induction of plant resistance response (Conn et al., 2008; Lehr et al., 2008; Kurth et al., 2014). Therefore, *Streptomyces* species are important resources of biofungicides or biofertilizers for agricultural use.

Attempts to use *Streptomyces* species to control plant diseases can be traced back to 1927, when Millard and Taylor (1927) found that *Streptomyces praecox* (an obligate saprophyte, formerly known as *Actinomyces praecox*) effectively suppressed potato scabies when it was applied in soil either alone or together with green manures. Streptomycin produced by *S. griseus* was the first antibiotic successfully used for control of *Erwinia amylovora*, the causal agent of pear fire blight (Beer et al., 1984). Since then, extensive studies have been carried out to exploit *Streptomyces* as biofungicides and/or biofertilizers. Many *Streptomyces* species have been successfully developed into commercial biofungicides either based on their live spores such as Mycostop® (Minuto et al., 2006) and Rhizovit® (Berg et al., 2010), or based on their bioactive metabolites such as blasticidin S, kasugamycin, polyoxins, and validamycins (Kim and Hwang, 2007). Due to these successful applications, discovering new *Streptomyces* strains and identifying novel antibiotics from *Streptomyces* species continue to be a hot and attractive research area. Many novel antibiotics such as bafilomycin K produced by *Streptomyces flavotrichini* Y12-26 (Zhang et al., 2011), elaiomycins B produced by *Streptomyces* sp. BK190 (Kim et al., 2011) and novonestmycins produced by *S. phytohabitans* (Wan et al., 2015) are being evaluated as new biofungicides.

Reveromycins are the antibiotics first isolated from the cultures of *Streptomyces reveromyceticus* SN-593 in the early 1990s (Osada et al., 1991). Reveromycin A showed inhibitory effect on growth and proliferation of the human pathogen *Candida albicans* and the human cancer cell lines KB and K562 (Osada et al., 1991). Osada et al. (1991) indicated that reveromycin A had antifungal activity against plant pathogenic fungi. However, they did not provide the detailed information

about the plant pathogenic fungi inhibited by reveromycin A in that report (Osada et al., 1991). Therefore, whether or not reveromycin A and other reveromycins can be used to control plant fungal diseases remains unknown.

Wan et al. (2008) isolated an antagonistic strain (F-1) of *S. platensis* from a healthy leaf of rice (*Oryza sativa* L.). They found that *S. platensis* F-1 is an effective biocontrol agent for suppression of many plant pathogenic fungi, including *Botrytis cinerea*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*, the causal agents of tomato gray mold, rice sheath blight, and *Sclerotinia* stem rot of oilseed rape, respectively (Wan et al., 2008). In order to improve the antifungal activity of *S. platensis* F-1, Che (2011) conducted a study to mutate the wild type strain F-1 by combined treatments of the spores of strain F-1 with UV-C and LiCl. A putative mutant named strain 3-10 was found to have enhanced antifungal activity by approximately 100 times compared to that of the original strain F-1 (Che, 2011). However, the chemical identity of the antifungal metabolites produced by the putative mutant strain 3-10 remains unknown. Moreover, strain 3-10 differed greatly from the original strain F-1 in colony morphology on potato dextrose agar, implying that it may not be derived from *S. platensis* F-1 (Che, 2011). Therefore, the taxonomic status of strain 3-10 needs to be clarified.

The objectives of this study are: (i) to determine the antifungal spectrum of the crude extract from the cultures of *Streptomyces* sp. 3-10; (ii) to purify and identify the antifungal metabolites produced by *Streptomyces* sp. 3-10; (iii) to evaluate the efficacy of the metabolites from strain 3-10 in suppression of strawberry fruit rot caused by *B. cinerea*, *Mucor hiemalis*, *Rhizopus stolonifer*, and *S. sclerotiorum*; and (iv) to clarify the taxonomic status of *Streptomyces* sp. 3-10.

MATERIALS AND METHODS

Microbial Species and Cultural Media

Five species of bacteria and 24 species of fungi and Oomycetes were used in this study and their origins were listed in Table S1. Among these microbial species, *Streptomyces* sp. 3-10 was used for production of antifungal metabolites. It was isolated from a culture containing spores of *S. platensis* F-1 jointly treated by UV-irradiation and LiCl (Che, 2011). *Aspergillus niger* A-1 (a soil saprophyte) was used as indicator in bioassays to detect the antifungal activity of the metabolites of strain 3-10 (Shakeel et al., 2016). The remaining two species of Oomycetes, four species of bacteria, and 21 species of fungi were used to test the antimicrobial spectrum of the metabolites of *Streptomyces* sp. 3-10.

A total of 22 cultural media (BM, CDM, GA, GS-1, ISM, ISP-1 to ISP7, ISP-9, KB, NA, NB, OCD, PDA, PDB, SDM, TDM, and TYM) were used in this study. The full names of these media and their ingredient compositions were listed in Table S2.

Preparation of the Crude Extract

Spore suspension (1×10^8 spores/ml) of *Streptomyces* sp. 3-10 was inoculated in 250-ml Erlenmeyer flasks each containing 100 ml potato dextrose broth (PDB), 1 ml spore suspension per flask. The flasks were incubated on a rotary shaker (150 rpm)

at 28°C for 3 days for production of antifungal metabolites. The culture supernatant was collected by centrifugation at 6,000 × g for 10 min, and then extracted with ethyl acetate. The ethyl acetate phase was then dried by vacuum using R-250 Rotavapor® (BUCHI Corporation, New Castle, USA) at 37°C and, a kind of brownish colloidal substance was obtained. It was treated as the crude extract of the antifungal metabolites of *Streptomyces* sp. 3–10.

Determination of the Antimicrobial Spectrum of the Crude Extract

Inhibition of fungi and Oomycetes was done using the PDA-amendment method. The crude extract (CE) dissolved in methanol at 12.5 mg/ml (w/v) was used as stock solution, which was then amended to PDA in Petri dishes (9 cm diameter, 20 ml per dish) to the final concentrations of 1 or 5 µg/ml. PDA amended with methanol alone (0.2%, v/v) was treated as control. Mycelial agar plugs (5 mm diameter) were removed from the margin area of the actively-growing colonies of the target organisms, and inoculated on the PDA amended with either the crude extract or methanol alone, one mycelial plug per dish, and three dishes (as three replicates) for each treatment. The resulting cultures were incubated at 20°C, 25°C, or 28°C for 1–7 days depending on the mycelial growth rates of the target organisms (Table 1). Diameter of the colony in each dish was measured. Inhibition of mycelial growth (IMG) was calculated using the formula: $IMG (\%) = (D_{CK} - D_{CE})/D_{CK} \times 100$, where the D_{CK} and D_{CE} represent the colony diameters in the treatments of control (CK) and CE, respectively.

Inhibition of the bacteria by the CE of *Streptomyces* sp. 3–10 was done using the agar diffusion method (Bonev et al., 2008). The four investigated bacteria (Table S1) were separately shake-incubated at 28°C (150 rpm) in nutrient broth for 48 h. Then, aliquots (100 µl) of the liquid culture of each bacterium were evenly spread on the nutrient agar in Petri dishes (9 cm diameter, 20 ml medium per dish). Sterilized stainless-steel Oxford cups (10 × 6 × 8 mm, height × inner diameter × outer diameter) were placed in the dishes, three cups per dish and three dishes for each bacterium. The three concentrations (10, 50, and 100 µg/ml) of the CE of *Streptomyces* sp. 3–10 were pipetted into the three Oxford cups in each dish, respectively, 200 µl per cup. For the control treatment, three Oxford cups in a bacteria-inoculated Petri dish were loaded with 1% methanol (v/v), 200 µl per cup. The cultures were incubated at 28°C for 48 h and formation of the clear zones around the cups was used as an indicator of antibacterial activity.

Suppression of Fungal Spore Germination by the Crude Extract

Rhizopus stolonifer and *Botrytis cinerea* were incubated on PDA at 20°C for 3 and 7 days, respectively. Spores of *R. stolonifer* (sporangiospores) and *B. cinerea* (conidia) were harvested from the respective cultures by washing with sterile distilled water and the spore suspensions (1×10^6 spores/ml) were then prepared. Aliquots (100 µl) of the spore suspensions were pipetted to and evenly spread on the D-glucose agar (GA) medium amended

TABLE 1 | Antifungal spectrum of the metabolites of *Streptomyces* sp. 3–10 (PDA, pH 5.3).

Fungus/omycetes	% Inhibition of growth (Means ± S.D.)		Cultural conditions (temperature, duration)
	1 µg/ml	5 µg/ml	
OOMYCOTA			
<i>Pythium apanidermatum</i>	40.5 ± 2.8	73.2 ± 0.9	20°C at 4 dpi ^a
<i>Pythium ultimum</i>	20.1 ± 1.7	48.2 ± 0.5	20°C at 1 dpi
ZYgomycota			
<i>Mucor hiemalis</i>	80.8 ± 2.5	100.0	20°C at 2 dpi
<i>Rhizopus stolonifer</i>	91.4 ± 2.8	100.0	20°C at 1 dpi
ASCOMYCOTA			
<i>Amphibotrys ricini</i>	69.3 ± 0.9	83.7 ± 1.4	20°C at 7 dpi
<i>Alteraria alternata</i>	38.7 ± 0.6	74.5 ± 7.5	25°C at 5 dpi
<i>Aspergillus flavus</i>	84.1 ± 2.7	95.2 ± 0.6	28°C at 3 dpi
<i>Aspergillus niger</i>	76.6 ± 1.3	97.5 ± 0.7	28°C at 3 dpi
<i>Aspergillus parasiticus</i>	23.6 ± 7.2	45.8 ± 1.4	28°C at 3 dpi
<i>Bipolaris maydis</i>	27.7 ± 8.1	52.7 ± 0.6	28°C at 5 dpi
<i>Botrytis cinerea</i>	86.0 ± 0.2	97.0 ± 0.6	20°C at 3 dpi
<i>Colletotrichum siamense</i>	79.8 ± 0.6	87.1 ± 1.5	28°C at 3 dpi
<i>Curvularia lunata</i>	17.2 ± 2.7	85.3 ± 0.7	25°C at 3 dpi
<i>Drechslera graminea</i>	82.7 ± 0.9	100.0	25°C at 3 dpi
<i>Fusarium graminearum</i>	0.2 ± 0.1	1.5 ± 0.1	25°C at 3 dpi
<i>Fusarium moniliforme</i>	28.9 ± 5.3	57.2 ± 4.7	25°C at 3 dpi
<i>Fusarium oxysporum</i>	83.6 ± 1.0	100.0	20°C at 5 dpi
<i>Monilia fructigena</i>	75.5 ± 3.3	94.3 ± 0.8	25°C at 3 dpi
<i>Pestalotia theae</i>	57.5 ± 5.0	87.7 ± 2.9	25°C at 3 dpi
<i>Pyricularia oryzae</i>	3.6 ± 2.5	9.4 ± 5.6	28°C at 3 dpi
<i>Sclerotinia minor</i>	92.5 ± 0.3	100.0	20°C at 2 dpi
<i>Sclerotinia sclerotiorum</i>	97.2 ± 0.3	100.0	20°C at 2 dpi
BASIDIOMYCOTA			
<i>Rhizoctonia solani</i>	16.6 ± 4.5	84.7 ± 2.4	28°C at 3 dpi
<i>Sclerotium rolfsii</i>	57.1 ± 1.8	91.8 ± 0.4	28°C at 3 dpi

^adpi, days post-incubation.

with the crude extract of *Streptomyces* sp. 3–10 at 0 (control), 1, 5, 10, 50, and 100 µg/ml, six dishes (as six replicates) for each treatment. The cultures were incubated at 20°C in the dark for 6, 9, and 12 h. At each time point, the number of germinated spores among randomly-selected at least 100 spores in each culture were counted under a compound light microscope. Additionally, length of the germ tubes of randomly-selected 20 germinated spores was measured in each replicate culture at 12 h post-incubation.

Isolation of the Antifungal Metabolites from the Crude Extract

The crude extract (10 g) of *Streptomyces* sp. 3–10 was dissolved in 15 ml methanol and the solution was mixed with 15 g 60-mesh silica-gel granules (Qingdao Haiyang Chemical Co., Ltd., China). The mixture was then loaded as the top layer in a chromatography column (4.5 × 80 cm, inner diameter × length) containing 400 g silica gel (200-mesh, Qingdao Haiyang Chemical Co., Ltd.). The column was eluted with gradient

chloroform/methanol solutions (from 99/1 to 0/100, v/v). The resulting fractions were individually assayed for antifungal activity against *A. niger* using the Oxford cup-agar diffusion method described by Shakeel et al. (2016). The fractions that had antifungal activity were combined, concentrated, and loaded in a Sephadex LH-20 chromatography column (GE Healthcare), which was eluted with methanol to remove impurities. The fractions that had antifungal activity were again combined, dried, and subjected to semi-preparative high performance liquid chromatography (HPLC). Finally, two pure compounds, designated as Compound Nos. 1 and 2, were obtained.

Identification of the Purified Compounds

Electrospray ionization mass spectrometric (ESI-MS) analysis and nuclear magnetic resonance spectroscopy (NMR) were used to determine the chemical structure of the two pure compounds from *Streptomyces* sp. 3–10. The ESI-MS analysis was done on a Waters ACQUITY UPLC *H-Class* system coupled to the XEVO TQ-S tandem quadrupole (Waters Cooperation, Milford, MA, USA). The compounds were separately dissolved in methanol to the final concentration of 1 µg/ml. An aliquot (1 µl) of each solution was injected into the instrument. The operating parameters were set as: capillary voltage at 1.0 kV, cone voltage at 30 kV, Z-spray source temperature at 100°C, desolvation temperature (N₂) at 400°C, desolvation gas flow at 800 L/h, mass range of *m/z* from 50 to 1,200. The mass spectra were collected both in the positive mode and in the negative mode, and compared with those in Chapman Combined Chemical Dictionary on CD-ROM version 6.1 (Chapman and Hall, 2003) for determination of the chemical identity of the compounds. Moreover, synthetic reveromycin A (purity > 98%) purchased from Abcam Trading (Shanghai) Co. Ltd. (Shanghai, China) was used as a reference chemical in the UV-Vis spectrum analysis on Waters ACQUITY UPLC *H-Class* system.

In the NMR analysis, the two pure compounds were dissolved in methanol-d₄ (CD₃OD, Sigma-Aldrich®), and were then determined in a 400 MR DD2 spectrometer (Agilent Technologies, USA) for spectra of ¹H-NMR and ¹³C-NMR. Tetramethylsilane (TMS) was used as the internal standard in the NMR analysis.

Determination of the Content of Reveromycin A in the Crude Extract

Synthetic reveromycin A (purity > 98%) purchased from Abcam Trading (Shanghai) Co. Ltd. was used as standard in quantitative determination of the content of reveromycin A in the CE from strain 3–10. Five solutions of the synthetic reveromycin A with the concentrations of 0.08, 0.16, 0.31, 0.63 and 1.25 µg/ml in methanol were prepared. An aliquot (1 µl) of each solution of the synthetic reveromycin A or the solution of the CE of strain 3–10 at 3.125 µg/ml was injected into Waters ACQUITY UPLC *H-Class* system. The peak area for reveromycin A in each solution sample was measured. A standard curve was plotted based on the peak areas for the solutions of the synthetic reveromycin A and the concentrations of those solutions. The content of reveromycin A in the CE of strain 3–10 was then calculated

based on that standard curve. The quantitative determination was repeated for three times.

Antifungal Activity of the Purified Compounds

The purified compounds were separately dissolved in methanol to 10 mg/ml (w/v) as stock solutions, which were used in the following two trials, a mycelial growth trial and a spore germination trial. In the mycelial growth trial, the stock solution of each compound was amended in PDA, which was adjusted to pH 4.5, 5.5, or 7.0 with 1 mol/L HCl or 1 mol/L NaOH. The final concentrations in PDA ranged from 0.3125 to 50.0 µg/ml for Compound No. 1, and from 0.3125 to 100 µg/ml for Compound No. 2 (Table S3). Meanwhile, methanol solution (0.2%, v/v) was added to PDA at pH 4.5, 5.5, or 7.0 as controls. Mycelial agar plugs of *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum* (5 mm diameter) were individually inoculated on PDA, three dishes for each treatment (fungus × compound × concentration × pH). The cultures were incubated at 20°C in the dark for 1 day for *R. stolonifer*, and for 3 days for the other three fungi. The colony diameter was measured and the percentage value of inhibition of mycelial growth (IMG) under each pH was calculated using the formula mentioned above. EC₅₀-values (effective concentrations that gave 50% inhibition) for each compound under each pH were estimated based on the IMG data and the concentrations of that compound applied in PDA (Van Ewijk and Hoekstra, 1993).

In the spore germination trial, the stock solution of each compound was amended in GA with pH-values of 4.5, 5.5, or 7.0. The final concentrations in GA ranged from 0.0625 to 50.0 µg/ml for Compound No. 1, and from 0.3125 to 100 µg/ml for Compound No. 2 (Table S3). Meanwhile, methanol solution (0.2%, v/v) was added to GA with pH 4.5, 5.5, and 7.0 as controls. Aliquots (100 µl) of the spore suspension (1 × 10⁶ spores/ml) of *B. cinerea*, *M. hiemalis*, or *R. stolonifer* were pipetted on GA alone or on GA amended with a purified compound, three dishes for each treatment (fungus × compound × concentration × pH). The cultures were incubated at 20°C for 12 h. Spore germination in each culture was observed under light microscope. Percent inhibition of spore germination (ISG) by each compound under each pH was calculated using the formula: ISG (%) = (P_{CK} - P_C)/P_{CK} × 100, where the P_{CK} and P_C represent the percentages of germinated spores in the treatments of control (CK) and a pure compound, respectively. Finally, EC₅₀-values for each compound under each pH were estimated based on the ISG data and the concentrations of that compound applied in GA (Van Ewijk and Hoekstra, 1993).

Control of Strawberry Fruit Rot by Antifungal Metabolites from Strain 3–10

Mature strawberries (*Fragaria × ananassa* cultivar “Jing Yu”) of similar size (3.0–3.5 × 2.0–2.5 cm, length × width, 16–18 g per berry) were collected from strawberry plants grown in a plastic tunnel. They were surface sterilized in 70% ethanol (v/v) for 1 min, followed by washing in sterile distilled water and blotting on sterilized paper towels to remove the excess

water on the fruit surface. Finally, they were placed in glass Petri dishes (9 cm diameter), six berries per dish. For each pathogen, there were 10 treatments, including one negative control treatment with 1% methanol (v/v), three CE treatments with the concentrations of 10, 50, and 100 µg/ml (pHs at 5.4, 4.3, and 4.2, respectively), three treatments of reveromycin A (from strain 3–10) with the concentrations at 10, 50, and 100 µg/ml (pH at 4.5), and three fungicide treatments (positive controls) with the concentrations of an appropriate fungicide (either captan, pyrimethanil, or dimetachlone) at 10, 50, and 100 µg a.i./ml. Captan (3a, 4, 7, 7a-tetrahydro-2-[(trichloromethyl) thio]-1 H-isoindole-1,3(2H)-dione) was used as the positive control for the pathogens *M. hiemalis* and *R. stolonifer*, whereas pyrimethanil (2-anilino-4,6-dimethylpyrimidine) and dimetachlone [N-(3,5-dichlorophenyl) succinimide] were used as positive controls for the pathogens *B. cinerea* and *S. sclerotiorum*, respectively. Both captan (50% wettable powder) and pyrimethanil (80% water dispersible granule) were purchased from Hebei Guan Long Agrichemical Co. Ltd. (Hengshui, China). Dimetachlone (40% wettable powder) was purchased from Zhejiang SPACE Agrichemical Co. Ltd. (Wenzhou, China).

The six strawberries in a Petri dish were immersed for 1 min either in the 1% methanol solution, or in a solution containing either the CE, reveromycin A or a fungicide. Then, they were maintained in a laminar flow hood for 15–30 min, re-placed in the dish, and inoculated with one of the four pathogens. *B. cinerea*, *M. hiemalis*, and *R. stolonifer* were spray-inoculated with the spore suspensions (1×10^6 spores/ml) amended with 0.5% D-glucose (w/v), approximately 10 ml spore suspension on the six strawberries in a Petri dish. *S. sclerotiorum* was inoculated with mycelial agar plugs (5 mm diameter), one mycelial agar plug on each berry. The dishes with the treated strawberries were placed in plastic boxes (80 × 60 × 50 cm, length × width × height), which were individually covered with a transparent plastic film (0.1 mm thick, Gold Mine Plastic Industry Ltd., Jiangmen, China) to maintain the high humidity condition. The boxes were placed in a growth room at 20°C under the light regime of 12-h light/12-h dark. After incubation for 3 and 5 days for the inoculation treatments with *R. stolonifer* and *M. hiemalis*, respectively, and for 7 days for the inoculation treatments with *B. cinerea*, and *S. sclerotiorum*, disease severity on the strawberries was individually rated using a numeric scale from 0 (completely healthy) to 8 (completely rotten) according to the description by Huang et al. (2011). Disease severity index (DSI) was then calculated using the following formula:

$$\text{DSI} = 100 \times \sum_{i=0}^n (S_n \times n) / 8 \times \sum_{i=0}^n (S_n)$$

Where n represents the rating scale (0–8) and S_n represents the number of strawberries corresponding to the rating scale n . This experiment was repeated two more times.

Determination of Phytotoxicity

The strawberry seedlings (*Fragaria × ananassa* cultivar “Jing Yu”) were transplanted in a plastic tunnel (Length × Width × Height, 29 × 6.5 × 2 m) in September 28 of 2016. The

plants were carefully managed (watering, weeding) as required. The toxicity experiment was performed from December 11 of 2016 to December 25 of 2016, when most strawberry plants became bloomed. There were four treatments in this experiment: (i) control (CK); (ii), (iii), and (iv) the crude extract of strain 3–10 (CE) at 10, 50, and 100 mg/ml, respectively. Twenty strawberry plants for each treatment were randomly selected in the field and the leaves on each selected plants were individually immersed for 1 min either in 1% methanol (v/v) (CK) or in each aqueous solution of CE. At 0, 1, 3, 7, and 14 days post treatment (dpt), the treated plants were observed for the toxicosis symptoms on the leaves (yellowing, necrosis and malformation on leaves and flowers). Three plants for each treatment were randomly selected and the three leaves on each treated plant were detached as a leaf sample, one leaf from each plant. The leaves were immediately taken to laboratory for determination of the chlorophyll content (a, b and total). For chlorophyll extraction, the three-leaf sample for each treatment was homogenized in 95% ethanol. The homogenate was centrifuged at 10,000 × g and the supernatant was transferred out for determination of the absorbance values in DU730 Beckman spectrophotometer at 663 and 645 nm, respectively (Rout et al., 1998). The resulting absorbance values A_{663} and A_{645} were used to calculate the content of chlorophyll a (Chl a), chlorophyll b (Chl b) and the total chlorophyll (Chl T) using the following formula:

$$\text{Chl a (mg/g. F.W.)} = [12.72 \times A_{663} - 2.59 \times A_{645}] \times V/W$$

$$\text{Chl b (mg/g. F.W.)} = [22.88 \times A_{645} - 4.67 \times A_{663}] \times V/W$$

$$\text{Chl T (mg/g. F.W.)} = [20.29 \times A_{645} + 8.05 \times A_{663}] \times V/W$$

where V represents volume of 95% ethanol used for chlorophyll extraction in homogenization; W represents weight of the leaf sample; F. W. represent fresh weight of the leaf sample.

Additionally, at 0 or 14 dpt, five other plants for each treatment were randomly selected, uprooted and again taken to laboratory. They were washed under the running tap water and dried in an oven at 50°C. The upper part of that plant (the roots were trimmed) was then weighed.

Morphological and Physiological Characterization

Streptomyces sp. 3–10 was streak-inoculated on various agar media (Tables S2, S5). The cultures were incubated at 28°C in the dark for 14 days for observation of the colony morphology (shape, size, color of the substrate mycelium and the aerial mycelium, soluble pigments in media). For morphological observation of the substrate mycelium, a sterilized glass slide (7.5 × 2.5 × 0.1 cm, length × width × thickness) was placed on a PDA culture of strain 3–10 at 28°C for 14 days. Then, the slide was removed from the culture, and the hyphae on the slide were stained with 1% methyl green and observed under a compound light microscope (Ruan and Huang, 2011). For observation of the spore morphology, a sterilized cellophane film was placed on PDA. Strain 3–10 was inoculated on the cellophane film and the culture was incubated at 28°C for 14 days. Then, the cellophane film was removed, and cut to small pieces (approximately 3 × 3 mm, length × width). The resulting

film pieces with the colonies of strain 3–10 were immediately fixed in the glutaraldehyde fixative, followed by dehydration with ethanol, drying in a Critical Point Dryer (Model: 13200E-AB, SPI SUPPLIES, West Chester, PA, USA), and gold-coating in a sputter coater (Model: JFC-1600, NTC, Tokyo, Japan) using the conventional procedures. Finally, the specimens on the film pieces were observed under a scanning electron microscope (Model: JSM-6390/LV, NTC, Tokyo, Japan). To characterize physiological features, strain 3–10 was inoculated on specific media (Tables S2, S6, S7) using related procedures described by Ruan and Huang (2011).

Phylogenetic Analysis

Streptomyces sp. 3–10 was shake-incubated at 28°C for 2 days in the liquid ISP-2 medium (Table S2). The mycelia in the cultures were harvested by centrifugation at 7,000 × g for 5 min. Genomic DNA (gDNA) was extracted from the mycelium using the reagents in the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). It was used as template in PCR for amplification of the 16S rDNA sequence using the universal primers 27f (5'-AGAGTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGCTACCTTGACGACTT-3'; Zhang et al., 2016). The 25-μl PCR reaction system contained 1 μl of gDNA (approximately 50 ng), 2.5 U *Taq* DNA polymerase (TaKaRa Biotechnol. Co., Ltd., Dalian, China), 2.5 μl 10× PCR buffer, 1 μl of each primer (20 μmol) and 0.5 μl of dNTPs mixture (10 mmol/L). The PCR was performed in a S1000™ thermal cycler (Bio-Rad, USA) with the following thermal program: initial denaturation at 95°C for 5 min, followed by 35 cycles (95°C for 30 s, 56°C for 30 s, 72°C for 1.5 min), and final extension at 72°C for 5 min. The resulting PCR product was purified from the agarose gel after electrophoresis and cloned into the pMD18-T vector (TaKaRa Biotechnol Co. Ltd., Dalian, China), which was subsequently transformed into *E. coli* DH5α. A positive *E. coli* clone with the correct DNA insert size was sequenced at Sangon Biotechnol. Co. Ltd. (Shanghai, China). The sequence was submitted to GenBank at NCBI and was assigned with the accession number KX811537.

For phylogenetic analysis, a dataset was established based on the 16S rDNA sequences of *Streptomyces* sp. 3–10, 39 other taxa of *Streptomyces* species, and strain DSM44928 of *Catenulispora acidiphila* (Table S4). They were aligned using the Clustal W program in the MEGA 7.0 software and phylogenetic analysis was done based on the alignment using the maximum likelihood (ML) methods. All the nucleotides in the DNA sequences were treated as un-ordered and un-weighted, and the gaps were treated as the missing data. The bootstrap consensus trees were inferred from 1,000 replicates.

Statistical Analysis

Data on spore germination rate, length of germ tubes, disease incidence and disease severity index in related experiments were separately analyzed for ANOVA (analysis of variance) using the PROC ANOVA procedure (SAS Institute, Cary, NC, USA, version 8.0, 1999). Treatment means in each experiment were separated using Least significant Different (LSD) test at $\alpha = 0.05$. Before ANOVA, the percentage data on spore

germination rate and disease incidence were transformed to numerical data by multiplication with 100. After analysis, the data were back-transformed to the percentage values. Data on content of chlorophyll a, chlorophyll b, the total chlorophyll and dry weight of strawberry plant between the treatments of control and the crude extract of *Streptomyces* sp. 3–10 was compared at $\alpha = 0.05$ using the PROC TTEST in the SAS software.

RESULTS

Antifungal Spectrum of the Crude Extract

The crude extract from *Streptomyces* sp. 3–10 showed a wide antifungal spectrum (Table 1). It inhibited mycelial growth of two species of *Pythium* and 20 species of fungi. The percentages of inhibition of mycelial growth varied greatly among the target species, ranging from 16.6 to 97.2% at 1 μg/ml, and from 45.8 to 100% at 5 μg/ml. Among the 20 fungal species, *M. hiemalis*, *B. cinerea*, *R. stolonifer*, and *S. sclerotiorum* are the causal agents of strawberry fruit rot. They were inhibited by 80.8, 86.0, 91.4, and 97.2%, respectively, at 1 μg/ml, and by the rates higher than 97% at 5 μg/ml (Table 1). In contrast, the crude extract weakly inhibited (<10%) mycelial growth of *Fusarium graminearum* (the causal agent of wheat head blight) and *Pyricularia oryzae* (the causal agent of rice blast) at two concentrations (Table 1).

Results from the antibacterial experiment showed that the crude extract of *Streptomyces* sp. 3–10 had no antibacterial activity (Figure S1). It failed even at 100 μg/ml to inhibit growth and proliferation of all the four investigated bacteria, including *Acidovorax citrulli* (the causal agent of watermelon bacterial fruit blotch), *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (the causal agent of bacterial wilt of beans), and *Erwinia carotovora* (the causal agent of potato soft rot), and *Bacillus subtilis* (a saprophyte).

Suppression of Fungal Spore Germination by the Crude Extract

The crude extract effectively inhibited spore germination of *B. cinerea* and *R. stolonifer* in GA at 20°C (Table 2). For *B. cinerea*, the inhibitory efficacy depended greatly on concentration of the crude extract. In the treatments of the crude extract at 1 and 5 μg/ml, the conidia germinated by 93–99% at 6–12 h post-incubation (hpi), not significantly different ($P > 0.05$) from the germination rate of 99% in the control treatment. The average values of germ-tube length reached 146.6 and 106.5 μm for the two concentrations of the crude extract, respectively. In the treatments of the crude extract at 10, 50, and 100 μg/ml, the percentages of germinated conidia at 12 hpi were reduced by 12, 90, and 99%, respectively, and the average germ tube length was reduced by 51, 89, and 92%, respectively, compared to the control treatment (Table 2). For *R. stolonifer*, while the control treatment had percentages of germinated sporangiospores of 0, 8.3 and 63.1% at 6, 9, and 12 hpi, respectively, and the average germ-tube length reached 67.4 μm at 12 hpi, the treatments of the crude extract at 1, 5, 10, 50, and 100 μg/ml completely inhibited sporangiospore germination at 6, 9, and 12 hpi, just as the fungicide treatment with captan at 100 μg a.i./ml (Table 2).

TABLE 2 | Effect of the crude extract (CE) of the antifungal substances produced by *Streptomyces* sp. 3–10 on spore germination and germ tube extension of conidia of *B. cinerea* and sporangiospores of *R. stolonifer* (20°C, GA).

Fungus	Treatment ^a	Spore germination (%)			Length of germ tubes (μm, 12 hpi)
		6 hpi ^b	9 hpi	12 hpi	
<i>B. cinerea</i>	Control	95.1 a ^c	96.6 a	98.7 a	147.3 a
	CE 1 μg/ml	94.5 a	95.7 a	98.8 a	146.6 a
	CE 5 μg/ml	93.5 a	96.5 a	98.2 a	106.5 b
	CE 10 μg/ml	39.3 b	81.5 b	86.7 b	72.3 c
	CE 50 μg/ml	7.5 c	10.9 c	9.9 c	17.0 d
	CE 100 μg/ml	0.8 d	0.9 d	1.0 d	11.4 d
	Pyrimethanil 100 μg a.i./ml	0.8 d	0.9 d	1.1 d	9.3 d
	LSD (0.05)	2.2	1.7	2.4	7.9
<i>R. stolonifer</i>	Control	0.0 a	8.3 a	63.1 a	67.4 a
	CE 1 μg/ml	0.0 a	0.0 b	0.0 b	0.0 b
	CE 5 μg/ml	0.0 a	0.0 b	0.0 b	0.0 b
	CE 10 μg/ml	0.0 a	0.0 b	0.0 b	0.0 b
	CE 50 μg/ml	0.0 a	0.0 b	0.0 b	0.0 b
	CE 100 μg/ml	0.0 a	0.0 b	0.0 b	0.0 b
	Captan 100 μg a.i./ml	0.0 a	0.0 b	0.0 b	0.0 b
	LSD (0.05)	0.0	0.3	4.6	3.2

^apH-values in GA alone, and in GA amended with the crude extract of *Streptomyces* sp. 3–10 at 1, 5, 10, 50, and 100 μg/ml were 5.8, 5.9, 5.8, 5.4, 4.8, and 4.6, respectively.

^bhpi, hours post-incubation.

^cMeans within the same column for each fungus followed by the same letter are not significantly different ($P > 0.05$) according to least significance test.

Chemical Identity of the Purified Compounds

Two compounds (Nos. 1 and 2) were purified from the crude extract. Compound No. 1 is a white amorphous powder. ESI±MS (100 kV) of this compound showed a molecular ion peak at m/z 683 ($M+Na$)⁺, 659 ($M-H$)⁻ and HR-ESI (positive) MS at m/z 683.3394 ($M+Na$)⁺ (calc., 683.3047). Based on these data, the molecular formula of this compound was inferred to be C₃₆H₅₂O₁₁. The UV-Vis spectrum in methanol gave the UV maximum absorbance at 237.9 nm (Figure S2). The ¹H-NMR spectrum (CD₃OD, 400 MHz; Figure S3) showed five methyl protons at δ_H 2.26 (3H, s), 1.75 (3H, s), 1.08 (3H, d, $J = 6.8$ Hz), 0.85 (3H, t, $J = 6.9$ Hz), and 0.79 (3H, d, $J = 5.6$ Hz), eight olefinic protons at δ_H 6.97 (1H, dd, $J = 15.7, 7.7$ Hz), 6.44 (2H, m), 6.25 (1H, d, $J = 15.6$ Hz), 5.88 (1H, s), 5.81 (1H, d, $J = 15.7$ Hz), 5.59 (1H, t, $J = 7.0$ Hz), and 5.53 (1H, dd, $J = 15.6, 7.3$ Hz), and three oxymethylene protons at δ_H 4.62 (1H, d, $J = 7.3$ Hz), 4.07 (1H, m), and 3.45 (1H, m; Table 3). The ¹³C NMR spectrum (CD₃OD, 100 MHz; Figure S4) showed 36 signals, including five methyl groups at δ_C 14.6, 15.3, 13.0, 18.0, and 14.2, 10 methylene carbons at δ_C 32.7, 28.7, 36.9, 35.2, 25.4, 34.8, 23.8, 23.4, 31.2, and 29.9, eight olefinic carbons at δ_C 122.7, 152.6, 127.9, 137.8, 129.5, 134.1, 139.3, and 121.9, two methine carbons at δ_C 44.1 and 36.3, two olefinic quaternary carbons at δ_C 135.5 and 152.2,

TABLE 3 | ¹H and ¹³C NMR spectra of reveromycins A and B from *Streptomyces* sp. 3–10.

Position	Reveromycin A		Reveromycin B		
	δ_H [m, J(Hz)]	δ_C	Position	δ_H [m, J(Hz)]	δ_C
1		170.3	1		170.4
2	5.81, d (15.7)	122.7	2	5.79, d (15.6)	121.2
3	6.97, dd (15.7, 7.7)	152.6	3	6.99, dd (15.8, 7.5)	152.9
4	2.52, m	44.1	4	2.51, m	44.0
5	4.07, m	76.9	5	4.08, dd (7.4, 5.4)	77.1
6	5.53, dd (15.6, 7.3)	127.9	6	5.47, dd (15.7, 7.6)	127.2
7	6.25, d (15.6)	137.8	7	6.39, d (15.7)	138.6
8		135.5	8		135.2
9	5.59, t (7.0)	129.5	9	5.77, m	130.9
10	2.40, m	32.7	10	2.57, m	32.6
		2.33, m		2.17, m	
11	3.45, m	76.3	11	3.45, m	78.5
12	1.39, m	36.3	12	1.38, m	35.7
13	1.46, m	28.7	13	1.61, m	30.3
14	1.72, m	36.9		1.51, m	
	1.46, m		14	1.72, m	35.3
15		97.0	15		108.6
16	1.84, m	35.2	16	1.99, m	39.8
	1.60, m			1.80, m	
17	2.31, m	25.4	17	1.99, m	32.9
	2.02, td (13.6, 4.0)			1.84, m	
18		84.2	18		88.8
19	4.62, d (7.3)	79.7	19	5.57, d (3.7)	80.4
20	6.44, m	134.1	20	6.24, dd (16.0, 3.8)	132.6
21	6.44, m	139.3	21	6.28, d (16.1)	136.1
22		152.2	22		152.5
23	5.88, s	121.9	23	5.78, s	122.5
24		170.4	24		170.2
25	1.84, m	34.8	25	1.59, m	35.6
	1.68, m			1.47, m	
26	1.25, m	23.8	26	1.31, m	26.6
	1.23, m				
27	1.27, m	23.4	27	1.31, m	24.4
	1.22, m				
28	0.85, t (6.9)	14.6	28	0.92, t (6.9)	14.5
4-Me	1.08, d (6.8)	15.3	4-Me	1.01, d (6.9)	15.1
8-Me	1.75, s	13.0	8-Me	1.74, s	12.7
12-Me	0.79, t (5.6)	18.0	12-Me	0.89, d (6.5)	18.2
22-Me	2.26, s	14.2	22-Me	2.24, s	14.0
1'		173.4	1'		173.1
2'	2.59, m	31.2	2'	2.60, m	30.4
3'	2.59, m	29.9	3'	2.52, m	29.8
4'		176.2	4'		175.9

four oxymethylene carbons at δ_C 76.9, 76.3, 84.2, and 79.7, three carboxyl carbonyl carbons at δ_C 170.3, 170.4, and 176.2, one ester carbonyl carbon at δ_C 173.4, and one quaternary spiroketal carbon at δ_C 97.0 (Table 3). Compound No. 1 was identified as reveromycin A (Figure 1) by comparing the data on the spectra

of ^1H NMR, ^{13}C NMR, MS and UV-Vis of this compound with the related spectra of reveromycin A reported by Fremlin et al. (2011). The average content of reveromycin A in the crude extract of strain 3–10 was 37.7% (Figure S5).

Compound No. 2 was also a white amorphous powder. ESI \pm MS (100 kV) of this compound had a molecular ion peak at m/z 683 ($\text{M}+\text{Na}$) $^+$, 659 ($\text{M}-\text{H}$) $^-$, HR-ESI (positive) MS at m/z 683.3041 ($\text{M}+\text{Na}$) $^+$ (calc., 683.3047). Based on these data, the molecular formula of this compound was inferred to be $\text{C}_{36}\text{H}_{52}\text{O}_{11}$. The UV spectrum in methanol gave a UV maximum absorbance at 238.9 nm (Figure S6). The ^1H -NMR spectrum (CD_3OD , 400 MHz; Figure S7) showed five methyl protons at δ_{H} 2.24 (3H, s), 1.74 (3H, s), 1.01 (3H, d, $J = 6.9$ Hz), 0.92 (3H, t, $J = 6.9$ Hz) and 0.89 (3H, d, $J = 6.5$ Hz), eight olefinic protons at δ_{H} 6.99 (1H, dd, $J = 15.8, 7.5$ Hz), 6.39 (1H, d, $J = 15.7$ Hz), 6.28 (1H, d, $J = 16.1$ Hz), 6.24 (1H, dd, $J = 16.0, 3.8$ Hz), 5.79 (1H, d, $J = 15.6$ Hz), 5.78, (1H, s), 5.77 (1H, m), 5.47 (1H, dd, $J = 15.7, 7.6$ Hz), and three oxymethine protons at δ_{H} 5.57 (1H, d, $J = 3.7$ Hz), 4.08 (1H, dd, $J = 7.4, 5.4$ Hz), 3.45, (1H, m; Table 3). The ^{13}C NMR spectrum (CD_3OD , 100 MHz; Figure S8) also showed 36 signals, including five methyl groups at δ_{C} 14.5, 15.1, 12.7, 18.2, and 14.0, 10 methylene carbons at δ_{C} 32.6, 30.3, 35.3, 39.8, 32.9, 35.6, 26.6, 24.4, 30.4, and 29.8, eight olefinic carbons at δ_{C} 121.2, 152.9, 127.2, 138.6, 130.9, 132.6, 136.1, and 122.5, two methine carbons at δ_{C} 44.0 and 35.7, two olefinic quaternary carbons at δ_{C} 135.2 and 152.5, four oxymethine carbons at δ_{C} 77.1, 78.5, 88.8, and 80.4, three carboxyl carbonyl carbons at δ_{C} 170.4, 170.2, and 175.9, one ester carbonyl carbon at δ_{C} 173.1 and one quaternary spiroketal carbon at δ_{C} 108.6 (Table 3). Compound No. 2 was identified as reveromycin B (Figure 1) by comparing the data on the spectra of ^1H NMR, ^{13}C NMR, MS, and UV-Vis of this compound with related spectra of reveromycin B reported by Fremlin et al. (2011).

Antifungal Activity of the Purified Compounds

Reveromycins A and B from *Streptomyces* sp. 3–10 effectively suppressed mycelial growth of *B. cinerea*, *M. hiemalis*,

R. stolonifer, and *S. sclerotiorum*, and spore germination of *B. cinerea*, *M. hiemalis*, and *R. stolonifer* (Table 4). The suppressive efficacy was greatly affected by ambient pH. For reveromycin A, the EC₅₀-values ranged from 0.10 to 0.88 $\mu\text{g}/\text{ml}$ at pH 4.5, from 0.42 to 1.76 $\mu\text{g}/\text{ml}$ at pH 5.5, and from 14.04 to 53.35 $\mu\text{g}/\text{ml}$ at pH 7.0. For reveromycin B, the EC₅₀-values ranged from 1.15 to 5.49 $\mu\text{g}/\text{ml}$ at pH 4.5, from 6.12 to 35.46 $\mu\text{g}/\text{ml}$ at pH 5.5, and >100 $\mu\text{g}/\text{ml}$ at pH 7.0.

Control Efficacy against Strawberry Fruit Rot

At 3–7 days post inoculation (20°C), the strawberries in the control treatment were severely diseased (Figure 2, Table 5). The percentages of diseased strawberries reached 100, 88.9, 100, and 94.4% for the control inoculations with *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum*, respectively. The disease severity index values reached 78.5, 62.5, 88.1, and 82.6 in these treatments, respectively. In contrast, most strawberries in the treatments of the crude extract and reveromycin A from strain 3–10 at 10, 50, and 100 $\mu\text{g}/\text{ml}$, as well as in the treatments of the fungicides at 10, 50, and 100 $\mu\text{g a.i.}/\text{ml}$ appeared healthy (Figure 2, Table 5). The percentages of diseased strawberries were lower than 40% and the disease severity index values were lower than 35 in these treatments. Reveromycin A at 50 and 100 $\mu\text{g}/\text{ml}$ completely suppressed strawberry fruit rot caused by all the four fungi. Statistical analysis indicated that for each pathogen, the treatments of the CE, reveromycin A and fungicide differed significantly ($P < 0.05$) from the control treatment both in disease incidence and in disease severity index. Under the same concentration, the crude extract and reveromycin A from strain 3–10 did not significantly ($P > 0.05$) differ from the corresponding fungicide.

Phytotoxicity

The treatments of the strawberry leaves with the crude extract of *Streptomyces* sp. 3–10 at 10, 50 and 100 $\mu\text{g}/\text{ml}$ did not produce any visible toxic symptoms (yellowing, necrosis and malformation) on leaves and flowers (Figure S9). They grew normally and did not significantly differ ($P > 0.05$) from the control treatment (water) both in leaf chlorophyll content and in dry weight of the upper part of strawberry plants.

Taxonomic Identity of *Streptomyces* sp. 3–10

Streptomyces sp. 3–10 grew and sporulated on nine agar media (BM, GS-1, ISP-1, ISP-2, ISP-3, ISP-5, ISP-6, ISP-7, PDA) after incubation at 28°C for 14 days with formation of whitish to grayish colonies. They hardly grew on the ISP-4 medium (Figure S10, Table S5). In the PDA cultures, the single colonies of *Streptomyces* sp. 3–10 were averagely sized by 1.6 ± 0.3 mm in diameter, dome-shaped in the colony center, and the saw tooth-shaped at the colony margin (Figure S11). The substrate mycelium was pale yellowish to orange in color and the aerial mycelium was whitish to grayish in color (Figure S11). The spore chains were rectiflexible in shape with the spores being short rod in shape, $0.9 \times 0.6 \mu\text{m}$ (length \times width) in size, and smooth surfaced (Figure S11). The cultural and morphological

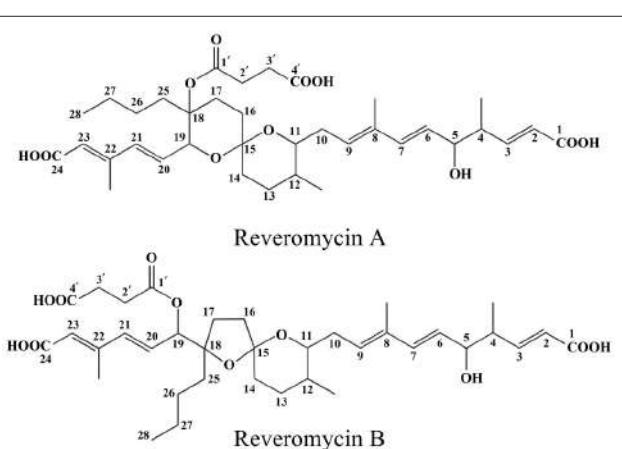


FIGURE 1 | Chemical structures of reveromycins A and B produced by *Streptomyces* sp. 3–10.

TABLE 4 | 50% effective concentrations (EC_{50}) of reveromycins A and B produced by *Streptomyces* sp. 3–10 against *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum*.

Fungus	Reveromycin A (Means \pm S.D., $\mu\text{g/ml}$)			Reveromycin B (Means \pm S.D., $\mu\text{g/ml}$)		
	pH 4.5	pH 5.5	pH 7.0	pH 4.5	pH 5.5	pH 7.0
EC_{50} FOR MYCELIAL GROWTH^a						
<i>B. cinerea</i>	0.88 \pm 0.01	1.76 \pm 0.07	53.35 \pm 1.01	5.37 \pm 0.11	30.60 \pm 0.87	>100.00
<i>M. hiemalis</i>	0.74 \pm 0.02	1.49 \pm 0.05	21.31 \pm 0.32	4.07 \pm 0.18	22.12 \pm 0.11	>100.00
<i>R. stolonifer</i>	0.67 \pm 0.03	1.45 \pm 0.08	21.45 \pm 3.62	4.48 \pm 0.04	24.61 \pm 0.35	>100.00
<i>S. sclerotiorum</i>	0.65 \pm 0.02	1.27 \pm 0.04	34.03 \pm 0.44	5.49 \pm 0.13	35.46 \pm 3.92	>100.00
EC_{50} FOR SPORE GERMINATION^b						
<i>B. cinerea</i>	0.57 \pm 0.01	1.45 \pm 0.03	49.33 \pm 2.11	4.01 \pm 0.09	23.62 \pm 0.18	>100.00
<i>M. hiemalis</i>	0.11 \pm 0.01	0.45 \pm 0.01	15.17 \pm 0.56	1.59 \pm 0.03	8.03 \pm 0.21	>100.00
<i>R. stolonifer</i>	0.10 \pm 0.01	0.42 \pm 0.01	14.04 \pm 0.61	1.15 \pm 0.01	6.12 \pm 0.11	>100.00

^aThe cultures on reveromycin A- or reveromycin B-amended PDA were incubated at 20°C for 24 h for *R. stolonifer*, whereas for 72 h for *B. cinerea*, *M. hiemalis* and *S. sclerotiorum*.

^bThe cultures with the conidia of *B. cinerea*, and sporangiospores of *M. hiemalis* and *R. stolonifer* on GA were incubated at 20°C for 12 h.

characteristics of *Streptomyces* sp. 3–10 matched the description for *Streptomyces yanglinensis* 1307^T (Xu et al., 2006).

Results of the physiological determination showed that strain 3–10 was similar to *S. yanglinensis* 1307^T in most of the measured features, but differed from *S. yanglinensis* 1307^T in utilization of myo-inositol, growth response to pH 3.5, degradation of Tween 80 and sensitivity to streptomycin sulfate and sulfamethoxazole. Strain 3–10 could utilize myo-inositol, whereas strain 1307^T could not. Strain 3–10 could grow on ISP-3 at pH 3.5, whereas strain 1307^T could not. Strain 3–10 could not degrade Tween 80, whereas strain 1307^T could. Strain 3–10 showed sensitive to streptomycin sulfate (10 $\mu\text{g/ml}$), but resistant to sulfamethoxazole (25 $\mu\text{g/ml}$). In contrast, strain 1307^T showed resistant to streptomycin sulfate (10 $\mu\text{g/ml}$), but sensitive to sulfamethoxazole (25 $\mu\text{g/ml}$; Tables S6, S7).

The close relationship between *Streptomyces* sp. 3–10 and *S. yanglinensis* 1307^T was further confirmed by phylogenetic analysis of the 16S rDNA sequences. *Streptomyces* sp. 3–10 is distantly related to strains F-1, JCM4662^T and NRBC13818^T of *S. platensis*, but is closely related to *S. yanglinensis* 1307^T (Figure 3). Therefore, *Streptomyces* sp. 3–10 might represent a novel phylotype of *S. yanglinensis*.

DISCUSSION

Reveromycins are spiroacetal polyketide compounds produced by *Streptomyces* species (Osada et al., 1991; Koshino et al., 1992; Takahashi et al., 1992a,b; Fremlin et al., 2011). So far, three strains of *Streptomyces* have been reported to produce reveromycins. They are strain SN-593 of *S. reveromyceticus* isolated from a soil sample in Japan (Osada et al., 1991; Koshino et al., 1992; Takahashi et al., 1992a,b), and strains MST-MA568 and MST-RA7781 of *Streptomyces* spp. isolated from a sediment sample and a soil sample, respectively, in Australia (Fremlin et al., 2011). Fremlin et al. (2011) reported that the frequency of reveromycins-producing actinomycete isolates is very low. Two (e.g., MST-MA568 and MST-RA7781) out of

400,000 actinomycete isolates were found to be able to produce reveromycins (Fremlin et al., 2011). The present study found that *Streptomyces* sp. 3–10 can produce reveromycins A and B. This finding enriched the reveromycins-producing *Streptomyces* resource and will be important for further exploitation of the *Streptomyces*-derived reveromycins for pharmaceutical and agricultural use in the future.

Biological activities of reveromycin A have been well elucidated in previous studies (Osada, 2016). It is a multifunctional chemical, owning strong capabilities to inhibit mitogenic activity induced by epidermal growth factor (Osada et al., 1991), to suppress hormone-dependent tumors like ovarian cancer and prostate (Takahashi et al., 1997), to alleviate osteoporosis by inducing apoptosis specifically in osteoclasts (Woo et al., 2006), and to inhibit proliferation of *Candida* species (Takahashi et al., 1992b; Fremlin et al., 2011). Miyamoto et al. (2002) found that the molecular target of reveromycin A in *Saccharomyces cerevisiae* is isoleucyl-transfer RNA (tRNA) synthetase. Although Osada et al. (1991) indicated that reveromycin A has antifungal activity against plant pathogenic fungi with the minimum inhibitory concentration ranging from 16 to 64 $\mu\text{g/ml}$. However, detailed information about the inhibited plant pathogenic fungi was not mentioned in that report (Osada et al., 1991). Therefore, whether or not reveromycin A and other reveromycin analogs can inhibit plant pathogens like *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum*, and Oomycetes such as like species of *Pythium* remains unknown. Fremlin et al. (2011) reported that reveromycins A to M had antifungal activity against *Candida* species. They did not report the antifungal activity of these reveromycins against plant pathogenic fungi.

In this study, we found that the crude extract from *Streptomyces* sp. 3–10 at 5 $\mu\text{g/ml}$ had a wide antifungal spectrum, including many important plant pathogens such as, *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum* (Table 1). We also demonstrated that the crude extract and reveromycin A from *Streptomyces* sp. 3–10 at 10, 50, and 100 $\mu\text{g/ml}$ was highly effective in suppression of strawberry fruit rot caused

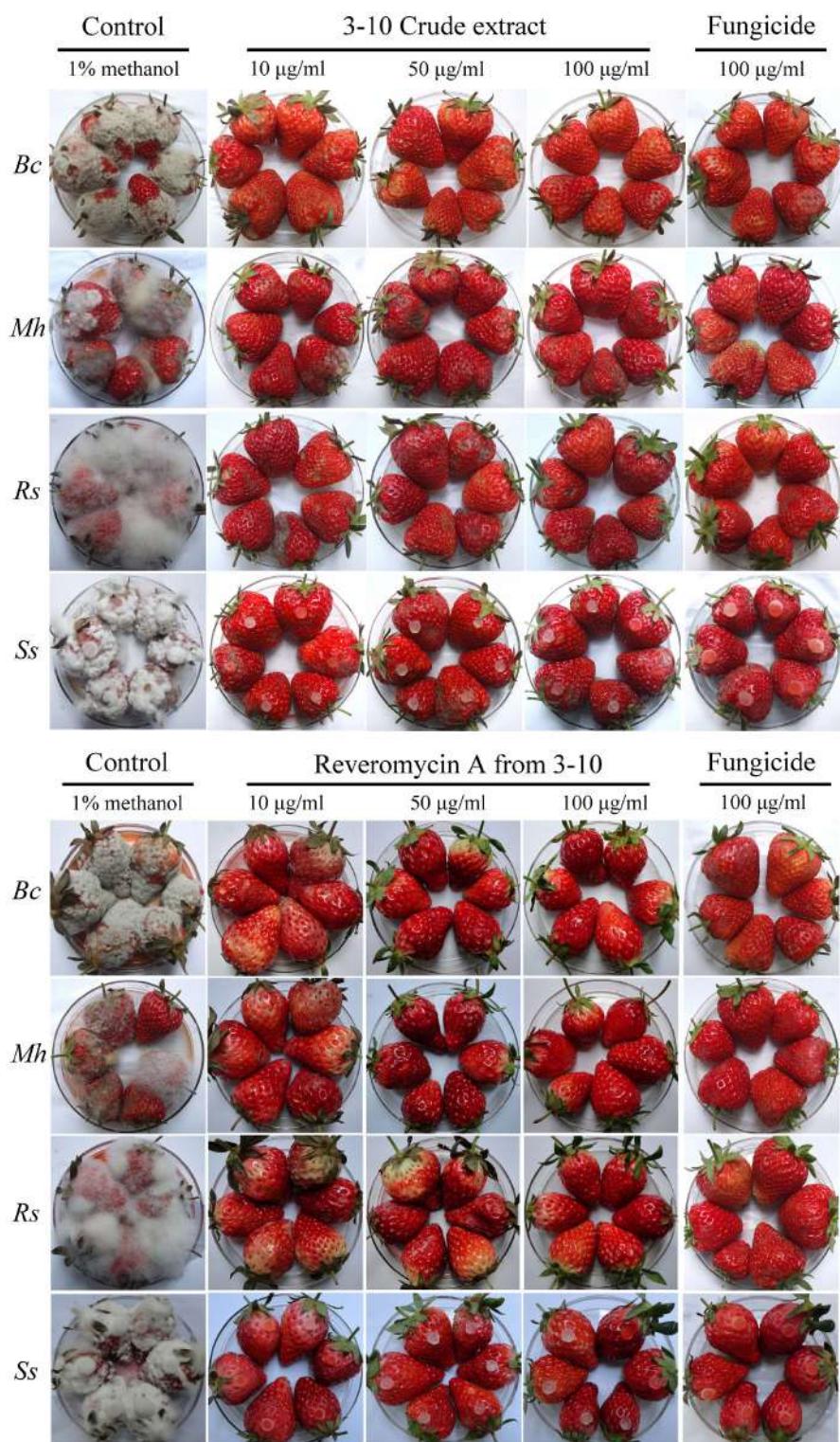


FIGURE 2 | Efficacy of the crude extract, reveromycin A from *Streptomyces* sp. 3–10 and fungicides in suppression of strawberry fruit rot caused by *Botrytis cinerea* (*Bc*), *Mucor hiemalis* (*Mh*), *Rhizopus stolonifer* (*Rs*), and *Sclerotinia sclerotiorum* (*Ss*). *B. cinerea*, *M. hiemalis*, and *R. stolonifer* were inoculated with spores. *S. sclerotiorum* was inoculated with mycelial agar plugs. The strawberries were maintained at 20°C under humid conditions for 3 days for *R. stolonifer*, 5 days for *M. hiemalis*, and 7 days for *B. cinerea* and *S. sclerotiorum*.

TABLE 5 | Efficacy of the crude extract (CE) and reveromycin A (RA) from *Streptomyces* sp. 3–10 in suppression of strawberry fruit rot caused by *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum* in comparison with respective fungicides.

Treatment ^a	<i>B. cinerea</i>		<i>M. hiemalis</i>		<i>R. stolonifer</i>		<i>S. sclerotiorum</i>	
	DI ^b	DSI ^b	DI	DSI	DI	DSI	DI	DSI
Control	100.0 a ^c	78.5 a	88.9 a	62.5 a	100.0 a	88.1a	94.4 a	82.6 a
CE 10 µg/ml	22.2 b	4.9 b	11.1 c	8.3 cd	27.7 b	7.6 c	5.6 b	1.4 b
CE 50 µg/ml	11.1 bc	4.2 b	5.6 c	2.1 de	11.1 c	2.8 d	0.0 b	0.0 b
CE 100 µg/ml	0.0 c	0.0 c	0.0 c	0.0 e	0.0 c	0.0 d	0.0 b	0.0 b
RA 10 µg/ml	22.2 b	4.2 b	11.1 c	2.1 e	11.1 c	2.7 d	0.0 b	0.0 b
RA 50 µg/ml	0.0 c	0.0 c	0.0 c	0.0 e	0.0 c	0.0 d	0.0 b	0.0 b
RA 100 µg/ml	0.0 c	0.0 c	0.0 c	0.0 e	0.0 c	0.0 d	0.0 b	0.0 b
FU 10 µg a.i./ml	11.1 bc	3.5 bc	27.7 b	34.7 b	38.8 b	22.8 b	5.6 b	2.1 b
FU 50 µg a.i./ml	5.6 c	2.1 bc	11.1 c	10.4 c	11.1 c	3.5 cd	0.0 b	0.0 b
FU 100 µg a.i./ml	0.0 c	0.0 c	0.0 c	0.0 e	0.0 c	0.0 d	0.0 b	0.0 b
LSD (0.05)	11.6	3.9	12.7	6.3	11.6	4.6	9.0	2.4

^aFungicides (FU): Pyrimethanil for *B. cinerea*, captan for *M. hiemalis* and *R. stolonifer*, and dimetachlone for *S. sclerotiorum*.

^bDI, Disease incidence (%); DSI, Disease severity index (0–100).

^cMeans within the same column followed by the same letter are not significantly different ($P > 0.05$) according to least significance test.

by these pathogens and the efficacy was comparable to that of the corresponding commercial fungicides (Table 5). It is well recognized that *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum* are the necrotrophic plant pathogens. They can aggressively infect mature strawberry fruit under cool and humid conditions, thereby causing severe economic losses for strawberry production. Nowadays, control of *B. cinerea* as well as three other fungi depends largely on repeated application of fungicides. In most cases, the fungicide application can suppress infection by these fungi. However, frequent application of the fungicides may cause some undesirable side effects, such as fungicide residues in strawberry fruit, pollution to environment, and development of fungicide-resistant fungal strains. The results about the wide antifungal spectrum and high antifungal activity for the metabolites of *Streptomyces* sp. 3–10 suggest that they have a promising potential to be exploited for control of strawberry fruit rot caused by these four fungi. This study found that the crude extract of *Streptomyces* sp. 3–10 at 10, 50 and 100 µg/ml did not produce any visible toxic symptoms on leaves of strawberry (Figure S9). This result suggests that application of the metabolites of *Streptomyces* sp. 3–10 on strawberry plants at the flowering stage might be a safe measure for control of strawberry fruit rot.

Among the fungi and Oomycetes inhibited by the metabolites from *Streptomyces* sp. 3–10, *Fusarium oxysporum*, *Pythium aphanidermatum*, *P. ultimum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Sclerotinia minor*, and *S. sclerotiorum* are soilborne plant pathogens. Antifungal activity against these organisms suggests that inoculation of *Streptomyces* sp. 3–10 into soil may yield suppressive effect on these organisms. This study found that *Streptomyces* sp. 3–10 can grow at pH 3.5 and 4.5, but failed to grow at pH 7.5 (Table S6). This acidophilic characteristic of *Streptomyces* sp. 3–10 may help it to adapt to the acidic soil conditions in south of China. Moreover, this study found that the antifungal activity of reveromycins A and B from *Streptomyces*

sp. 3–10 is high at pH 4.5 and 5.5, whereas is decreased at pH 7.0 (Table 4), implying that application of *Streptomyces* sp. 3–10 in acidic soil may achieve high antifungal efficacy in suppression of these soil-dwelling fungi.

Previous studies showed that many plant pathogenic fungi such as *B. cinerea* and *S. sclerotiorum* can secrete oxalic acid to facilitate their infection and colonization of plant tissues (Choquer et al., 2007; Williams et al., 2011). Oxalic acid can acidify the surrounding environment. In this study, we found that reveromycins A and B from *Streptomyces* sp. 3–10 showed higher antifungal activity at pH 4.5 than at pH 5.5 and 7.0 (Table 4). Thus, the acidic environment created by oxalic acid produced by *B. cinerea* and *S. sclerotiorum* may enhance the antifungal activity of *Streptomyces* sp. 3–10 against the two pathogens. This may be one of the reasons responsible for the high antifungal activity of reveromycins A and B in inhibition of *B. cinerea* and *S. sclerotiorum*.

Streptomyces sp. 3–10 was previously thought to be a mutant *S. platensis* F-1, as it was isolated from a PDA culture of strain F-1 treated with UV-C and LiCl (Che, 2011). Due to the dramatic difference between strains 3–10 and F-1 in colony morphology, this study tried to clarify the taxonomic status of *Streptomyces* sp. 3–10. The results showed that strain 3–10 is closely related to *S. yanglinensis* 1307^T (possibly representing a novel phylotype of *S. yanglinensis*), but is distantly related to *S. platensis* F-1 (Figure 3). This result suggests that *Streptomyces* sp. 3–10 is probably a contaminant, rather than a derivative from *S. platensis* F-1.

Streptomyces yanglinensis was established by Xu and colleagues in 2006 with strain 1307^T as the type strain (Xu et al., 2006). Results of this study showed that strain 3–10 was similar to strain 1307^T in the majority of the measured physiological characteristics, but is different from strain 1307^T in utilization of myo-inositol, in growth response to pH 3.5, in degradation of Tween 80, as well as in sensitivity to streptomycin sulfate and

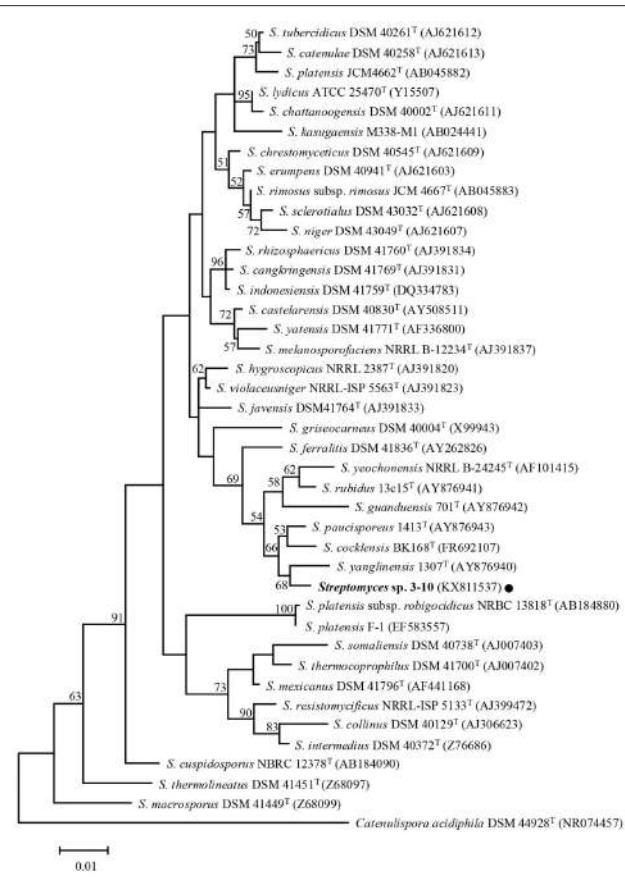


FIGURE 3 | A maximum-likelihood tree showing the relationship between *Streptomyces* sp. 3-10 and 39 other taxa of *Streptomyces*.

The phylogenetic tree was inferred based on 16S rDNA sequences with *Catenuliospora acidiphila* as the out-group. The bootstrap values ($n = 1,000$) higher than 50% are shown at the internodes in the tree. The NCBI GenBank accession numbers are given in parentheses following the strain names. The scale bar indicates 1% nucleotide substitution per site.

sulfamethoxazole (Tables S6, S7). Therefore, strain 3-10 may represent a physiological type different from strain 1307^T.

It is quite interesting that among the four reveromycins-producing strains of *Streptomyces* (SN-593, MST-MA568, MST-RA7781, 3-10) so far known, three strains of *Streptomyces* (MST-MA568, MST-RA7781, 3-10) are closely related to *S. yanglinensis* based on phylogeny of 16S rDNA sequences (Figure 3; Fremlin et al., 2011). This result suggests that

S. yanglinensis-related species may have a conserved pathway for biosynthesis of reveromycins. Several other strains of *S. yanglinensis* from soil samples collected in China (e.g., strains 317, 913 and 1,307) were readily reported (Xu et al., 2006). Whether or not they can produce reveromycins remains unknown and additional studies are necessary to clarify this point.

CONCLUSIONS

This study demonstrated that the crude extract of *Streptomyces* sp. 3-10 could effectively inhibit mycelial growth of 22 species of fungi and spore germination of *B. cinerea*, *M. hiemalis*, and *R. stolonifer*. Two antifungal compounds, reveromycins A and B, were purified from the cultures of *Streptomyces* sp. 3-10. Both the crude extract of strain 3-10 and reveromycin A purified from that strain showed high efficacy in suppression of strawberry fruit rot caused by *B. cinerea*, *M. hiemalis*, *R. stolonifer*, and *S. sclerotiorum*. The efficacy was comparable to that of corresponding commercial fungicides. The findings of this study are useful for further exploitation of reveromycins to control plant fungal diseases.

AUTHOR CONTRIBUTIONS

AL, HL, HC, LY designed research; AL and HL performed research and analyzed the spectra of ^1H NMR and ^{13}C NMR; JZ and MW provided new agents and analyzed the data; AL, WC, and GL wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00550/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fermentation Conditions that Affect Clavulanic Acid Production in *Streptomyces clavuligerus*: A Systematic Review

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The β-lactamase inhibitor, clavulanic acid is frequently used in combination with β-lactam antibiotics to treat a wide spectrum of infectious diseases. Clavulanic acid prevents drug resistance by pathogens against these β-lactam antibiotics by preventing the degradation of the β-lactam ring, thus ensuring eradication of these harmful microorganisms from the host. This systematic review provides an overview on the fermentation conditions that affect the production of clavulanic acid in the firstly described producer, *Streptomyces clavuligerus*. A thorough search was conducted using predefined terms in several electronic databases (PubMed, Medline, ScienceDirect, EBSCO), from database inception to June 30th 2015. Studies must involve wild-type *Streptomyces clavuligerus*, and full texts needed to be available. A total of 29 eligible articles were identified. Based on the literature, several factors were identified that could affect the production of clavulanic acid in *S. clavuligerus*. The addition of glycerol or other vegetable oils (e.g., olive oil, corn oil) could potentially affect clavulanic acid production. Furthermore, some amino acids such as arginine and ornithine, could serve as potential precursors to increase clavulanic acid yield. The comparison of different fermentation systems revealed that fed-batch fermentation yields higher amounts of clavulanic acid as compared to batch fermentation, probably due to the maintenance of substrates and constant monitoring of certain entities (such as pH, oxygen availability, etc.). Overall, these findings provide vital knowledge and insight that could assist media optimization and fermentation design for clavulanic acid production in *S. clavuligerus*.

Keywords: clavulanic acid, clavulanate, *Streptomyces clavuligerus*, fermentation, systematic review

INTRODUCTION

Microorganisms serve as attractive resources, owing to their ability to synthesize structurally-diverse substances with various bioactivities (Demain, 1999; Newman et al., 2000; Bérdy, 2005; Demain and Sanchez, 2009). These microbial natural products may be used as effective drug(s) or act as drug lead compounds that could be further modified and developed for higher efficacy. Within the Bacteria domain, actinomycetes showed unprecedented ability to produce potentially novel, clinically useful, secondary metabolites such as anticancer, antioxidants, antivirals and antibacterials (Ara et al., 2014; Lee et al., 2014a,b; Manivasagan et al., 2014; Azman et al., 2015; Ser et al., 2015a,b; Tan et al., 2015). These filamentous bacteria produce around 8700 antibiotics, with the majority of them derived from members of the *Streptomyces* genus (Bérdy, 2005; Demain and Sanchez, 2009; de Lima Procópio et al., 2012). As the largest antibiotic-producing genus, *Streptomyces* species are capable of producing different classes of antibiotics including aminoglycosides (e.g., streptomycin by *S. griseus*), macrolides (e.g., tylosin from *S. fradiae*), and β -lactams (e.g., cephalexin and clavulanic acid by *S. clavuligerus*) (Waksman et al., 1944; Brown et al., 1976; Reading and Cole, 1977; Okamoto et al., 1982).

The β -lactam antibiotics are one of the most popular classes of antibacterial agents, whose mechanism of action is via inhibition of bacterial cell wall synthesis (Page, 2012). Soon after the utilization of β -lactam antibiotics, a number of bacteria have been found to exhibit resistance to this class of drugs. One of the strategies deployed by this group of bacteria to survive against β -lactam antibiotics is by the production of a β -lactam-hydrolyzing enzyme – β -lactamase; which functions to neutralize these antibiotics by cleaving the β -lactam ring (Wilke et al., 2005; Toussaint and Gallagher, 2015). Thus, to overcome this resistance, β -lactamase inhibitors are often used in conjunction with β -lactam antibiotics as these compounds prevent the degradation of these antibiotics and increase the efficacy of these drugs (Saudagar et al., 2008).

Clavulanic acid was first purified as a novel β -lactamase inhibitor from *S. clavuligerus* ATCC 27064, which was isolated from South American soil in 1971 (Higgins and Kastner, 1971; Brown et al., 1976). This compound presents with a nucleus similar to that of penicillin, with notable differences such as lacking anacylamo side chain, containing oxygen in place of sulfur, and having a β -hydroxyethylidene substituent in the oxazolidine ring (Brown et al., 1976; Saudagar et al., 2008). Clavulanic acid or clavulanate, is commercially used along with amoxicillin (Augmentin) and this combination has been listed as an important antibacterial agents in the WHO list of essential medicines (2015) (Toussaint and Gallagher, 2015). This compound was first recovered from the fermentation process, which remains as one of the most frequently used strategies to manufacture important drugs and their intermediates for medicinal use. In order to facilitate the higher production of valuable compound as such, advanced fermentation technologies were subsequently developed, which included fed-batch fermentation systems (Thiry and Cingolani, 2002; Schmidt, 2005).

At the same time, researchers began to look into the biosynthesis pathway of clavulanic acid in an attempt to maximize its production (Figure 1). These efforts then resulted in the identification of two important precursors for clavulanic acid—arginine (C5 precursor) and glutaraldehyde-3-phosphate (C3 precursor) (Romero et al., 1986; Kanehisa and Goto, 2000; Kanehisa et al., 2016). Apart from clavulanic acid, *S. clavuligerus* is known to produce other clavams and cephalexin; as illustrated in Figure 1. As *S. clavuligerus* is unable to assimilate glucose, various compounds have been studied as C3 precursor candidates to ensure proper formulation of fermentation media and improve the yield of clavulanic acid (Aharonowitz and Demain, 1978; Garcia-Dominguez et al., 1989; Pérez-Redondo et al., 2010). Thus, this systematic review examined the effect of different fermentation conditions on the production of clavulanic acid in *S. clavuligerus*. Based on the available literature, our objective was to describe how additional supplements in basal medium, pH, as well as temperature affect the production of the β -lactamase inhibitor in *S. clavuligerus*, which in turn could assist and improve the development of fermentation media and/or systems for the production of this valuable antibiotic, clavulanic acid.

METHODS

This systematic review was carried out in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (Moher et al., 2009).

Database Search

Systematic searches were performed in the following databases: PubMed, Medline, ScienceDirect, and EBSCO. MeSH terms were “*Streptomyces*,” “*clavuligerus*” combined with “clavulanic acid” or “clavulanate.” We included studies from database inception to June 30th, 2015.

Study Selection and Data Extraction

Two reviewers (H-LS and JW-FL) independently screened and evaluated all titles and abstracts retrieved from the comprehensive search, based on the inclusion and exclusion criteria. The bibliographies of relevant studies were checked for additional publications. Full text of selected original articles were then obtained and reviewed. Any disagreements between the two reviewers were resolved by consensus. Studies providing data of clavulanic acid production in *Streptomyces clavuligerus* were included. Other inclusion criteria were: (1) studies must involve wild-type *Streptomyces clavuligerus*; (2) studies must describe fermentation conditions using batch and/or fed-batch fermentation strategies; and (3) studies must report the specific amount of clavulanic acid produced by *Streptomyces clavuligerus*. Studies conducted using *S. clavuligerus* mutants, studies conducted in organisms other than *S. clavuligerus*, and studies reporting only specific production of clavulanic acid; were excluded. We also excluded solid phase extraction studies, immobilization studies, and all reviews, conference abstracts, systematic reviews, meta-analyses, comments, and letters to the editor. The following information was extracted independently

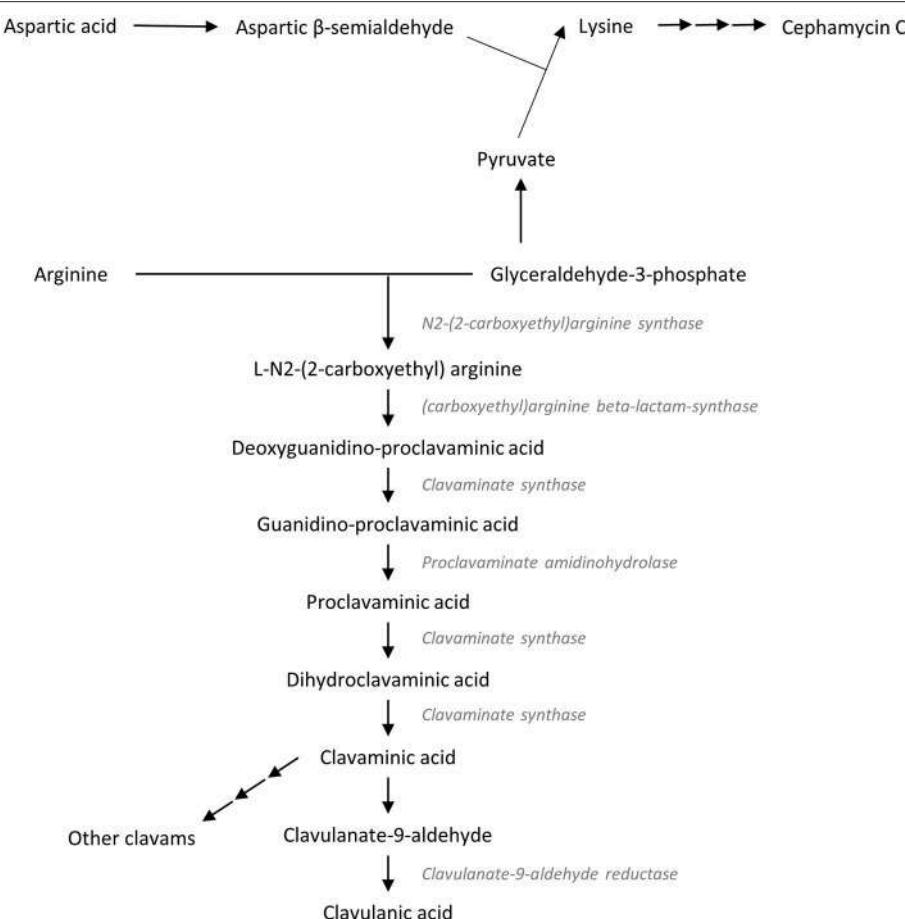


FIGURE 1 | Biosynthesis pathways of clavulanic acid (Kanehisa and Goto, 2000; Lynch and Yang, 2004; Kanehisa et al., 2016).

by the two reviewers from each study (Table 1): (1) study and year of publication, (2) fermentation type and volume, (3) oxygen control and/or airflow control, (4) fermentation/production media composition and pH, (5) addition of supplements (e.g., glycerol, starch, and vegetable oils), and (6) maximum clavulanic acid produced. Any discrepancies were discussed between both authors.

RESULTS

Literature Search

The search yielded a total of 627 articles, while an additional 11 articles were obtained from other sources (Figure 2). After the removal of duplicate records, a total of 474 articles were accessed, out of which 432 articles were excluded based on their titles and abstracts. 42 full text articles were reviewed, out of which 29 studies were eligible for the qualitative analysis according to the inclusion criteria (Table 1). The analysis was divided into several categories based on the design of the experiments: (a) utilization of glycerol or starch as sole carbon source, (b) addition of glycerol and different oil in batch fermentation, (c) amino acids as supplements in basal medium, (d) other factors

affecting clavulanic acid production in batch fermentation, and (e) comparison between batch and fed-batch fermentations.

Utilization of Glycerol, Starch, or Sucrose as Sole Carbon Source

Thakur et al. (1999) and Maranesi et al. (2005) described that different media compositions resulted in different levels of clavulanic acid production. When comparing between glycerol and sucrose as a sole carbon source, Lee and Ho (1996) observed no production of clavulanic acid in media added with glycerol, but higher production of the compound in media with sucrose (3.63 mg/L). Similar findings were also reported by Ives and Bushell (1997) where no production of clavulanic acid was observed in glycerol-containing C-limited media. Meanwhile, another study by Thakur et al. (1999) demonstrated that the addition of dextrin or glycerol as a sole carbon source neither improved nor decreased the production of clavulanic acid. On the contrary, two studies reported a totally different observation—basal media containing glycerol exhibited higher maximum amounts of clavulanic acid as compared to starch (Saudagar and Singhal, 2007a; Bellão et al., 2013). Indeed the maximum amount of clavulanic acid observed in media containing glycerol

TABLE 1 | Effect of fermentation conditions on clavulanic acid production in *Streptomyces clavuligerus* (Max CA, maximum clavulanic acid; NBD, neutralized, bleached and deodorized; NB, neutralized and bleached; TN, total nitrogen; N.A., not available).

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	O ₂ control	pH	Temperature, °C		Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L	
							Fixed	Gradient				
1 Romero et al., 1986	NRRL 3585	Glycerol, sucrose, proline, NaCl, K ₂ HPO ₄ , CaCl ₂ , MnCl ₂ ·4H ₂ O, FeCl ₃ ·6H ₂ O, ZnCl ₂ , MgSO ₄ ·7H ₂ O	Batch	N.A.	N.A.	No	7	N.A.	N.A.	Arginine, Ornithine	16 µg/mg DW [*] 9 µg/mg DW [*]	
2 Lebiri et al., 1987	N.A.	Glycerol, L-asparagine, MgSO ₄ , K ₂ HPO ₄ , FeSO ₄ ·7H ₂ O, MnCl ₂ ·4H ₂ O, ZnSO ₄ ·7H ₂ O, CaCl ₂	Batch	1000/3000	200/600	No	6.9	28	–	250	Phosphate (2 mM) Phosphate (75 mM)	
3 Belmar-Beney and Thomas, 1991	N.A.	Glycerol, malt extract, bacteriological peptone, polypropylene glycol (antifoam)	Batch	7000	5000	0.5 vvm	7	26	–	490 990 1300	– – –	
4 Lee and Ho, 1996	NRRL 3585	Proline, glutamic acid, NaCl, K ₂ HPO ₄ , CaCl ₂ , MnCl ₂ ·4H ₂ O, FeCl ₃ ·6H ₂ O, AnO ₂ , MgSO ₄ ·7H ₂ O	Batch	N.A.	40	No	8.3	27	–	220	Glycerol Sucrose Crude palm oil NBD palm olein NBD palm stearin NB palm-kernel olein NB palm-kernel stearin Oleic acid	
5 Ives and Bushell, 1997	NRRL 3585	Glycerol, NH ₄ Cl, K ₂ HPO ₄ , KH ₂ PO ₄ , MgSO ₄ ·7H ₂ O, FeSO ₄ ·7H ₂ O, CuCl ₂ , CoCl ₂ , CaCl ₂ ·2H ₂ O, ZnCl ₂ , MnO ₂ , Na ₂ MoO ₄	Batch	N.A.	1500	N.A.	30	–	750	C-limited (glycerol, 15 g/L) N-limited (NH ₄ Cl, 1.5 g/L) P-limited (No K ₂ HPO ₄ , replaced with MOPS at 21 g/L)	0 34 35	
6 Kwon and Kim, 1998	ATCC 27064	Starch-asparagine medium	Batch	50	20	No	7	27	–	250	Methyl viologen (added after 38 h)	39.83–49.79 9.96–25.89

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	O ₂ control	pH	Temperature, °C	Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
			Fixed	Gradient						
7 Gouveia et al., 1999	NRRL 35655	Glycerol, sucrose, proline, glutamic acid	Batch	500	50	No	6.5	28	—	47.80-67.71
		Glycerol, sucrose, proline, arginine							menadione (added after 38 h)	55.76-79.66
		Glycerol, peptone, K ₂ HPO ₄ , soy meal							plumbagin (added after 38 h)	63.73-103.56
		Glycerol, peptone, K ₂ HPO ₄ , Samprosoy 90NB							phenazine methosulfate (added after 38 h)	
									H ₂ O ₂ (added after 38 h)	51.78-69.71
8 Thakur et al., 1999	NRRL 35655	Yeast extract, malt extract, peptone, glycerol K medium	Batch	250	27	No	7	30	—	250
		Dextrin, soyabean meal, casein hydrolysate, Fe ₂ SO ₄								—
		Glycerol, sucrose, proline, glutamic acid								—
9 Gouveia et al., 2001	N.A.	Glycerol, K ₂ HPO ₄	Batch	500	55	No	6.5	28	—	250
									Soybean protein extract	1120
									Corn steep liquor, soybean protein extract	490
									Yeast extract, soybean protein extract	700
									Bacteriological peptone, soybean protein extract	900
10 Chen et al., 2002	ATCC 27064	Soymeal extract, peptone, K ₂ HPO ₄	Batch	N.A.	100	No	7	28	—	200
									Glycerol(10 g/L)	11.36
									Glycerol(15 g/L)	11.79
									Glycerol(20 g/L)	10.93

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	Fermentation volume, mL	O_2 control	pH	Temperature, °C		Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
								Fixed	Gradient			
11 Chen et al., 2003	ATCC 27064	Glycerol, soy meal extract, peptone, K_2HPO_4	Batch	5000	3000	1 vvm	7	28	–	200	Glycerol(30 g/L) Glycerol(40 g/L)	4.61 2.89
			Fed-batch	N.A.	100	No	7	28	–	–	Glycerol (added up to 84 h, every 12 h) Glycerol (added up to 108 h, every 12 h) Glycerol (added up to 132 h, every 12 h)	115 230 255 270
12 Lynch and Yang, 2004	N.A.	Glycerol, $MgSO_4 \cdot 7H_2O$, K_2HPO_4 , $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot H_2O$, $CdCl_2$	Batch	2000	200	1 vvm	6.9	30	–	700	L-lysine(1 g/L) L-lysine(20 g/L) L-lysine(1 g/L), degraded clavulanic acid	0 27 42
13 Lin et al., 2005	ATCC 27064	Glycerol, soy meal extract, peptone, KH_2PO_4	Batch	500	110	No	7	28	–	200	–	90

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	O ₂ control	pH	Temperature, °C		Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
							Fixed	Gradient			
14 Maranesi et al., 2005	N.A.	Dextrose, soybean flour, malt extract, FeSO ₄ ·7H ₂ O, MOPS buffer	Batch	3000	500	No	7	28	—	250	—
		Glycerol bacto peptone, soybean flour, MOPS buffer							—	—	200
		Starch, soybean flour, soybean oil, malt extract, K ₂ HPO ₄ , MOPS buffer, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O							—	—	100
		Glycerol, soybean flour, Samprosoy 90NB, malt extract, K ₂ HPO ₄ , MOPS buffer, MnCl ₂ ·4H ₂ O, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O, CaCl ₂							—	—	458
		Soybean flour, soybean oil, malt extract, K ₂ HPO ₄ , MOPS buffer, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O							—	—	170
		soybean flour, malt extract, K ₂ HPO ₄ , MOPS buffer, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O							—	—	478
15 Neto et al., 2005	ATCC 27064	Glycerol, Samprosoy 90NB, malt extract, K ₂ HPO ₄ , MgSO ₄ ·7H ₂ O, MgCl ₂ ·4H ₂ O, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O	Batch	3000	500	No	7	28	—	250	Soybean oil (16 g/L)
											Soybean oil (23 g/L)
											Soybean oil (30 g/L)
											Corn oil (23 g/L)
											Sunflower oil (23 g/L)
											660
											420
											753
											722
											680
											660
											194

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	O ₂ control	pH	Temperature, °C	Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
					Fixed	Gradient				
16 Rosa et al., 2005	ATCC 27064	Glycerol, sambrosoy 90NB, malt extract, yeast extract, K ₂ HPO ₄ , MgSO ₄ ·7H ₂ O, MnCl ₂ ·4H ₂ O, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O	Fed-batch	N.A.	2500	0.5 w/v	6.8	28	—	800 Same composition as media with lower glycerol concentration (10 g/L)
17 Wang et al., 2005 ^{\$}	ATCC 27064	Soy flour, glycerol, ornithine, K ₂ HPO ₄ , FeSO ₄ , MgSO ₄	Batch	300	30	No	N.A.	28	—	250 Varied concentration of each components
18 Bushell et al., 2006	NRRL 3685	Glycerol, NH ₄ Cl, K ₂ HPO ₄ , MOPS, MgSO ₄ ·7H ₂ O, FeSO ₄ ·7H ₂ O, CuCl ₂ , OoCl, CaCl ₂ ·2H ₂ O, ZnCl ₂ , MnCl ₂ , Na ₂ MoO ₄	Batch	2500	1000	2 w/v	6.8±0.2	30	—	750 Varied concentration of each components
19 Teodoro et al., 2006	ATCC 27064	Glycerol, bacto peptone, malt extract, yeast extract, K ₂ HPO ₄ , MgCl ₂ ·4H ₂ O, MnCl ₂ ·4H ₂ O, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O, silicone antifoam	Batch	4000	4000	0.5 w/v	6.8±0.1	28	—	800 Samprosoy 90NB (10 g/L)
20 Ortiz et al., 2007	ATCC 27064	Glycerol, soybean oil (SO), K ₂ HPO ₄ , MnCl ₂ ·4H ₂ O, FeSO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O	Batch	500	50	No	6.8	28	—	250 Soybean flour Soy protein isolate
				N.A.	4000	No	6.8±0.1	28	—	800 Soybean flour (TN: 1.6 g/L, SO: 16 g/L)
										380 Samprosoy 90NB (20 g/L)
										290 Samprosoy 90NB (30 g/L)

(Continued)

TABLE 1 | Continued

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermenter volume, mL	Fermentation volume, mL	O ₂ control	pH	Temperature, °C	Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
								Fixed	Gradient		
222 Saundager and Singhal, 2007 ^b	ATCC 27064	Glycerol, sucrose, proline, glutamic acid, CaCl ₂ , 2H ₂ O, FeCl ₃ , MnCl ₂ , NaCl, MgSO ₄ .7H ₂ O, ZnCl ₂ , KH ₂ PO ₄	Batch	N.A.	N.A.	N.A.	7.0± 0.2	N.A.	—	N.A.	600-1100
										L-arginine (1-100 mM)	650-850
										L-proline (1-100 mM)	650-750
										L-ornithine (1-100 mM)	650-900
										L-lysine (1-100 mM)	30-100
										L-leucine (1-100 mM)	750-1700
										L-threonine (1-100 mM)	500-800
										L-typtophan (1-100 mM)	600-620
										L-cysteine (1-100 mM)	420-430
										L-valine (1-100 mM)	20-50
	Batch	N.A.	N.A.	N.A.	N.A.	N.A.	7.0± 0.2	N.A.	N.A.	KH ₂ PO ₄ (1 mM)	724
										KH ₂ PO ₄ (10 mM)	878
										KH ₂ PO ₄ (100 mM)	779
										KH ₂ PO ₄ (200 mM)	524
	Fed-batch	N.A.	N.A.	N.A.	N.A.	N.A.	7.0± 0.2	N.A.	N.A.	Control	1100
										Glycerol (added every 12 h till 60 h)	1200
										Glycerol (added every 12 h till 72 h)	1280
										Glycerol (added every 12 h till 120 h)	1300
	Fed-batch	N.A.	N.A.	N.A.	N.A.	N.A.	7.0± 0.2	N.A.	N.A.	Arginine (added every 12 h)	1310
										Theanine (added every 12 h)	1863

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	Fermentation volume, mL	O ₂ control	pH	Temperature, °C		Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
								Fixed	Gradient			
23 Eftimiu et al., 2008	ATCC 27064	NH ₄ Cl, KH ₂ PO ₄ , MOPS	Batch	N.A.	50	No	6.8	N.A.	N.A.	Glycerol	Sunflower oil	25
										Soybean oil	Flaxseed oil	18
										Rapeseed oil	Olive oil	N.A.
												N.A.
												47
24 Kim et al., 2009	NRRL 3585	Soya flour, phosphate, MgCl ₂ ·4H ₂ O, Fe ₂ SO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O	Batch	7000	4500	No	7.0	N.A.	N.A.	Olive oil	Palm oil	820
										Corn oil	Triclein	700
										Triplamitin	Trilinolein	380
												989
												410
												220
25 Salem-Berkhit et al., 2010	ATCC 27064	Starch, soybean flour, phosphate, ZnSO ₄ ·H ₂ O, Fe ₂ SO ₄ ·7H ₂ O, MnCl ₂ ·4H ₂ O	Batch	500	50	No	7.0	28	—	Glycerol	Olive oil	564
										Cotton seed oil	Corn oil	1120
										Castor oil	Castor oil	740
										Coconut oil	Coconut oil	911
										Palm oil	Palm oil	300
										Sunflower oil	Sunflower oil	380
										Linseed oil	Linseed oil	580
												600
												700
26 Teodoro et al., 2010	ATCC 27061	Glycerol, bacto peptone, malt extract, yeast extract, K ₂ HPO ₄ , MgCl ₂ ·4H ₂ O, MnCl ₂ ·4H ₂ O, Fe ₂ SO ₄ ·7H ₂ O, ZnSO ₄ ·7H ₂ O, silicone antifoam, soybean oil	Batch	500	50	No	6.0	28	—	Glycerol	Glycerol	25
										Glycerol	Glycerol	564
												300
												117
												1120
												500
												509

(Continued)

TABLE 1 | Continued

References	Strain	Basal medium	Fermentation type	Fermentation volume, mL	O ₂ control	pH	Temperature, °C	Shaking/Stirrer speed, rpm	Supplement	Max CA concentration [#] , mg/L
					Fixed	Gradient				
27 Cerri and Badino, 2012	N.A.	Glycerol, bactopeptone, K ₂ HPO ₄ , MgSO ₄ .7H ₂ O, MnCl ₂ .4H ₂ O, FeSO ₄ .7H ₂ O, ZnSO ₄ .7H ₂ O, MOPS	Batch	N.A.	5000	3 wvm 4.1 wvm	6.8±0.1	N.A.	N.A.	—
28 Costa and Badino, 2012	ATCC 27064	Glycerol, soybean protein isolate, K ₂ HPO ₄ , MgSO ₄ .7H ₂ O, MOPS	Batch	500	50	No	6.8±0.1	N.A.	N.A.	—
29 Bellão et al., 2013	DSM 41826	Soybean meal L-lysine, yeast extract, K ₂ HPO ₄ , MgSO ₄ .7H ₂ O, CaCl ₂ .2H ₂ O, NaCl, FeSO ₄ .7H ₂ O, MnCl ₂ .4H ₂ O, ZnSO ₄ .7H ₂ O	Batch	5000	4000	No	6.8±0.1	28	—	800 Glycerol Starch
										348.5 125.2
										1051.9-1186.6 200.0-440.1
										982.1 469.0

*The study by Romero et al. (1986) expressed maximum clavulanic acid production in a different unit, µg of clavulanic acid per mg of biomass dry weight.

[#]Values estimated from original studies.

^{\$}For media optimization, studies used factorial design matrix (Wang et al., 2005) and L₂₅ orthogonal array (Saudegar and Singhvi, 2007a).

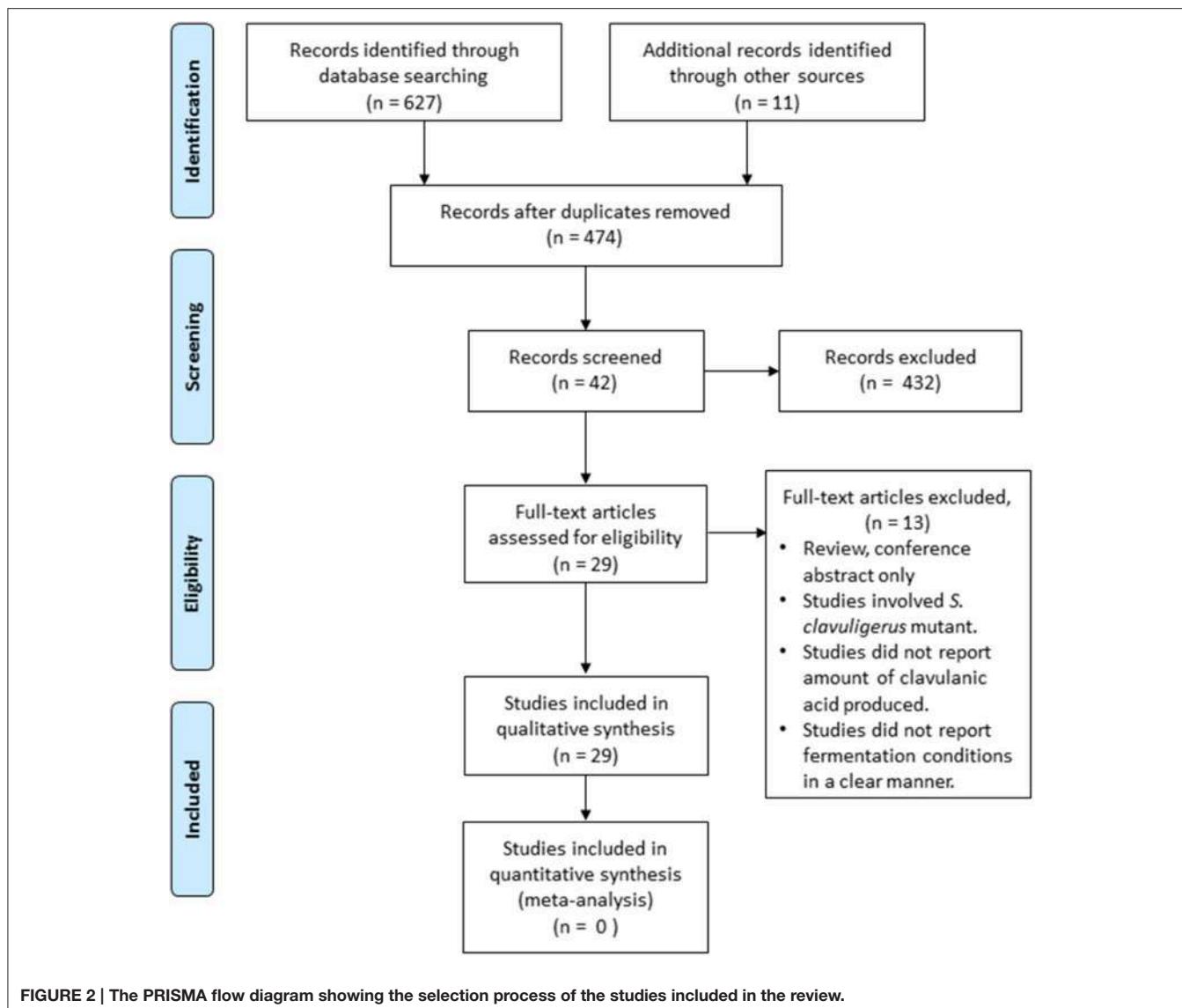


FIGURE 2 | The PRISMA flow diagram showing the selection process of the studies included in the review.

was found to be 348.5 mg/L, nearly two times higher compared to media with starch as a sole carbon source (Bellão et al., 2013). Additionally, two other studies by Chen et al. (2002) and Saudagar and Singhal (2007a) revealed a biphasic dose response of glycerol; whereby clavulanic acid production was inhibited at concentrations which were either too high or too low.

Addition of Glycerol and Different Oil in Batch Fermentation

Apart from glycerol, several studies (*n* = 6) have also looked at how other oil and unsaturated fatty acids affect the production of clavulanic acid in *S. clavuligerus*. An earlier study revealed a relatively low production of clavulanic acid in media containing different fractions of palm oil or its purified, major constituents (e.g., palmitic acid, stearic acid, lauric acid, oleic acid) (Lee and Ho, 1996). A more recent study in 2009 by Kim et al. (2009) reported maximum clavulanic acid production of 700 mg/mL

in media supplemented with palm oil, an intermediate value as compared to other oil sources. The highest clavulanic acid production reported in the same study was observed in media containing triolein (which is a major constituent of palm oil) at 989 mg/L.

Different vegetable oils may stimulate the production of clavulanic acid, as demonstrated by two different studies. In batch fermentation, Efthimiou et al. (2008) described increased clavulanic acid production when olive oil was used in place of glycerol as a sole carbon source; with a maximum concentration of 47 mg/L being recorded, which is nearly double that observed in media containing glycerol (25 mg/L). These results were consistent with another recent study, whereby the addition of olive oil improved clavulanic acid production as compared to glycerol; with a maximum concentration at 1120 and 564 mg/L, respectively (Salem-Berkhit et al., 2010). Relatively high production was also observed in media supplemented with

corn oil (911 mg/L), followed by cotton seed oil (740 mg/L), and linseed oil (700 mg/L). Media with castor oil was found to yield the lowest amount of clavulanic acid (300 mg/L). Given that different vegetable oils may have a slight difference in fatty acids and lipid composition, Maranesi et al. (2005) described that when the same concentration of oil was used, the production of clavulanic acid differed slightly between soybean oil, corn oil, and sunflower oil. The greatest concentrations of clavulanic acid using soybean oil, corn oil, and sunflower oil was recorded to be between the ranges of 660–753 mg/L. These results were consistent with a study by Saudagar and Singhal (2007a), as similar concentrations of clavulanic acid was observed in media containing palm oil and soybean oil.

Protein and Amino Acids as Supplements in Basal Medium

The choice of soybean flour or soy protein isolate in fermentation media affects the production of clavulanic acid by *S. clavuligerus* (Gouveia et al., 1999; Wang et al., 2005; Ortiz et al., 2007). The difference in the type of protein as a source of nitrogen was found to affect the production of clavulanic acid as studied by Gouveia et al. (2001). Increased production of clavulanic acid was reported in media containing soybean flour (698 mg/L) rather than soy protein isolate (338 mg/L). Further investigation with soybean flour revealed that different amounts of nitrogen with varied amounts of soybean oil, produced different concentrations of clavulanic acid (660 mg/L – 906 mg/L). Similar findings were reported by Teodoro et al. (2006) as different clavulanic acid concentrations were observed (135–380 mg/L) in media containing varied concentrations of soy protein isolate.

The investigation on the role of amino acids as a source of nitrogen in the production of clavulanic acid began in 1986 (Romero et al., 1986). Several studies focusing on the effects of the amino acids, arginine and ornithine; toward the production of clavulanic acid showed inconsistent results. Romero et al. (1986) reported maximum concentrations of clavulanic acid in a slightly different manner, where 16 µg/dry weight of biomass was observed with arginine; while 9 µg/dry weight of biomass was observed with ornithine. Differing results were recorded by Chen et al. (2003) as media supplemented with ornithine contained higher concentrations of clavulanic acid at 200 mg/L, compared with arginine at 100 mg/L. Nevertheless, recent studies supported the results of Romero et al. (1986); where higher amounts of clavulanic acid were seen in media supplemented with different concentrations of arginine compared to ornithine (Saudagar and Singhal, 2007a,b). One of the studies also tested the effect of other amino acids such as L-proline, L-lysine, L-leucine, L-glutamine, L-threonine, L-tryptophan, L-cysteine, and L-valine (Saudagar and Singhal, 2007b). Among these amino acids, the highest concentration of clavulanic acid was observed in media supplemented with L-threonine; which was not reported in other literature included in this systematic review. Lynch and Yang (2004) tested the influence of L-lysine further by adding degraded clavulanic acid into the fermentation broth. The study suggested that L-lysine is one of the most important amino acids for the production

of clavulanic acid, given that fermentation broths with low concentrations of L-lysine (1 g/L) failed to yield any clavulanic acid. Moreover, the addition of degraded clavulanic acid showed improved clavulanic acid production (maximum clavulanic acid concentration at 42 mg/L) as compared to fermentation broths with L-lysine alone (20 g/L, clavulanic acid concentration at 27 mg/L).

Other Factors Affecting Clavulanic Acid Production in Batch Fermentation

Aside from sole carbon or nitrogen sources, other factors that may affect the production of clavulanic acid include the addition of phosphate, pH, temperature, and agitation or shaking speed. The potential repressive effect of phosphate on clavulanic acid production in *S. clavuligerus* was demonstrated by two selected studies (Lebrihi et al., 1987; Bushell et al., 2006; Saudagar and Singhal, 2007b). The study by Lebrihi et al. (1987) tested two levels of phosphate, 2 and 75 mM. Based on HPLC measurements, the lower concentration of phosphate (2 mM) in fermentation media was found to contain significantly higher levels of clavulanic acid; with maximum concentration observed at 90 mg/L, as compared to 3 mg/L observed in fermentation media containing high concentrations of phosphate (75 mM). Without changing the pH of the media, Saudagar and Singhal (2007b) demonstrated that the addition of phosphate in the production medium showed a biphasic response. At the highest tested concentration of KH₂PO₄ (200 mM), the maximum concentration of clavulanic acid dropped drastically to 524 mg/L. Thus the optimum concentration of KH₂PO₄ for clavulanic acid production was determined to be 10 mM (with a maximum clavulanic acid concentration recorded at 878 mg/L).

Furthermore, the pH of fermentation media was described as having a profound effect on clavulanic acid yield (Saudagar and Singhal, 2007a; Salem-Berkhit et al., 2010). By using fermentation media with different pH, different levels of clavulanic acid were seen; with maximum concentrations reported at pH 7. Similar patterns of clavulanic acid production were seen in the tested fermentation media regardless of the sole carbon sources used (i.e., glycerol or olive oil). In addition, Costa and Badino (2012) reported that the temperature at which fermentation was carried out may lead to variation in clavulanic acid production. Low fermentation temperature (20°C) resulted in maximum clavulanic acid concentration as high as 1266.2 mg/L as compared to 631.6 mg/L at 25°C and 168.7 mg/L at 30°C.

Aeration or agitation speed throughout cultivation and production was described to affect clavulanic acid yield as well. In fact, the effect of aeration on the production of clavulanic acid can be tested with a direct experiment involving the use of the Erlenmeyer flask with different baffle heights (Lin et al., 2005). The results showed that the flask with a higher baffle height had a slight increase in the production of clavulanic acid (180 mg/L), as compared to a normal Erlenmeyer flask (90 mg/L). Another study showed that agitation speed has a positive correlation with clavulanic acid production (Rosa et al., 2005). At 800 rpm, two flasks with different oxygen flow rates showed similar levels of maximum clavulanic acid concentration, with 475 mg/L obtained from the flask with the low oxygen flow rate and 482 mg/L

from the flask with the low oxygen flow rate. A study by Cerri and Badino (2012) also supported the view that an increase in agitation speed leads to higher production of clavulanic acid, but not oxygen flow. When the oxygen flow was increased to 4.1 vvm, the maximum concentration of clavulanic acid was observed to be 269 mg/L, which is approximately half of the maximum concentration observed with an oxygen flow of 3 vvm (454 mg/L). However, these results were contradictory with a previous study by Belmar-Beiny and Thomas (1991) which showed that there is no significant difference in clavulanic acid production as a result of different stirring speeds, even with same oxygen flow rate.

Besides that, the presence of redox-cycling agents in the production media may influence the production of clavulanic acid (Kwon and Kim, 1998). Five redox-cycling agents were tested—methyl viologen, menadione, plumbagin, phenazine methosulfate, and hydrogen peroxide (H_2O_2). All of the redox-cycling agents promoted the production of clavulanic acid, except methyl viologen (9.96–25.89 mg/L). The highest maximum clavulanic acid concentration was described with phenazine methosulfate (63.73–103.56 mg/L), followed by plumbagin (55.76–79.66 mg/L), and H_2O_2 (51.78–69.71 mg/L).

Comparison between Batch and Fed-Batch Fermentations

Among the selected studies, there were a total of seven fed-batch fermentation experiments, with the majority looking at the effect of adding glycerol into the fermenter over a period of time. Most of the studies took similar approaches to study the effect of glycerol in fed-batch fermentations: (a) by maintaining glycerol at a certain level throughout the fermentation period and/or (b) by adding fixed amounts of glycerol at fixed time points (Chen et al., 2002; Neto et al., 2005; Saudagar and Singhal, 2007b; Teodoro et al., 2010; Costa and Badino, 2012). Regardless of the methods, the study showed higher levels of maximum clavulanic acid concentration than control in the fed-batch fermentation. Comparing batch and fed-batch fermentation, fed-batch fermentation systems seemed to generate a higher yield of clavulanic acid (Chen et al., 2002; Neto et al., 2005; Bellão et al., 2013). A study by Bellão et al. (2013) observed a higher maximum clavulanic acid concentration in the latter method at 982.1 mg/L, as compared to 348.5 mg/L. Apart from the addition of glycerol, lower fermentation temperatures also resulted in a higher yield of clavulanic acid, which was also observed in both batch and fed-batch fermentation.

Besides that, the effects of amino acid was also studied using the fed-batch fermentation approach. Following batch fermentation that revealed increased clavulanic acid production by arginine and threonine, Saudagar and Singhal (2007b) showed that by using fed-batch fermentation technologies; the production of the compound could be further increased. Nevertheless, another study reported lower clavulanic acid yield in fed-batch fermentation with the addition of ornithine as compared to batch fermentation (Chen et al., 2003). On top of that, an increase in the amount of clavulanic acid produced was seen in other fed-batch experiments with the addition of glycerol and arginine. Chen et al. (2003) reported that fed-batch fermentation using glycerol, ornithine, and arginine;

yielded different amounts of clavulanic acid, whereby the highest amount was demonstrated with glycerol (300 mg/L), followed by arginine (210 mg/L), and the lowest with ornithine (110 mg/L). The addition of glycerol together with either of the amino acids resulted in intermediate values, where the combination of glycerol and arginine produced 130 mg/L; while glycerol and ornithine resulted in 200 mg/L. Meanwhile, Teodoro et al. (2010) investigated the influence of the presence of ornithine in batch and fed-batch fermentation systems on clavulanic acid production. The study did not find any significant changes in maximum clavulanic acid production due to ornithine, regardless of batch or fed-batch fermentation systems. Interestingly, the same study also revealed an insignificant difference in maximum clavulanic acid concentration when the feeding media (which possesses the same composition as the production media) was replaced with distilled water containing only glycerol and ornithine (at same concentrations as the production media).

DISCUSSION

The microbial fermentation system is important for the discovery and development of pharmaceutical drugs. Clavulanic acid as a β -lactamase inhibitor was initially isolated from *S. clavuligerus* ATCC 27064 using the traditional fermentation system (Higgins and Kastner, 1971; Brown et al., 1976). β -lactamase inhibitors help to prevent drug resistance against β -lactam antibiotics, and allows successful eradication of harmful pathogens. Considering its therapeutic value against infectious diseases, the biosynthesis pathways of clavulanic acid in *S. clavuligerus* have been studied extensively over the years; beginning around 1980s by a research group led by Romero et al. (1986) (Figure 1). The two precursors involved in clavulanic acid biosynthesis, arginine and glyceraldehyde-3-phosphate; undergo a series of enzymatic processes to form the β -lactam inhibitor. Given that both arginine and glyceraldehyde-3-phosphate play an important role in primary metabolism, the production of clavulanic acid could be improved by refining the composition of the fermentation media (Kirk et al., 2000). It is also presumed that *S. clavuligerus* produces higher amount of clavulanic acid when there is adequate supply of these precursors. The wild type strain of *S. clavuligerus* is unable to metabolize glucose, and further molecular studies revealed that the strain lacks the expression of the glucose permease gene (Garcia-Dominguez et al., 1989; Pérez-Redondo et al., 2010).

Based on literature obtained in this study, glycerol was found to be the most popular choice of carbon source in clavulanic acid production; in order to ensure an efficient supply of glyceraldehyde-3-phosphate Once glycerol is metabolized into glyceraldehyde-3-phosphate, it can either enter the clavulanic acid biosynthesis pathway, or be involved in glycolytic or gluconeogenesis reactions (Figure 3). The inclusion of glycerol enhances the production of clavulanic acid as compared to carbohydrates, as glycerol provides a higher energy content on a weight-by-weight basis (Efthimiou et al., 2008). As glycerol serves as a backbone for triglycerides, its utilization by *S. clavuligerus* has prompted researchers to study the potential of other oils

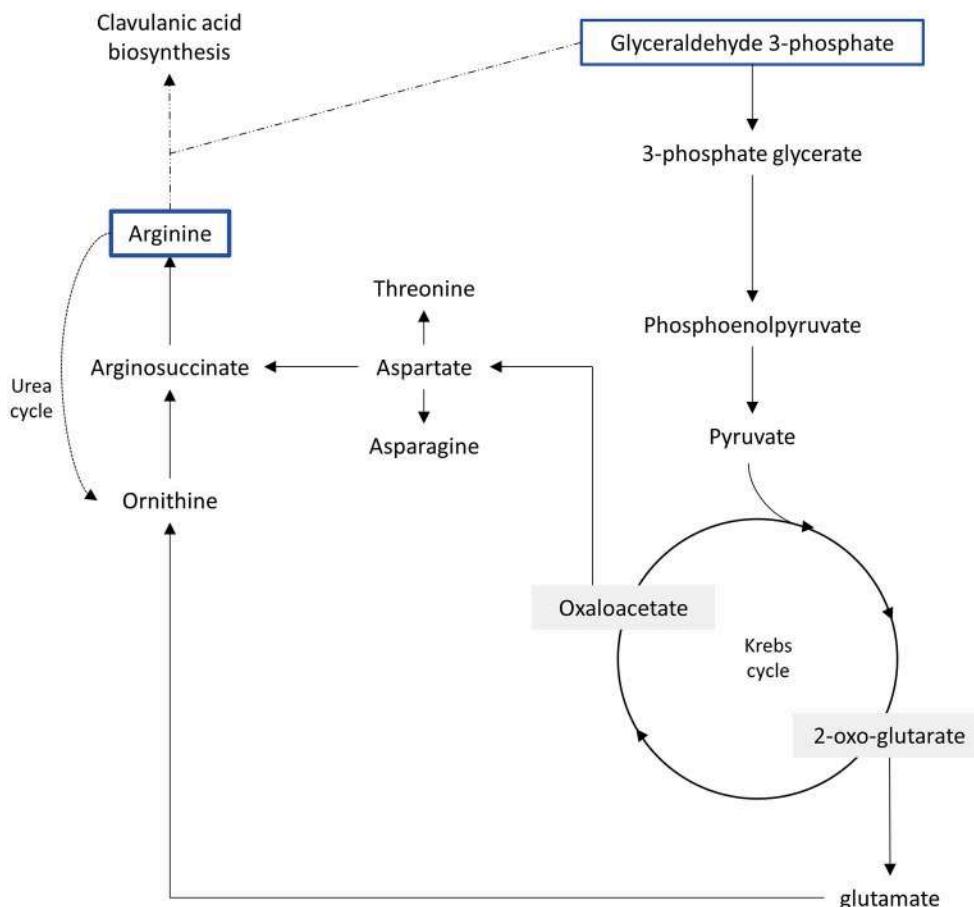


FIGURE 3 | Important pathways that could affect metabolic pool of clavulanic acid precursors - arginine and glutaraldehyde-3-phosphate (Kirk et al., 2000; Bushell et al., 2006).

to be used as a source of carbon. Vegetable oils such as olive oil and corn oil may serve as cost-effective options as they are readily available at a lower cost compared to carbohydrate substrates. As compared to glycerol, vegetable oils seem to be a more attractive source of energy; as a typical oil contains about 2.4 times more energy than glycerol (Stowell, 1987). Other than preventing carbon catabolite regulation, these oils could act as an antifoam, preventing the formation of foam in the media that may impede gas exchange and negatively affect bacteria growth (Friberg et al., 1989; Görke and Stölke, 2008). Overall, the utilization of vegetable oils might be a better choice for clavulanic acid production in fermentation processes compared to glycerol, as these natural oils may further enrich the media with the presence of various polyunsaturated fatty acids (e.g., oleic acid, linoleic acid) (Park et al., 1994). As the addition of olive oil has been shown to greatly improve clavulanic acid production, further investigations on the effect of vegetable oils could suggest the potential of these compounds to be exploited as a cheaper alternative source of carbon in fermentation processes.

On the other hand, many studies have shown the potential of arginine and ornithine as specific precursors for clavulanic acid. These two amino acids are believed to be interconvertible

by the urea cycle via the enzymatic action of arginase (Figure 3). Even though presence of the urea cycle in prokaryotes was once considered unusual, several recent studies have reported arginase activity in *S. clavuligerus* (Mendz and Hazell, 1996; Bushell et al., 2006). These reports then further suggest an important role of this pathway in clavulanic acid biosynthesis. Thus, the addition of arginine or ornithine in fermentation media would increase the flow of C5 precursors into the biosynthesis pathway, which then subsequently increase the production of clavulanic acid (Romero et al., 1986; Lynch and Yang, 2004). From the included studies, one study showed that L-threonine improved the production of clavulanic acid (Saudagar and Singhal, 2007b). Indeed the supplementation of L-threonine was found to prevent the anaplerotic flux on pyruvate to synthesize amino acids such as isoleucine, which in turn increased the availability of C3 precursors and eventually enhanced clavulanic acid production (Ives and Bushell, 1997; Bushell et al., 2006; Saudagar and Singhal, 2007b). Even though the study by Saudagar and Singhal (2007b) has emphasized the role of L-threonine in clavulanic acid production, arginine and ornithine have demonstrated relatively strong influence on clavulanic acid production in *S. clavuligerus*. Thus, further studies on L-threonine could eventually shed

some light on the importance of this amino acid in clavulanic acid production, particularly using the metabolic flux analysis approach.

Apart from media composition, environmental stress is known to affect the production of secondary metabolites in microorganisms including members of *Streptomyces* genus. The availability of oxygen determines the growth and survival of the bacteria as well as production of secondary metabolites, which includes antibiotics in *S. clavuligerus* (Yegneswaran et al., 1991). Out of the selected studies, there were three studies which highlighted the importance of oxygen control and agitation speed. Fermentation in bioreactors is often paired with computers for precise control of reaction conditions. Rosa et al. (2005) showed that the high speed stirring promoted the production of clavulanic acid, which was associated with a lower amount of biomass production. It is believed that the high speed stirring prevents cell clumping and also causes cell shearing (Toma et al., 1991; Rosa et al., 2005). Additionally, Kwon and Kim (1998) demonstrated that the addition of redox-cycling agents increased clavulanic acid production. The presence of reactive oxygen species in the fermentation media leads to an imbalance in redox status, which could trigger stress and damage microbials, or even lead to cell death (Cabisco et al., 2010). The breakage and/or damage of bacterial cells upon exposure to such stress in turn, encourage the neighboring cells to produce secondary metabolites in an attempt to survive and protect against the challenge (Toma et al., 1991; Joshi et al., 1996; Rosa et al., 2005).

In addition, temperature and pH control are also crucial for the production of clavulanic acid and stability of the compound. *S. clavuligerus* ATCC 27064 was reported to have optimal growth from 26 to 30°C with no growth above 37°C (Higgins and Kastner, 1971), while most of the selected studies reported that fermentation temperature ranged between 20 and 30°C. Costa and Badino (2012) demonstrated a maximum clavulanic acid concentration of 1266.2 mg/L when fermentation was performed at 20°C. The study also mentioned that lower concentrations of clavulanic acid was observed with the increase in fermentation temperature. Even though a low temperature of 20°C was not favorable for the growth of *S. clavuligerus*, high production yield of clavulanic acid was observed. There are two possible explanations that could lead to this observation: (a) the low temperature places "cold" stress upon the organism which in turn promotes the production of secondary metabolites (including clavulanic acid); (b) the low temperature may have lowered degradation rate of clavulanic acid, thus ensuring the stability of the compound (Beales, 2004; Bersanetti et al., 2005; Jerzsele and Nagy, 2009; Santos et al., 2009; Feng et al., 2011). Similarly, pH of the medium could affect the growth of *S. clavuligerus*, as it is described to grow between pH 5.0 and 8.5, however sporulation is not observed from pH 7.0 to 8.5 (Higgins and Kastner, 1971). Hence, the determination of the optimum temperature and pH for the fermentation process is critical as slight changes in these factors could tip off the balance between the growth of the organism (biomass) and the production of secondary metabolite(s). On top of that, clavulanic acid was shown to be more stable at a neutral pH, as the decomposition

rate was described to be higher at acidic or alkaline pH (Jerzsele and Nagy, 2009). Taken altogether, the pH of the media and fermentation temperature may play important roles in clavulanic acid production as these factors could eventually lead to degradation of this valuable compound.

In this review, most of the selected papers used a one-factor-at-a-time method in batch fermentation to study the effect of carbon or nitrogen sources on clavulanic production in *S. clavuligerus*. However, two studies incorporated more complicated analyses in their studies to facilitate multifactorial comparisons. For instance, Wang et al. (2005) proposed using statistical methods to optimize the fermentation media for clavulanic acid production by *S. clavuligerus*. By combining factorial factorial design and response surface methodology, the study suggested optimal concentration of three of the most important components identified via factorial factorial design—soy meal powder (38.102 g/L), FeSO₄·7H₂O (0.395 g/L), and ornithine (1.177 g/L). Meanwhile, Saudagar and Singhal (2007a) designed a slightly different fermentation media by undertaking another statistical approach to optimize fermentation media for clavulanic acid production. By using the L₂₅ orthogonal array method, the study suggested optimum concentrations of soybean flour (8.8%), soybean oil (1.2%), dextrin (1.0%), yeast extract (1.5%), and KH₂PO₄ (0.2%); with an optimal pH of 7.0 ± 0.2. Thus in designing a fermentation media for clavulanic acid production, it is important to ensure that the media can support the proper growth of *S. clavuligerus*; as well as provide factors that could stimulate the production of valuable secondary metabolites. Following media optimization, several studies have also incorporated another strategy to maximize the production of clavulanic acid—by utilizing fed-batch fermentation systems which represent a high throughput platform as compared to traditional batch fermentation methods.

The main difference between batch and fed-batch fermentation systems is that the latter is frequently monitored with the assistance of sophisticated technologies and allows precise control of the entire fermentation process (Longobardi, 1994; Li et al., 2014). As a scale-up production process, the fed-batch fermentation system often allows an increase in productivity with a concomitant decrease in production cost. At the time of writing, the current report is one of the first that reviews and investigates the effects of fermentation conditions affecting the production of clavulanic acid in *S. clavuligerus*; and further compares the batch and fed-batch fermentation systems for clavulanic acid production. Costa and Badino (2012) and Bellão et al. (2013) reported the utilization of glycerol in fed-batch fermentation systems resulted in a surge in the production of clavulanic acid (observed as a 1.2–2.8-fold increase in maximum clavulanic acid amount, depending on other factors such as temperature). Compared to glycerol, the addition of amino acid (as a source of nitrogen) in fed-batch fermentation showed less of an effect on the production of clavulanic acid. Only three studies reported the effect of amino acid in fed-batch fermentation (Chen et al., 2003; Saudagar and Singhal, 2007b; Teodoro et al., 2010); where one of the studies discovered that the addition of arginine and ornithine did not affect the production of clavulanic acid in both batch and fed-batch

fermentation systems (Saudagar and Singhal, 2007b). Chen et al. (2003) discovered that ornithine increased the production of clavulanic acid in batch but not fed-batch fermentation, while arginine increased the production in fed-batch but not batch fermentation. Given that the fed-batch fermentation system entails a scale-up process, researchers would expect a higher yield of end-products (Modak et al., 1986; Thiry and Cingolani, 2002; Hewitt and Nienow, 2007). However, it has been suggested that sometimes a large-scale fed-batch fermentation may not generate similar results as observed in small-scale batch fermentation, as the fed-batch fermentation system involves a more dynamic environment (Hewitt and Nienow, 2007). Nevertheless, fed-batch fermentation appears to be a better fermentation strategy for clavulanic acid production as demonstrated in the selected studies. Likewise, further investigations using this fermentation method could upscale clavulanic acid production and ensure a better understanding of the biosynthesis pathways for this valuable compound.

FUTURE PROSPECT AND CONCLUSION

Over a span of 30 years, the research on the fermentation process for the production of clavulanic acid has gained remarkable interest from the scientific community. In this systematic review, a total of 29 studies was selected after a thorough literature search. It is worth mentioning that there were some inconsistencies in the measurement of clavulanic acid production in *S. clavuligerus* as some studies did not report the standard deviation even though the experiments were carried out in replicate. This then did not allow for results to be synthesized quantitatively and perform a meta-analysis. The majority of the articles highlighted the importance of media composition and supplements in the production of clavulanic acid, particularly glycerol, vegetable oils, and the amino acids, arginine and ornithine. In batch fermentation systems which are commonly used for laboratory-scale production, the utilization of various sugars (e.g., dextrin and sucrose) and glycerol as sole carbon sources in clavulanic acid production requires further investigation; as current studies have reported inconsistent results and the role of these compounds in the biosynthesis pathways is yet to be clearly defined. Further investigation into the role of carbohydrates and glycerol in clavulanic acid biosynthesis would greatly improve the

knowledge of media optimization. Nevertheless, the utilization of different oil sources as a sole carbon source and amino acids as a source of nitrogen in the fermentation media seems to have a strong influence on clavulanic acid production in *S. clavuligerus*; followed by other factors such as pH and temperature. Among the vegetable oils, media supplemented with olive oil showed the highest level of clavulanic acid production, which indicates that olive oil contains potentially important nutrients that could improve the production of the antibiotics. Furthermore, amino acids such as arginine and ornithine which could serve as C5 precursors, have also been shown to increase clavulanic acid yield. For the most part, the development of scale-up production tools such as fed-batch fermentation systems could offer a “budget-friendly” method for clavulanic acid production, as this is particularly important for the pharmaceutical industry where production cost is one of the major concerns. With the advancement of next generation sequencing technologies, researchers have identified numerous genes involved in clavulanic acid biosynthesis in *S. clavuligerus* including the clavaminate synthases genes (Medema et al., 2011). By combining this knowledge, further studies involve scale-up productions would be beneficial to identify biosynthetic roles, as well as determine the regulation of these carbon and nitrogen sources in clavulanic acid production in *S. clavuligerus*.

AUTHOR CONTRIBUTIONS

H-LS and JW-FL contributed to the literature database search, data collection, data extraction, data analysis and writing of the manuscript. H-LS, JW-FL, NC, SAJ, UDP, K-GC, B-HG and L-HL performed data analysis and rationalization of the results. The topic was conceptualized by B-HG and L-HL.

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***Streptomyces venezuelae* ISP5230 Maintains Excretion of Jadomycin upon Disruption of the MFS Transporter JadL Located within the Natural Product Biosynthetic Gene Cluster**

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JadL was identified as a Major Facilitator Superfamily (MFS) transporter (T.C. 2.A.1) through sequence homology. The protein is encoded by *jadL*, situated within the jadomycin biosynthetic gene cluster. JadL has, therefore, been assigned a putative role in host defense by exporting its probable substrates, the jadomycins, a family of secondary metabolites produced by *Streptomyces venezuelae* ISP5230. Herein, we evaluate this assumption through the construction and analysis of a *jadL* disrupted mutant, *S. venezuelae* VS678 (Δ *jadL::aac(3)IV*). Quantitative determination of jadomycin production with the *jadL* disrupted mutant did not show a significant decrease in production in comparison to the wildtype strain, as determined by HPLC and by tandem mass spectrometry. These results suggest that efflux of jadomycin occurs upon disruption of *jadL*, or that JadL is not involved in jadomycin efflux. Potentially, other transporters within *S. venezuelae* ISP5230 may adopt this role upon inactivation of JadL to export jadomycins.

Keywords: natural products, MFS transporters, streptomyces, drug efflux, jadomycins, major facilitator superfamily

INTRODUCTION

Secondary metabolite production is controlled by complex regulatory networks which are affected by environmental factors such as temperature, nutrient availability, and signaling. Given the complex nature of such regulatory systems, the expression of secondary metabolites is often difficult to replicate within a laboratory setting, and many clusters remain silent (Rutledge and Challis, 2015). The jadomycins (Figure 1A) are angucycline antibiotics (Ayer et al., 1991; Doull et al., 1993) with cytotoxic activities (Jakeman et al., 2009a; Dupuis et al., 2012) and unique drug efflux properties in drug resistant breast cancer cell lines (Issa et al., 2014; Hall et al., 2015) produced by *Streptomyces venezuelae* ISP5230 (ATCC10712) that are regulated by a “cryptic” pathway; expression is induced with the use of minimal media under stress conditions including ethanol shock, phage induction or co-culture with yeast (Doull et al., 1994; Jakeman et al., 2006). In the absence of these additional stress factors, chloramphenicol (Cam) is the major natural product

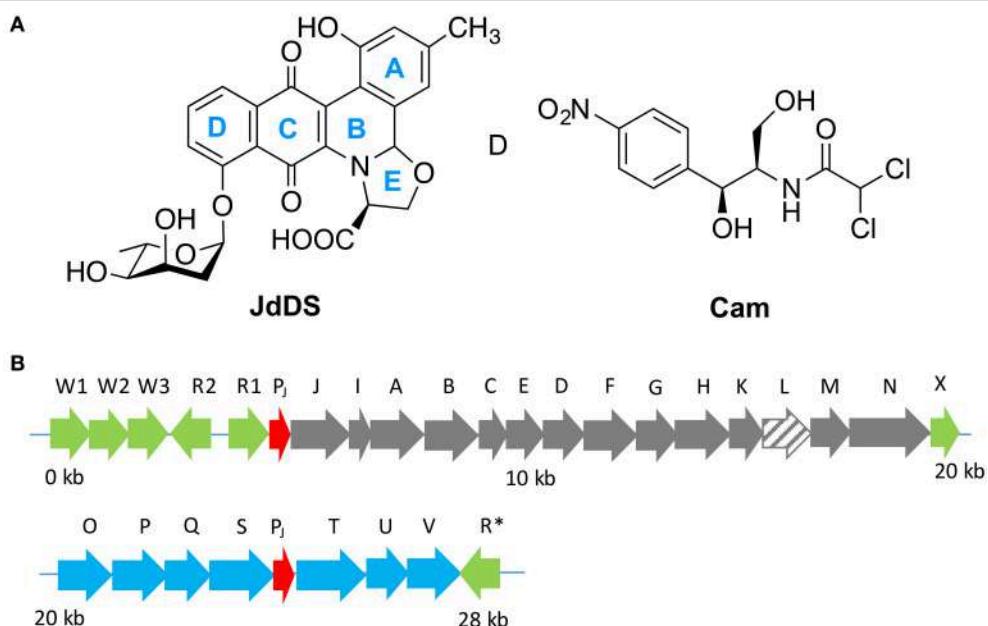


FIGURE 1 | (A) Extensive cross regulation has been reported between the biosynthetic pathways of jadomycins (i.e., **JdDS**) and chloramphenicol (**Cam**) within *S. venezuelae* ISP5230. **(B)** The jadomycin biosynthetic gene cluster. Regulatory genes are colored in green, predicted promoter regions in red, angucycline biosynthetic structural genes in gray, sugar biosynthetic structural genes in blue, and *jadL*, which codes a predicted transport protein, is hatched.

produced by *S. venezuelae* ISP5230. The E-ring present in the jadomycin angucyclic framework arises from a rare spontaneous biosynthetic step involving the incorporation of an amino acid. This chemistry enables a strategy for facile derivatization of the jadomycins using culture media containing a single amino acid (Jakeman et al., 2005, 2009b; Robertson et al., 2015). Recently, as a result of genome analysis a number of natural products have been discovered from *S. venezuelae* ISP5230, including gaburedin (Sidda et al., 2014), venezuelin (Goto et al., 2010), forxymithine (Kodani et al., 2015), (+)-isodauc-8-en-11-ol (Rabe et al., 2015), and venemycin (Thanapipatsiri et al., 2016).

JadL is a putative efflux protein coded within the jadomycin biosynthetic gene cluster (Figure 2). *JadL* is a member of the Major Facilitator Superfamily MFS, Transporter Classification (T.C. 2.A.1), a large and functionally diverse, although structurally and mechanistically conserved, family of transporters with substrates including sugars, amino acids, peptides, drugs, and small anions or cations (Quistgaard et al., 2016). Given the location of *jadL* within the context of the jadomycin gene cluster, we predicted that its primary role would be extracellular export of jadomycins. Herein, we have studied the effect of *jadL* disruption on jadomycin production, that we hypothesized would result in a reduction in the amount of excreted jadomycin.

MATERIALS AND METHODS

Bioinformatics Tools

BLAST searches were performed using the standard BlastP (RRID:SCR_001010) program with the *JadL* amino acid

sequence as input (GenBank: CCA59275.1). Searches excluded environmental and non-cultured strains. BlastP searches were also conducted against the data set from the Transporter Classification Database (TCDB, RRID:SCR_004490). Predictions for transmembrane domains were performed using the constrained consensus TOPology prediction server (CCTOP, <http://cctop.enzim.mta.hu>).

Strain Maintenance and Growth

Streptomyces venezuelae ISP5230 (ATCC10712) spores were maintained as solutions in 25% glycerol at -70°C (Kieser et al., 2000). For natural product productions, *S. venezuelae* strains were maintained at 30°C on maltose yeast malt extract (MYM, maltose 0.4% w/v, yeast extract, 0.4% w/v, malt extract 1% w/v, pH 7.0) agar for 1–2 weeks before inoculation in MYM broth. Media was supplemented with 50 µg mL⁻¹ apramycin for disruption strains.

Construction of the *jadL* Disruption Mutant: *S. venezuelae* VS678 (*ΔjadL::aac(3)IV*)

A 4 kb DNA fragment containing *jadL*, a SacI digest product from lambda clone LH7 (Han et al., 1994), was ligated into SacI linearized pHJL400 (Larson and Hershberger, 1986) to give pJV105A and pJV105B, in which inserts are oppositely oriented. pJV105A was digested with NcoI, situated 722 bp after the *jadL* start codon, and ligated with an apramycin resistance cassette bearing flanking NcoI sites to produce the disruption (*jadL::aac(3)IV*) vector pJV106. *S. venezuelae* ISP5230 protoplasts, prepared as described elsewhere (Yang et al., 1995; Kieser et al., 2000), were

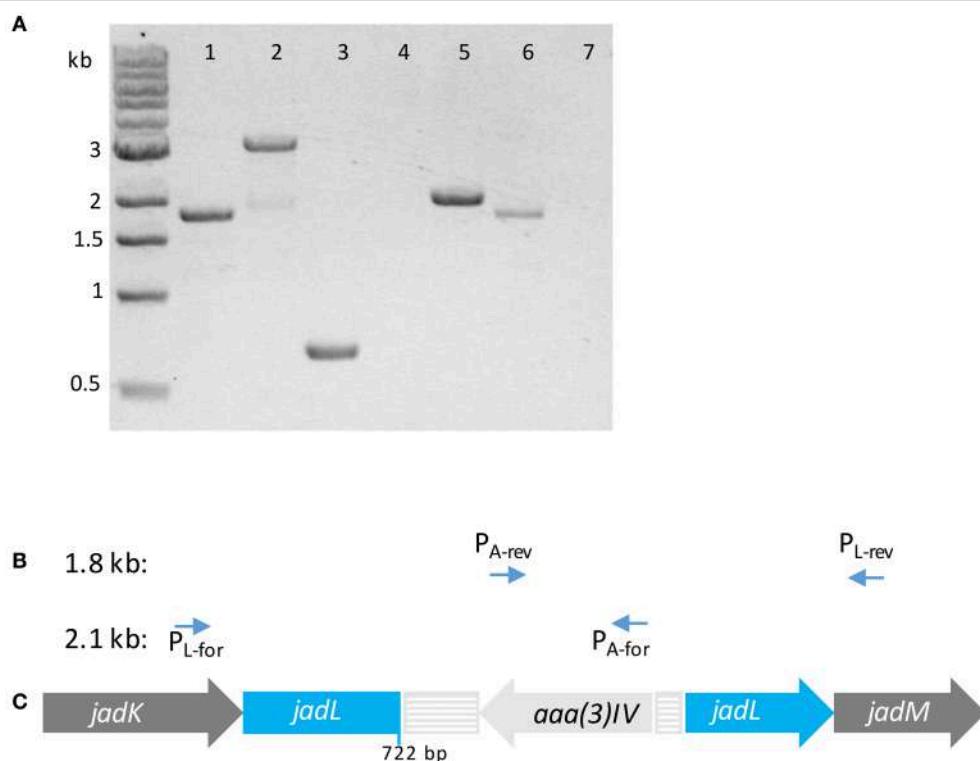


FIGURE 2 | (A) Tris-acetate-EDTA (TAE) agarose gel (1% w/v) of PCR products using template *S. venezuelae* ISP5230 gDNA (lane 1) and *S. venezuelae* VS678 gDNA (lanes 2–7) with indicated primers (1) $P_{L\text{-for}}/P_{L\text{-rev}}$; (2) $P_{L\text{-for}}/P_{L\text{-rev}}$; (3) $P_{Apr\text{-for}}/P_{Apr\text{-rev}}$; (4) $P_{L\text{-for}}/P_{Apr\text{-rev}}$; (5) $P_{L\text{-for}}/P_{Apr\text{-for}}$; (6) $P_{L\text{-rev}}/P_{Apr\text{-rev}}$; (7) $P_{L\text{-rev}}/P_{Apr\text{-for}}$. Successful amplification of fragments in lanes (5) and (6) indicate that the apramycin resistance gene (*aaa(3)IV*) within the resistance cassette is coded on the 3'-5' strand. **(B)** Primers pairs that amplified products of the indicated size. **(C)** The orientation of the resistance cassette containing *aaa(3)IV* in *S. venezuelae* VS678 as deduced from the PCR products. The disruption cassette is colored in pale gray and the disrupted gene (*jadL*) is colored in blue.

transformed with pJV106, regenerated on R5N agar (Aidoo et al., 1990) and overlaid with soft nutrient agar containing thiostrepton (25 $\mu\text{g mL}^{-1}$) after 10 h. Transformants were patched to MYM agar containing thiostrepton (25 $\mu\text{g mL}^{-1}$) then replica plated to MYM containing apramycin (50 $\mu\text{g mL}^{-1}$). Single crossover mutants, resistant to both thiostrepton and apramycin, were patched to MYM containing apramycin and carried through three rounds of sporulation. After this time, colonies with the appropriate phenotype for double crossover mutants, i.e., resistance to apramycin and sensitivity to thiostrepton, were screened by PCR. PCR screening to confirm insertion of the apramycin disruption cassette used a primer pair flanking *jadL*: $P_{L\text{-for}}$ 5'-ACCTTCGCCGAGTACGAGTC-3' and $P_{L\text{-rev}}$ 5'-TGTGCGACAGCGAGAAAG-3'. Primers used to determine the orientation of the apramycin resistance gene were $P_{Apr\text{-for}}$ 5'-TGCTGGTCCACAGCTCCTTC-3' and $P_{Apr\text{-rev}}$ 5'-GAGCGGCATCGCATTCTTC-3'. All PCR reactions were performed using Phusion DNA polymerase (New England Biosciences) and were supplemented with 10% dimethyl sulfoxide with reaction conditions following manufacturer protocols. Template genomic DNA was isolated from overnight *S. venezuelae* cultures (MYM, 30°C, 250 rpm) using standard commercial kits.

Culture Conditions for Jadomycin DS (JdDS) Production

Standard jadomycin production protocols were followed (Jakeman et al., 2006) with D-serine as sole amino acid in the production medium ensuring biosynthesis of JdDS. A 1 cm^2 patch of cells was harvested from 1 to 2 week old MYM-agar plates, with 50 $\mu\text{g mL}^{-1}$ apramycin for disruption strains, and used to inoculate MYM without antibiotics. The inoculum was shaken (250 rpm) at 30°C for 18–20 h. The cells were then harvested (5,000 rpm), the supernatant decanted, and the cells washed with minimal salt media [MSM, MgSO_4 0.4 g/L, MOPS 1.9 g/L, salt solution (1% w/v NaCl, 1% w/v CaCl_2) 9 mL/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2% w/v stock solution) 4.5 mL/L, trace mineral solution ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 880 mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 39 mg/L, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 6.1 mg/L, H_3BO_3 5.7 mg/L, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 3.7 mg/L) 4.5 mL/L, pH 7.5] twice. Cells suspended in MSM were used to inoculate fresh MSM supplemented with D-serine (60 mM), dextrose (33 mM), and phosphate (50 μM) to an initial $\text{OD}_{600} \sim 0.6$. Ethanol was added to the culture media to a final concentration of 30 μL per 1 mL media. Ethanol shocked cultures were shaken (250 rpm) at 30°C for 52 h. Cell growth was monitored by measuring the OD_{600} at the indicated time intervals by withdrawing 600 μL aliquots from

each of triplicate cultures; A_{526} measurements were recorded using the corresponding clarified aliquots. Absorbance data was plotted with GraphPad Prism 6.02 software.

Isolation of JdDS and Analysis of Natural Product Profile

All solvents used for natural product purification were high performance liquid chromatography (HPLC)-grade. After incubation for 52 h, jadomycin production cultures (50 mL) were pelleted by centrifugation (8,000 rpm). The supernatant was passed through 0.22 μM Millipore filters. Clarified culture media was passed through a 2 g Silica-phenyl column (Si-Ph, Silicycle) to which the jadomycins bind tightly. The column was washed with water, until the flow through was colorless, and HPLC-grade methanol was used to elute compounds bound to the column. After concentration by evaporation (Genevac EZ-Bio personal evaporator), the mass of the crude extract was determined. Analysis of the natural products produced by each strain was performed by analytical HPLC, using a previously described method (Robertson et al., 2016), and by thin layer chromatography (TLC), using glass-backed silica plates (SiliCycle, 250 μM , F₂₅₄ silica) and 5:5:1 ethyl acetate: acetonitrile:water as the eluent. Crude material was solubilized in methanol for TLC analysis. Visualization reagents were not required as jadomycins are deeply colored.

Quantification of JdDS by LCMS²

The concentration of **JdDS** produced by each of the wildtype and *S. venezuelae* VS678 strains was quantified by liquid chromatography coupled to mass spectroscopy (LCMS). A purified sample of **JdDS** was used to construct a calibration curve. Electrospray ionization in positive mode (ESI⁺) LCMS experiments were run using an HPLC (Agilent 1100) equipped with a reversed phase column (Phenomenex Kinetic 2.6 μM Hilic, 150 \times 2.1 mm) coupled to a hybrid triple quadrupole mass spectrometer (Applied Biosystems, 2,000 Qtrap). LCMS instrumentation and running conditions have been described elsewhere (Robertson et al., 2016). The following settings were applied for the acquisition of enhanced product ion scan (EPI) experiments: capillary voltage +4,500 kV, declustering potential +80 V, and curtain gas 10 (arbitrary units). To construct the standard curve, EPI experiments with 5 μL injections of purified **JdDS** at 2, 5, 10, and 15 μM in methanol were collected with m/z [M+H]⁺ m/z 524 as the parent ion. In the resulting spectra, the area beneath the peak for the parent ion at m/z 524 and for the fragment ion at m/z 394 were determined by integration using the LCMS software (Applied Biosystems, Analyst version 1.4.1). The relationship between **JdDS** concentration (μM) and area (unitless) was solved using linear regression. Crude samples (from the Si-Ph column methanol extract) were taken up in 1 mL methanol, then diluted 200-fold by serial dilution. After LCMS² analysis, the intensities under the m/z 524 and 394 peaks were used to determine the concentration of **JdDS** using the linear relationship described above. An average of the values obtained from each curve was used to calculate the total amount of **JdDS** per 50 mL bacterial culture.

RESULTS AND DISCUSSION

Predicted Properties of Jadl Based on Sequence Homology

The gene, *jadL*, is found amongst the structural genes within the jadomycin biosynthetic gene cluster (Han et al., 1994; Wang and Vining, 2003; Pullan et al., 2011). The gene encodes a protein with 459 amino acids that contains an MFS_1 and an H⁺ antiporter domains. The domains identified suggest an antiporter mode of substrate transport reliant on a proton gradient. MFS family transporters are ubiquitous; within the genome of *S. venezuelae*, a Pfam search for the MFS_1 domain identified 106 sequences. As is the case for most members of the MFS superfamily, *JadL* is predicted to have 12 transmembrane (TM) domains (Table 1), with the N- and C-terminal domains each comprising of 6 TM domains. The position of the disruption cassette begins at amino acid position 241 (from the N-terminus) and is situated at the beginning of predicted TM helix 7, which positions the disruption cassette between the C- and N-terminal domains. It is widely accepted that MFS family transporters have highly conserved structures and operate by the same “clamp and switch” mechanism (Quistgaard et al., 2016). MFS family transporters are active in a monomeric form, where the substrate binding occurs at the cleft between the N- and C-terminal domains; substrate and proton binding is mediated by a number of amino acids contacts located on the TM regions scattered over both the N- and C-terminal domains (Quistgaard et al., 2016). Such interactions have been demonstrated in several crystal structures, a few of the many examples include the well-studied *Escherichia coli* lactose permease (LacY) (Abramson et al., 2003), and multidrug transporters such as *E. coli* ErmD (Yin et al., 2006) and MdfA (Heng et al., 2015). Thus, we predict that the *jadL* disruption mutant will be unable to bind or transport its substrate(s).

In a query for genes homologous to *jadL* a BlastP search identified *kinJ* (69% identity), a gene found within the kinamycin, a diazo-containing glycosylated angucycline, biosynthetic pathway of *Streptomyces murayamaensis* (Gould et al., 1998). The top protein blast hits, all uncharacterized MFS family transporters from actinobacteria with high identity similarities (67–71%), are listed in Table 2. A BlastP search against the TCDB is summarized in Table 3. The majority of the homolog identified (20–30% identity) were from family 2.A.1.21, the drug: H⁺ antiporter-3 (12 spanner) (DHA3) family, which

TABLE 1 | *JadL* protein sequence with TM regions (CCTOP) shown in bold.

MVKARSNTFRSLSVRNFR**I**FAAGQV**V**SVAGTWTM**V**VQA**D**WLVLG**G**TGDSGT**A**
GAVTALQFAPMLLLTLYGG**R**LADRYDKRMLLTAA**N**LTAGALA**A**VLAVL**V**LTGG**R**
LWHIWLLALGIGVVNA**E**VVPTRMSV**F**GELVG**N**ELLPNASAL**S**AAYFSVARVAGPA
LAGLLITGFGTG**W**AIALNA**S**YLAT**V**AGL**R**MMRPEENPGGARG**R**PEAGQ**G**
KEERKDARVV**D**GLRYTASRAD**L**TLP**M**ALV**A**VG**I**LCGMNF**Q**LTLP**L**LA**K**TVFHADA
TS**F**GLLT**T**FAAG**S**LL**G**A**I**AG**T**RRSGRP**A**RT**V**IGS**A**LF**G**ALE**A**AG**W**AP**G**
FAV**V**LL**T**LG**F**AS**I**Y**F**QA**A**NH**R**QLGSD**P**AY**R**GR**I**LA**Y**TL**L**Q**G**STPL**G**ALL**V**
LTERLG**A**GLW**L**GG**V**LS**A**AL**V**AL**G**LEY**R**GR**T**RPARTAA**A**PDPSRG**P**SDSD**P**
PDSDPD**S**ERL**V**RDA**A**PEGR**R**

TABLE 2 | Top 10 BlastP results for JadL.

Hit	Protein accession	Organism	Amino acid length	Identity
1	OAR23019	<i>Streptomyces</i> sp. <i>ERV7</i>	429	67
2	AAO65354	<i>Streptomyces</i> <i>murayamaensis</i>	426	69
3	KPH97664	<i>Actinobacteria bacterium</i> <i>OK006</i>	454	68
4	WP_060900228	<i>Streptomyces</i> <i>diastatochromogenes</i>	418	68
5	WP_053676855	<i>Streptomyces</i> sp. <i>WM4235</i>	429	73
6	WP_054237237	<i>Actinobacteria bacterium</i> <i>OK006</i>	418	68
7	WP_051827886	<i>Streptomyces bicolor</i>	433	67
8	WP_035732764	<i>Frankia</i> sp. <i>Allo2</i>	419	71
9	WP_018961660	<i>Streptomyces</i> sp. <i>CNB091</i>	424	71
10	WP_051919510	<i>Streptomyces</i> sp. <i>NRRL F-5140</i>	424	68

TABLE 3 | Top 10 BlastP results for JadL against the TCDB.

Hit	Protein accession	Organism	Amino acid length	Identity	TC number
1	D3Q871	<i>Stackebrandtia nassauensis</i> (strain DSM 44728/NRRL B-16338/NBRC 102104/LLR-40K-21)	417	31	2.A.1.21.11
2	Q7BKK4	<i>Streptococcus pneumoniae</i>	405	24	2.A.1.21.22
3	Q0E7C5	<i>Listonella anguillarum</i> serovar O2	437	25	2.A.1.38.2
4	Q9X4X4	<i>Pseudomonas abietaniphila</i>	547	25	2.A.1.30.1
5	H5X1B8	<i>Saccharomonospora marina</i> XMU15	395	27	2.A.1.21.21
6	O32859	<i>Mycobacterium fortuitum</i>	409	25	2.A.1.21.4
7	P95827	<i>Streptococcus pyogenes</i> , and OS <i>Streptococcus pneumoniae</i>	405	22	2.A.1.21.1
8	P64783	<i>Mycobacterium tuberculosis</i>	419	25	2.A.1.21.12
9	O31137	<i>Mycobacterium smegmatis</i>	419	23	2.A.1.21.3
10	A6QJ21	<i>Staphylococcus aureus</i> (strain Newman)	397	21	2.A.1.21.7

is consistent with the hypothesized role of JadL. Members of the 2.A.1.38, the enterobactin (siderophore) exporter (EntS) family, and 2.A.1.30, the putative abietane diterpenoid transporter (ADT) family, were also amongst the top hits from the TCDB database.

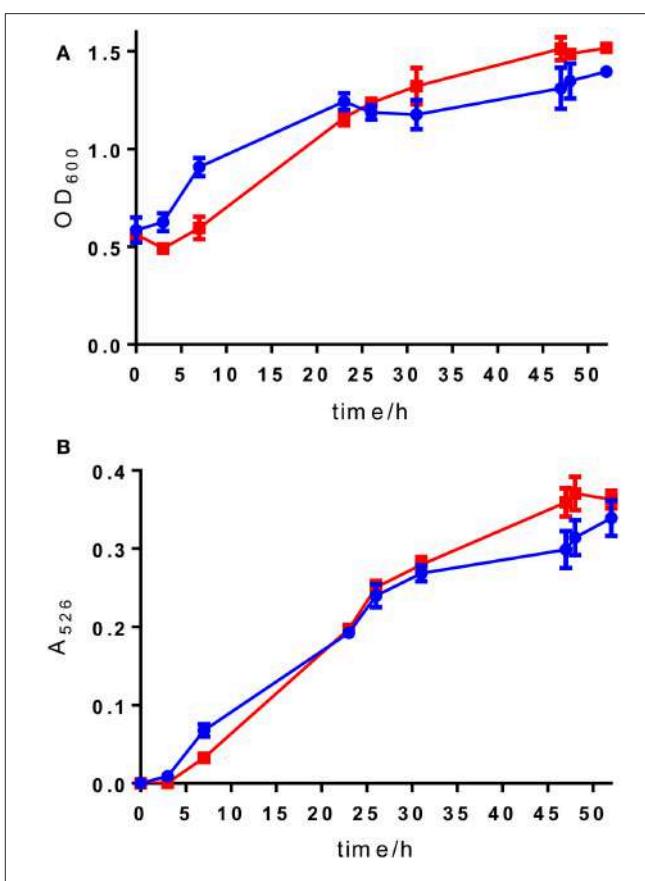


FIGURE 3 | (A) OD₆₀₀ readings and **(B)** A₅₂₆ readings for wild type *S. venezuelae* ISP5230 (blue circles) and *S. venezuelae* VS678 (red squares). Error bars show the standard deviation and squares/circles shown the mean of the data from triplicate samples.

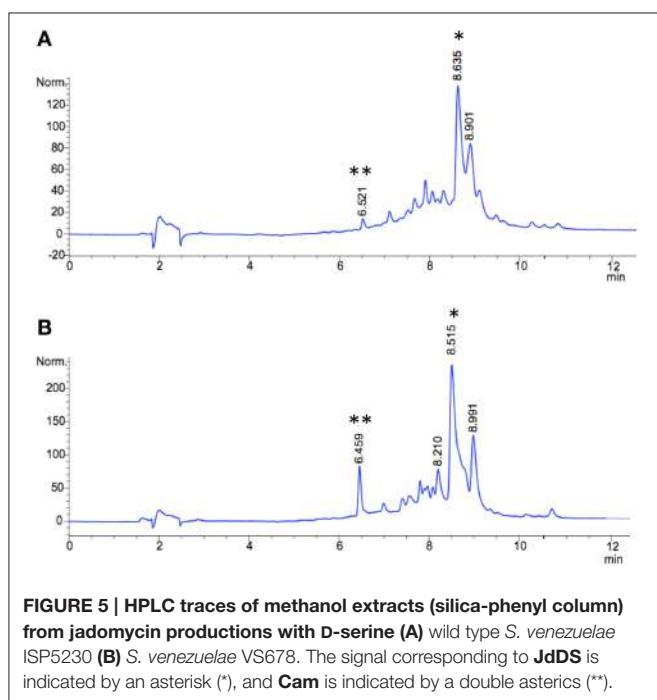
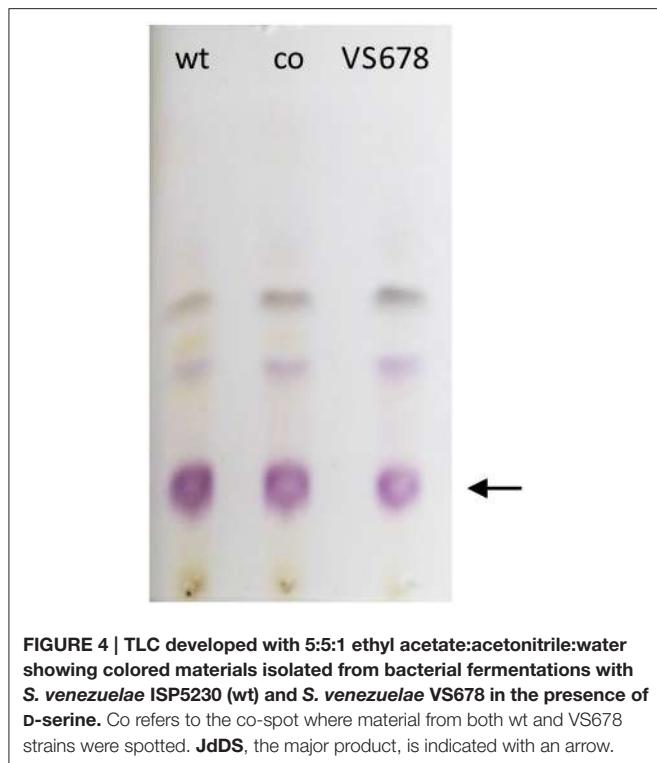
Construction and Confirmation of the *jadL* Disruption Mutant

S. venezuelae protoplasts were transformed with pJV106A bearing the *jadL* disruption cassette. After several rounds of sporulation to facilitate heterologous recombination, a double crossover mutant sensitive to thiostrepton and resistant to apramycin was confirmed to contain the disruption cassette by amplification of a 3 kb PCR product using primers flanking *jadL* (P_L-for/P_L-rev). This strain was designated *S. venezuelae* VS678. A corresponding 1.8 kb band was amplified from the wildtype strain (Figure 2). The orientation of the apramycin cassette was confirmed using PCR. Amplification was observed only when the primers pairs P_L-for/P_{Apr}-for and P_L-rev/P_{Apr}-rev were used indicating an opposite orientation of the apramycin resistance gene relative to *jadL* (Figure 2).

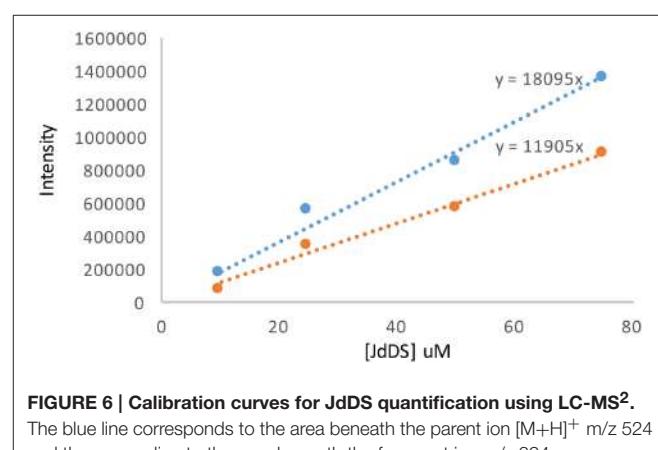
Analysis and Quantification of JdDS Production in *jadL* Disruption Strain *S. venezuelae* VS678

Selection of a single amino acid in the MSM culture media to produce a single E-ring variant was necessary to facilitate

quantification. We selected D-serine as the amino acid because these culture conditions and the final jadomycin product, **JdDS**, are well characterized and extensively used as a standard in our laboratory (Robertson et al., 2016). After induction of jadomycin production by ethanol shock, growth curves for the



disruption mutant and a wildtype control were monitored over 52 h (**Figure 3**). Cell growth in the disruption mutant cultures appeared to lag versus the wildtype over the first 24 h, but then caught up. The absorbance values at A₅₂₆, that provide an estimate for excreted colored natural products, showed similar values for both strains after 52 h. Initial values were lower in the disruption mutant, consistent with the growth curve. In order to quantify the final amount of **JdDS** obtained, the methanol extract from the Si-Ph column was concentrated to dryness yielding 3.1 mg crude material from the wildtype strain and 2.8 mg from the disruption strain. TLC analysis showed that the colored compounds produced by both strains were identical (**Figure 4**), and that **JdDS** was produced by both strains. HPLC analysis showed the peak corresponding the **JdDS** at R_t 8.5 min in both crude samples (**Figure 5**). By mass spectral analysis, it was determined that 1.1 mg **JdDS** was produced by the wildtype and 1.3 mg was produced by the disruption mutant per 50 mL culture using the calibration curves shown in **Figure 6**. Our results clearly show, contrary to our hypothesis, that disruption of the MFS family protein *jadL* does not significantly affect jadomycin natural product production. Over 52 h, the disruption strain *S. venezuelae* VS678 grew comparatively to the wildtype and produced a similar final concentration of **JdDS**, by both by qualitative (HPLC, TLC) and quantitative (LCMS²) analysis. That jadomycin production was not obviated rules out the possibility of polar effects arising from the insertion of the apramycin disruption cassette. The methodology used for jadomycin isolation in which the first step involves removal of bacterial cells through pelleting ensures that only materials excreted from the cells (the supernatant) are collected. Therefore, we believe that our data shows that **JdDS** is being effectively excreted from cells of the disruption mutants. The initially depressed growth rates observed in the disruption strain may reflect stress induced from the disruption of *JadL*, but that the growth improves subsequently, suggest that alternate, currently unknown, mechanisms of **JdDS** export may be induced. Additionally, an increase of **Cam** levels in the HPLC trace of the crude isolate from the disruption mutant supports that jadomycin biosynthesis may have been somewhat strained, resulting in the production of **Cam**. There have been a number of



studies on the regulatory crosstalk between jadomycins and **Cam** biosynthesis in *Streptomyces venezuelae* ISP5230 (Fernandez-Martinez et al., 2014; Robertson et al., 2015, 2016; Sekurova et al., 2016).

Discussion on the Role of **JadL** in Secondary Metabolite Production

Our data suggests that disruption of *jadL* does not have a substantive effect on **JdDS** production. These results were rather unexpected, as we anticipated that without functional **JadL**, **JdDS** would accumulate within *S. venezuelae* and disrupt growth and/or reduce jadomycin production significantly. It is noteworthy that depressed growth rates were observed in the first 24 h, after which the growth of the disruption mutant recovered relative to growth of the wildtype. Presumably, alternate export mechanisms are induced during the first 24 h. The appearance of **Cam** in the disruption mutant may be an indication of additional stress on *S. venezuelae* VS678. It is entirely plausible that *S. venezuelae* possesses other transporters capable of exporting jadomycins, indicating **JadL** is not essential for this role. This is consistent with the role of the MFS transporter, **SirA**, present in the sirodesmin biosynthetic gene cluster in the fungus, *Leptosphaeria maculans* (Gardiner et al.,

2005). **SirA**, whilst present within the biosynthetic gene cluster for sirodesmin, was determined not to be solely responsible for the efflux of endogenously produced sirodesmin, however, **SirA** did contribute toward self-protection. Our data showing the comparable growth for the wild-type and blocked mutant strains is suggestive that if **JadL** is responsible for self-resistance in *S. venezuelae* ISP5230, as was observed for **SirA**, that the concentrations of the jadomycin excreted into the media are insufficient to have a deleterious effect upon the growth of *S. venezuelae* ISP5230.

AUTHOR CONTRIBUTIONS

SF and JM performed experiments. SF, JM, LV, and DJ conceived the work. SF and DJ prepared the manuscript.

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A Mechanism of Synergistic Effect of Streptomycin and Cefotaxime on CTX-M-15 Type β -lactamase Producing Strain of *E. cloacae*: A First Report

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A *bla*_{CTX-M-15} gene is one of the most prevalent resistant marker found in member of enterobacteriaceae. It encodes cefotaxime hydrolysing β -lactamase-15 (CTX-M-15) causing resistance against beta lactam antibiotics. Since single antibiotic therapy fails to control infection caused by multidrug resistance strain, therefore combination therapy was came into practice as an effective treatment. We have first time explained the mechanism where two antibiotics of different classes work against resistant strains. Binding parameters obtained by spectroscopic approach showed significant interaction and complex formation between drugs and CTX-M-15 enzyme with decreased *K_SV* and *K_d* values. CD analysis showed altered conformation and significant changes in alpha helical content of CTX-M-15 enzyme on interaction with streptomycin in combination with cephalosporin. Steady state kinetics revealed decrease in hydrolytic efficiency of enzyme to about 27% by cooperative binding behavior upon sequential treatment of enzyme with streptomycin and cefotaxime. Therefore, the study concludes that combination therapy against CTX-M-15 producing strain with Cefotaxime/Streptomycin in 1:10 molar ratio, decreases CTX-M-15 efficiency significantly because of the fact that streptomycin induced structural changes in CTX-M-15 hence cefotaxime was not properly bound on its active site for hydrolysis rather available for the target to inhibit bacterial cells.

Keywords: antibiotic resistance, synergy, β -lactamase, streptomycin, cefotaxime

INTRODUCTION

β -lactamases are the group of enzymes that cleave amide bond in beta lactam rings of beta lactam antibiotics rendering them harmless to bacteria. Production of these enzymes is the predominant cause of gram negative bacterial resistance against β -lactam antibiotics which has become a major health concern (Bonnet, 2004; Bush, 2010a).

In Enterobacteriaceae, ESBLs (Extended spectrum beta lactamases) encoding CTX-M type markers on plasmid, are reported worldwide (Coque et al., 2008; Hawkey and Jones, 2009). It hydrolyzes the oxyimino-cephalosporin and cefotaxime with about 1000-fold higher catalytic efficiency than other class A β -lactamases (Bauernfeind et al., 1992; Bonnet, 2004). CTX-M type ESBLs display greater hydrolytic activity against Cefotaxime than Ceftazidime

(Bonnet, 2004). CTXM-15 is the widely spread ESBL in India and is reported in wide members of Enterobacteriaceae family (Karim et al., 2001). It has been reported that bacteria expressing ESBLs are resistant toward various β -lactam antibiotic groups such as Penicillins, different generations of Cephalosporins, Aztreonam and also to various antibiotic /inhibitor combinations (Faheem et al., 2013). The widespread dissemination of CTX-M-15 by *E. coli* and other enteric bacilli has a significant impact on hospital and community-acquired infections (Bush, 2010b; Chen et al., 2014).

It has been observed that treatment with single antibiotic fails to cure increasing microbial infections due to emergence of antibiotic resistance. For e.g., studies performed on other β -lactamases such as OXA-51 in *Acinetobacter baumannii* shows how carbapenem antibiotic is hydrolyzed by β -lactamases leading to the survival of the pathogen (Tiwari and Moganty, 2014) Therefore, it is an augmented need to employ combination therapy and to understand pharmacological and pharmacodynamic (Lin et al., 1987) behavior of multiple drugs for rational basis of antibiotics selection for effective treatment in order to avoid antagonism between certain antibiotics as demonstrated earlier (Gunnison et al., 1950).

A marked increase in bactericidal effect *in vitro* by synergistic treatment with penicillin and streptomycin has been reported earlier (Jawetz et al., 1951), compared to single drug treatment (Gunnison et al., 1950). An observation which is consistent with a hypothesis that streptomycin faces natural barrier while entering enterococci which can be prevailed by agents inhibiting cell wall synthesis such as penicillin and hence can produce synergistic effect (Moellering and Weinberg, 1971). We have already reported earlier that the synergistic effect of cefoxitin with streptomycin and cefotaxime proved an effective combination against multidrug resistant bacterial strains (Hasan et al., 2013).

The mechanism behind effective nature of drugs of two different classes in combination has not yet been explained. Therefore, this is the first time we have initiated this work to understand the molecular mechanism behind synergy of cephalosporin and aminoglycoside against multidrug resistance strains. The hypothesis proposed was that streptomycin might induce structural changes in CTX-M-15 enzyme on binding, hence may not allow cefotaxime to properly bind and hydrolyze, as a result cefotaxime is available for target site inhibition in bacterial cells.

MATERIALS AND METHODS

Protein/Enzyme Source

CTX-M-15 from *Enterobacter cloacae* clinical strain, EC-15 (Genebank accession no.: JN860195.1) (Chen et al., 2014) *E. coli* BL21 (DE3), pQE-2 (high copy cloning vector).

Antibiotics and Other Chemicals

Cefotaxime and Cefoxitin were purchased from Sigma chemical co. (St. Louis, MO), Streptomycin from Himedia (India), IPTG

Abbreviations: ESBLs, Extended spectrum beta lactamases; IPTG, Isopropyl- β -D-1-thiogalactopyranoside; PBS, phosphate buffer saline; CD, Circular Dichroism.

(isopropyl- β -D-1-thiogalactopyranoside) was purchased from Roche (Basel, Switzerland). Nitrocefin was purchased from Calbiochem (USA). Imidazole was purchased from Sigma-Aldrich. Other reagents and chemicals were of analytical grade and double distilled water was used throughout the study.

*bla*_{CTX-M-15} Cloning and Expression of CTX-M-15

The plasmid DNA carrying *bla*_{CTXM-15} gene cloned from clinical *E. cloacae* EC-15 strain (Genebank accession no.: JN860195.1), was extracted using Qiagen plasmid extraction kit, according to manufacturer's instructions. The *bla*_{CTX-M-15} was amplified by PCR using primers CTX-M-15-F (5' ATATCATATGGTTAAAAAATCACTG 3') containing Nde I restriction site and CTX-M-15-R (5' ATATAAGCTTTACAAACCGTCGGTGAC 3') containing Hind III restriction site. The PCR conditions used were 95°C for 30 s, 54°C for 25 s, 72°C for 40 s and the reaction process was carried out for 35 cycles (Faheem et al., 2013). The PCR product does not contain the promoter region of the gene. The PCR product and pQE-2 (high copy cloning vector), were double digested with NdeI and Hind III endonucleases, ligated and transformed into competent *E. coli* BL21 (DE3) cells by heat shock method (4°C for 30 min, 42°C for 90 s and 4°C for 10 min). Transformants harboring *bla*_{CTX-M-15} gene were selected on LB agar plates containing ampicillin (100 μ g/ml). The clones were confirmed by double restriction digestion of obtained transformed cells by NdeI and HindIII enzymes (Figure S1) and sequencing by standard procedures.

To express and purify CTXM-15 β -lactamase, the competent cells of *E. coli* BL21 (DE3) harboring pQE-2 vector carrying *bla*_{CTX-M-15} gene, a 10 ml overnight culture of these transformed cells in Luria-Bertani broth containing 100 μ g/ml ampicillin was used to inoculate 1 l of Luria-Bertani broth containing 100 μ g/ml ampicillin. Bacterial culture was grown at 37°C with shaking at 120 rpm, until an optical density of 0.6–0.8 was reached at 600 nm (Faheem et al., 2013). The culture was cooled and induced with 0.2 mM IPTG and placed at 16°C at 150 rpm for 12–16 h. The bacterial cells were collected by centrifugation and re-suspended in 20 ml lysis buffer containing 50 mM Sodium phosphate (pH 8.0), 300 mM NaCl and 10 mM Imidazole along with 0.1% β -mercaptoethanol per liter culture. The bacterial cells were ruptured by sonication at 35% amplitude for 10 min, and the cell debris obtained was removed by centrifugation at 12,000 rpm for 30 min. The clear supernatant was loaded onto a Ni-NTA column, which was pre-equilibrated by lysis buffer, and washed with lysis buffer supplemented with 50 mM imidazole. Protein was eluted with PBS (Phosphate buffer saline, 50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl) buffer containing 250 mM imidazole. Pure protein was obtained after dialysis in PBS. Purity of the purified protein was estimated to be more than 97% as determined by a single band of 31 kDa on SDS-PAGE (Figure S2). The final protein concentrations were determined by using the molar extinction coefficient of 25, 440 M⁻¹ cm⁻¹ at 280 nm.

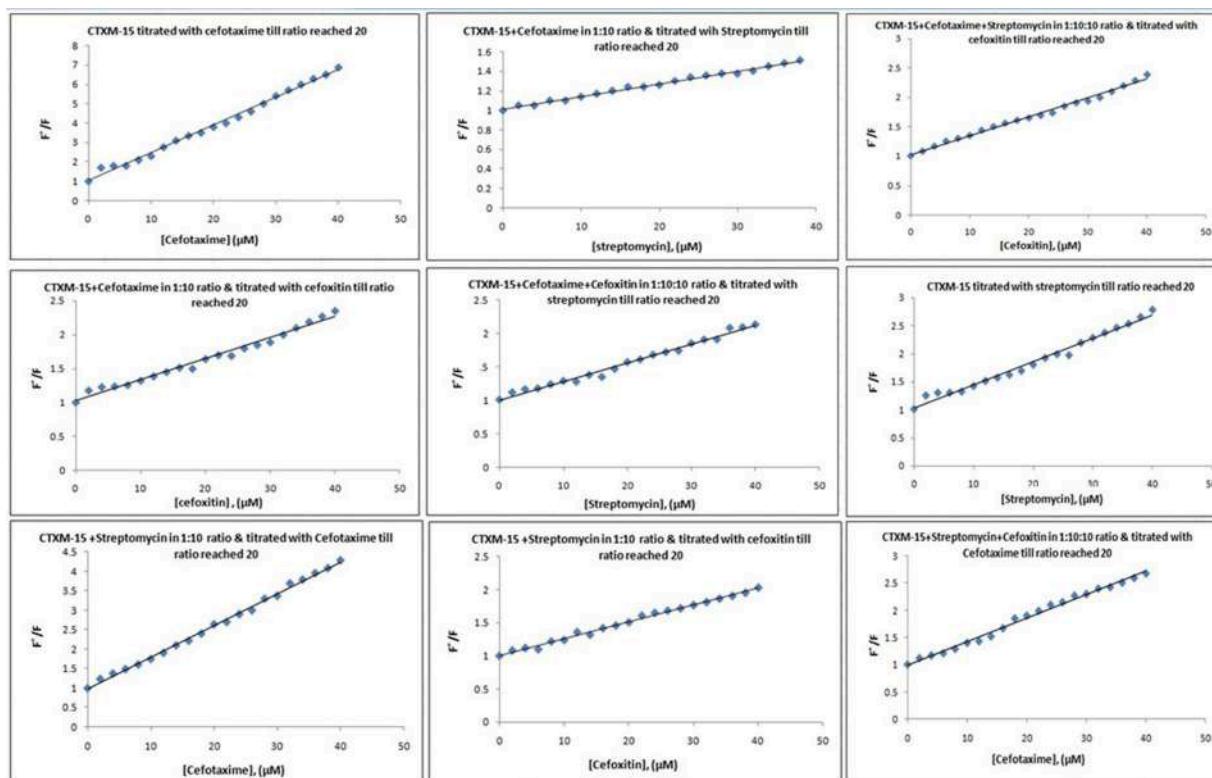


FIGURE 1 | Stern-Volmer plots for drug induced fluorescence quenching of CTX-M-15. At 298 K under different drug incubations, the Stern-Volmer plots for CTX-M-15- drug interactions is shown. The concentration of CTX-M-15 was 2 μM and the concentration of the bounded drug was 20 μM in 50 mM sodium phosphate buffer at pH 7.4.

TABLE 1 | Stern–Volmer quenching constants and binding parameters for CTX-M-15 and Cefotaxime/Streptomycin/Cefoxitin interactions.

	K _{sv} (M ⁻¹)	K _q (M ⁻¹ s ⁻¹)	K _a (M ⁻¹)	n	R ²
CTX-M-15+CTX	14.6×10^4	3.38×10^{13}	0.297×10^4	0.633	0.995
CTX-M-15+CTX+FOX	3.2×10^4	0.74×10^{13}	0.037×10^4	0.558	0.993
CTX-M-15+CTX+FOX+STR	2.8×10^4	0.64×10^{13}	0.361×10^4	0.794	0.991
CTX-M-15+CTX+STR	1.2×10^4	0.278×10^{13}	0.126×10^4	0.773	0.994
CTX-M-15+CTX+STR+FOX	3.2×10^4	0.742×10^{13}	1.253×10^4	0.904	0.991
CTX-M-15+STR	4.4×10^4	1.02×10^{13}	0.019×10^4	0.47	0.997
CTX-M-15+STR+CTX	8.2×10^4	1.902×10^{13}	2.904×10^4	0.916	0.993
CTX-M-15+STR+FOX	2.5×10^4	0.58×10^{13}	0.162×10^4	0.729	0.992
CTX-M-15+STR+FOX+CTX	4.2×10^4	0.974×10^{13}	2.023×10^4	0.944	0.994

CTX, cefotaxime; STR, streptomycin; FOX, cefoxitin.

Fluorescence Spectra Measurements

All the fluorescence study was done on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corporation, Kyoto,

Japan) which is equipped with a thermostatically controlled cell holder and attached to a water bath to maintain desired constant temperature. Fluorescence quenching was monitored by measuring intrinsic fluorescence quenching of protein to elucidate the mechanism of its interaction with drug molecule (Eftink and Ghiron, 1976; Lakowicz, 1988) between 300 and 450 nm after selectively exciting the sample at 295 nm, both the excitation and emission slits were set at 5 nm and the spectra were recorded at fast scanning mode. To a 3 mL sample containing 2 μM CTX-M-15 protein alone or incubated with 20 μM of cefotaxime, cefoxitin and streptomycin each in various combinations and by successive direct addition of 2 μM of each drug in such a manner that the total volume added was not more than 40 μL at 298 K. All the fluorescence intensities were corrected for the inner filter effect. The decrease in fluorescence intensity of protein at emission maxima was analyzed by using the Stern-Volmer equation (Lakowicz, 1988):

$$\frac{F^\circ}{F} = 1 + K_{sv}[Q] = 1 + K_q \tau^\circ [Q] \quad (1)$$

where F° and F are the fluorescence intensities in the absence and presence of drug (quencher), K_{sv} is the Stern-Volmer constant, $[Q]$ is the molar concentration of quencher, and k_q and τ° are the bimolecular quenching rate constant and the lifetime of the protein fluorescence in the absence of quencher, respectively. The

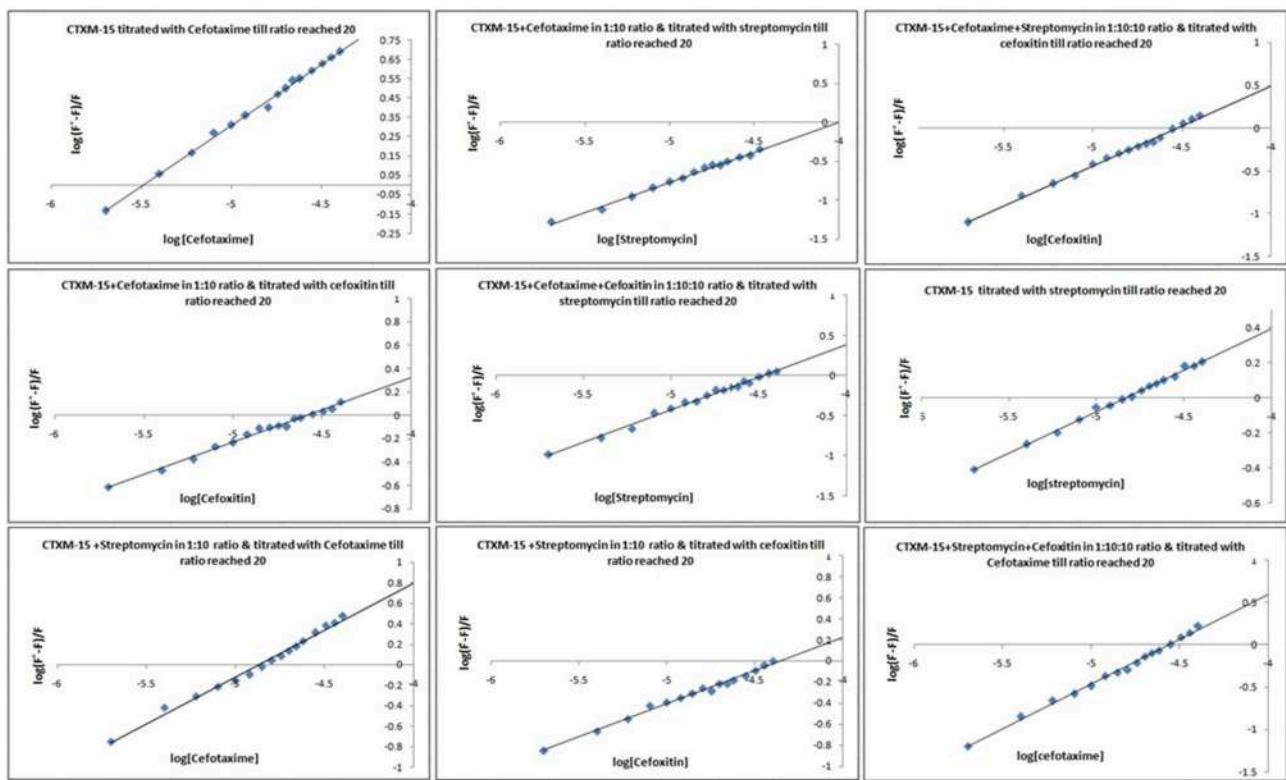


FIGURE 2 | Modified Stern–Volmer plots for the quenching of CTX-M-15. At 298 K under different drug incubations, the Modified Stern–Volmer plots for CTX-M-15–drug interactions is shown. The concentration of CTX-M-15 was 2 μ M and the concentration of the bounded drug was 20 μ M in 50 mM sodium phosphate buffer at pH 7.4.

bimolecular rate constant K_q was calculated from the relation:

$$K_q = K_{sv}/\tau^\circ \quad (2)$$

where τ° is the mean fluorescence life time of Trp which is $\sim 4.31 \times 10^{-9}$ s. The binding constant (K_a) and the number of binding sites (n) were calculated using the following modified Stern–Volmer equation (Kang et al., 2004):

$$\log \frac{F^\circ - F}{F} = \log K_a + n \log [Q] \quad (3)$$

CD Spectra Measurements

CD spectra were collected on a Jasco J-810 spectropolarimeter (Jasco International Co. Ltd., Tokyo, Japan) equipped with a Peltier-type temperature controller (PTC-423S/15) and attached to a water bath. Far-UV CD spectra of CTX-M-15 in the absence and presence of drugs (1:10) (Micro molar ratio) were taken at protein concentrations of 5 μ M in 0.1 cm path length cells, respectively and all the spectra were corrected for the appropriate blanks. The instrument was calibrated with (+)-10-camphorsulfonic acid. All the spectra were measured at 298 K using a scan speed of 100 nm/min and the response time of 1 s. The observed ellipticity obtained is converted to mean residual

ellipticity [MRE] in deg.cm².dmol⁻¹ by using the following equation (Rehman et al., 2015):

$$MRE = \frac{[\theta]_{obs}}{10ncl} \quad (4)$$

Where $[\theta]_{obs}$ is the observed ellipticity in mdeg, n is the total number of amino acid residues (291) in CTX-M-15 protein, c is the molar concentration of the protein, and l is the path length in cm. The α -Helical content of drug treated and untreated CTX-M-15 was calculated from the MRE values at 208 and 222 nm using the following equation (Chen et al., 1972):

$$\% \alpha - \text{helix} = \left[\frac{[MRE]_{208\text{nm}} - 4000}{33000 - 4000} \right] * 100 \quad (5)$$

$$\% \alpha - \text{helix} = \left[\frac{[MRE]_{222\text{nm}} - 2340}{30300} \right] * 100 \quad (6)$$

Steady-State Kinetics Experiments

Enzyme kinetics measurements were recorded on a Shimadzu UV-1800 double beam spectrophotometer (Shimadzu International Co. Ltd., Kyoto, Japan) at 298 K.

The hydrolysis activity of CTX-M-15 toward a chromogenic cephalosporin substrate Nitrocefin was studied (O'Callaghan et al., 1972). Steady-state enzyme kinetics was performed by directly monitoring the initial velocities of appearance

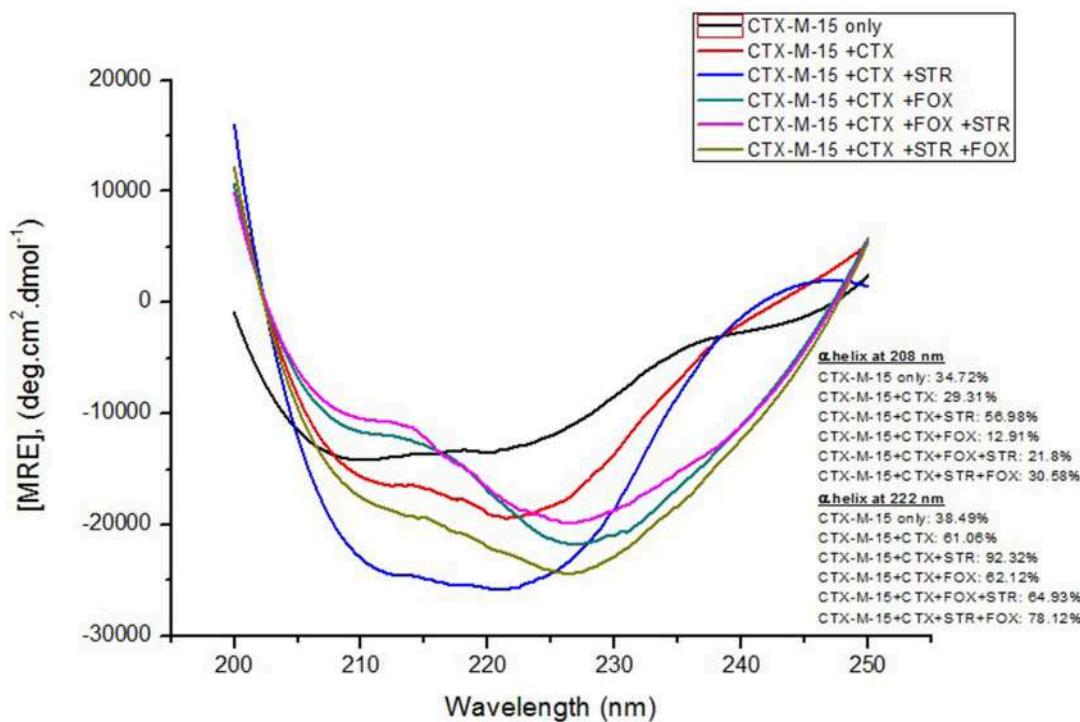


FIGURE 3 | Far-UV CD spectra of CTX-M-15. CD spectra of CTX-M-15 alone or in complex with cefotaxime alone or along with streptomycin/cefoxitin or both at 1:10, 1:10:10, and 1:10:10:10 molar ratio was taken. The concentration of CTX-M-15 and cefotaxime/streptomycin/cefoxitin was 5 and 50 μ M in 50 mM Sodium phosphate buffer pH 7.4 at 298 K.

or disappearance of chromophore, Nitrocefin. The effect of Cefotaxime, Streptomycin, and Cefoxitin binding on the catalytic activity of CTX-M-15 toward Nitrocefin was determined by steady state kinetics at pH 7.4 in 50 mM phosphate buffer and 298 K. The concentration of CTX-M-15 enzyme was kept constant at 8.75 nM, (for dilution of the enzyme and to prevent denaturation, BSA was added to a final concentration of 20 μ g/ml (Galleni et al., 1994), we found that BSA at the concentration used in the experiment did not show any effect on the hydrolytic ability of CTX-M-15), while the concentration of nitrocefin was varied from 0 to 650 μ M. CTX-M-15 activity in presence of 87.5 nM of each drug, cefotaxime, streptomycin and cefoxitin was obtained by incubating them for 1.5 h with enzyme at room temperature. The rate of Nitrocefin hydrolysis was determined by measuring the appearance of nitrocefin (red colored product) at 486 nm for 65 s. All the measurements were performed on Shimadzu UV-1800 double beam spectrophotometer. The concentration of nitrocefin was determined by measuring absorbance using a molar extinction coefficient value of 15,000 $M^{-1} cm^{-1}$ at 486 nm. The kinetic parameters (k_{cat} and K_m) were determined according to the Michaelis-Menten method by fitting the data to the following equations.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (7)$$

$$K_{cat} = \frac{V_{max}}{[E]} \quad (8)$$

RESULTS

The *bla*_{CTX-M-15} gene was cloned and transformants harboring *bla*_{CTX-M-15} gene were confirmed by double restriction digestion using NdeI and HindIII enzymes for its presence. Agarose gel of double digestion showed two bands of 4.8 kb corresponding to pQE-2 vector and 800 bp corresponding to *bla*_{CTX-M-15} gene (Figure S1). Purity of the protein obtained after dialysis in PBS was estimated to be more than 97% as determined by single band of 31 kDa on SDS-PAGE (Figure S2). The final protein concentrations were determined to be 1.5 mg/ml using the molar extinction coefficient of 25, 440 $M^{-1} cm^{-1}$ at 280 nm.

Fluorescence spectra measurements showed the effect of single and combination of drug binding on the fluorescence property of CTX-M-15 (Figures S3, S4). A progressive decrease in the fluorescence intensity was observed due to quenching of CTX-M-15 fluorescence. The data were analyzed according to the Stern-Volmer Equations (1) and (2) (Figure 1, Table 1). The binding constant (K_a) lying in the range of 10^2 – 10^4 M^{-1} and the number of binding sites (n) which was found to be one, were determined using modified Stern-Volmer Equation (3) (Figure 2, Table 1). K_{SV} values for interactions with different drugs were of the order of 10^4 M^{-1} . The k_q values were determined from the ratios of K_{SV}/t_0 after taking t_0 for CTX-M-15 = 4.31×10^{-9} s. The k_q values were of the order of 10^{12} – 10^{13} $M^{-1}s^{-1}$.

Far-UV CD spectrum characterizes the conformation of the peptide backbone to determine secondary structure (α -helices

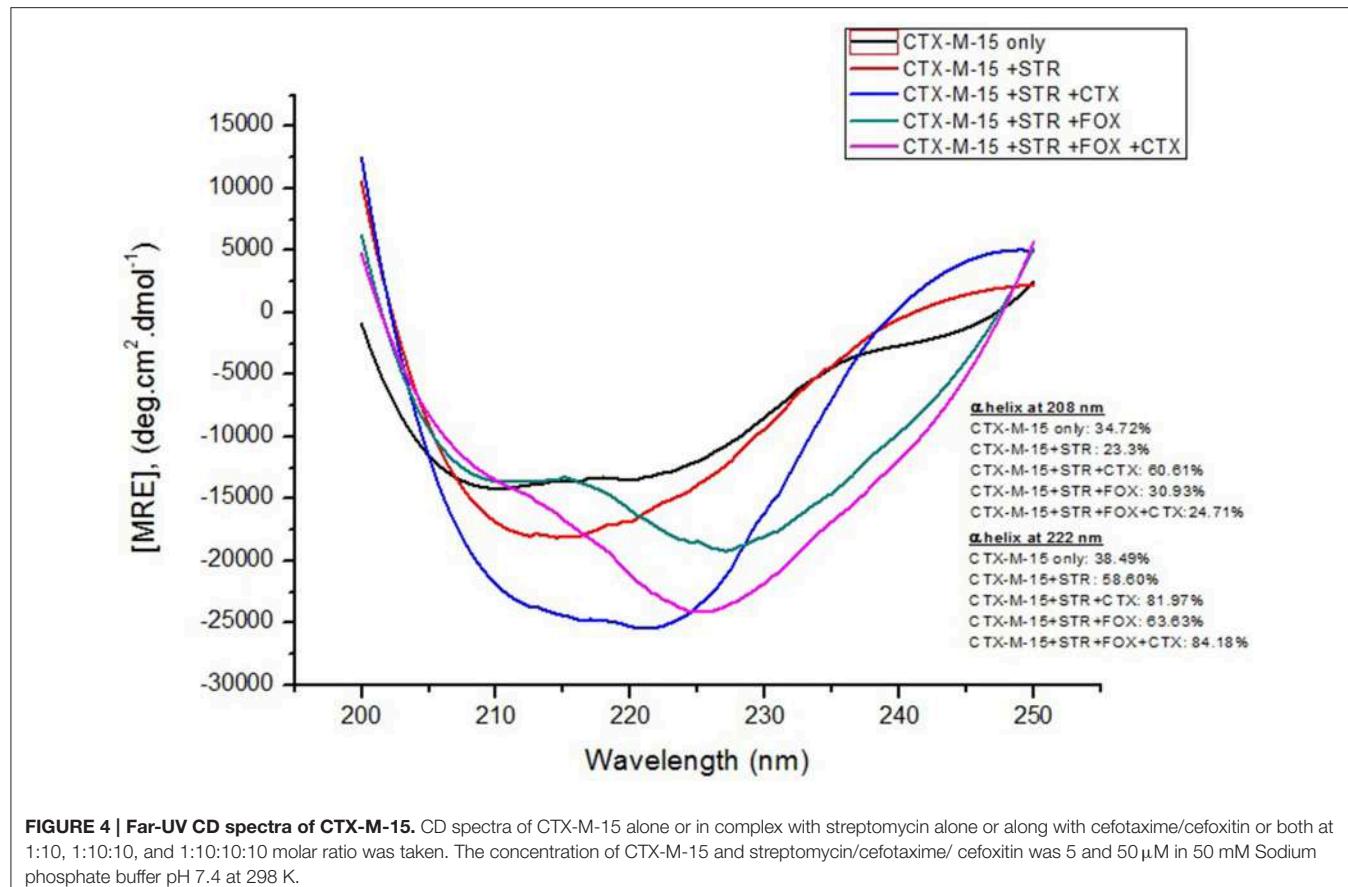


TABLE 2 | Spectral characteristics of CTX-M-15 under different drug binding conditions.

	MRE ₂₀₈ (deg.cm ² .dmol ⁻¹)	% α helix at 208	MRE ₂₂₂ (deg.cm ² .dmol ⁻¹)	% α helix at 222
CTX-M-15	$-14,070 \pm 210$	34.72%	$-14,004.9 \pm 145$	38.49%
CTX-M-15+CTX	$-12,500.1 \pm 161$	29.31%	$-20,842.9 \pm 132$	61.06%
CTX-M-15+CTX+STR	$-20,524.3 \pm 170$	56.98%	$-30,313.2 \pm 126$	92.32%
CTX-M-15+CTX+FOX	$-7,744.89 \pm 157$	12.91%	$-21,163.5 \pm 201$	62.12%
CTX-M-15+CTX+FOX+STR	$-10,322.8 \pm 190$	21.8%	$-22,061.1 \pm 138$	64.93%
CTX-M-15+CTX+STR+FOX	$-12,871 \pm 173$	30.58%	$-26,010.9 \pm 223$	78.12%
CTX-M-15+STR	$-10,760.6 \pm 102$	23.3%	$-20,096.1 \pm 139$	58.60%
CTX-M-15+STR+CTX	$-21,577.9 \pm 189$	60.61%	$-27,178.1 \pm 159$	81.97%
CTX-M-15STR+FOX	$-12,972.1 \pm 144$	30.93%	$-21,621.4 \pm 149$	63.63%
CTX-M-15+STR+FOX+CTX	$-11,166.3 \pm 204$	24.71%	$-27,848.7 \pm 187$	84.81%

CTX, cefotaxime; STR, streptomycin; FOX, cefoxitin.

MRE values are reported as the average of \pm standard error from three independent experiment.

and β -sheets) of the protein. Hence the possible effects of (single/combination) drug on the secondary structure of CTX-M-15 was monitored by CD spectroscopy in the far-UV region (250–200) and the results are shown in Figures 3, 4. CTX-M-15, in the absence of drug showed two negative bands at 208 and 222 nm which is a characteristic of the α -helix protein with minima at 208 and 222 nm. The far-UV CD spectra of CTX-M-15 closely resembled to that of the CTX-M-1 (Perez-Llarena et al., 2011).

MRE (mean residual ellipticity) and % alpha helical content of the protein calculated are shown in Table 2. The MRE₂₀₈ nm and MRE₂₂₂ nm of CTX-M-15 under native condition, without any drug treatment was found to be $-14,070$ and $-14,004.9$ deg cm² dmol⁻¹ respectively (Table 2), with alpha helical content of 34.72 and 38.49% as calculated from Equations (5) and (6). In the presence of drug combinations, CTX-M-15 showed remarkable distortion in the alpha helical content with

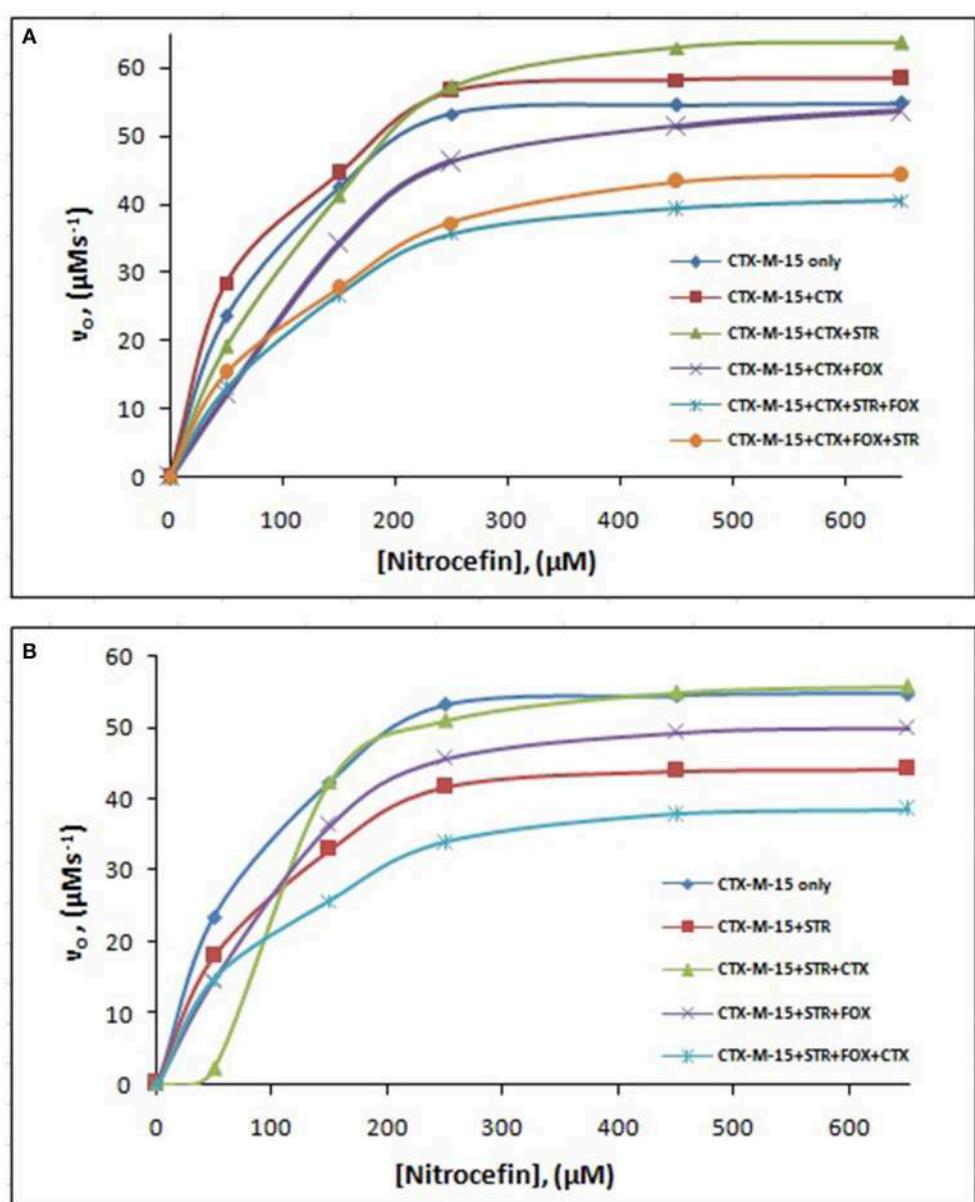


FIGURE 5 | Michaelis-Menten plot. Steady-state kinetics of Nitrocefin hydrolysis by CTX-M-15 in the absence and presence of different combinations of **(A)** Cefotaxime/Streptomycin/Cefoxitin and **(B)** Streptomycin/Cefotaxime/Cefoxitin at 298 K was carried out. The concentration of CTX-M-15 was 8.75 nM while the concentration of drugs were 87.5 nM in 50 mM sodium phosphate buffer, pH 7.4.

maximum disruption in streptomycin and cefotaxime bounded CTX-M-15.

The steady-state kinetics of the purified CTX-M-15 was carried out on nitrocefin, a chromogenic cephalosporin substrate which revealed a hydrolytic profile that is a characteristic of molecular class A beta lactamase. The representative Michaelis-Menten plots are shown in **Figure 5** and the deduced kinetic parameters (kcat, Km, and kcat/Km) are summarized in **Table 3**. The catalytic activity of CTX-M-15 was investigated on nitrocefin to ascertain the involvement of CTX-M-15 in drug binding. The Michaelis-Menten plots of nitrocefin hydrolysis

at 1:10, 1:10:10, 1:10:10:10 molar ratios of different CTX-M-15: cefotaxime/streptomycin/cefoxitin, drug combinations were analyzed to check if kcat and Km values in any case being reduced. The kinetic data were also plotted as Lineweaver-Burk plots to infer the kinetic parameters by which drug inhibited the hydrolysis activity of CTX-M-15 (**Figure 6**). We found increased Km values for all CTX-M-15 drug incubations except for cefotaxime alone with CTX-M-15, implying the decrease in the affinity of enzyme toward substrate nitrocefin in the presence of streptomycin and in all studied combination of streptomycin and cephalosporin drugs treatment. Similarly we found decreased

TABLE 3 | Steady-State Kinetic Parameters for Hydrolysis activity of CTX-M-15 in presence of various drugs.

	Km (μM)	Kcat (s^{-1})	Kcat/Km ($\mu\text{M}^{-1}\text{s}^{-1}$)
CTX-M-15 only	88.13 \pm 0.5	7618.97 \pm 1.4	86.451
CTX-M-15+ CTX	65.932 \pm 0.9	7618.97 \pm 0.85	115.557
CTX-M-15+ STR	93.789 \pm 1.0	6015.02 \pm 0.3	64.133
CTX-M-15+STR+CTX (Allosteric curve)	\sim 100 \pm 2.0	\sim 6285 \pm 3.1	\sim 62.85
CTX-M-15+STR+FOX	187.212 \pm 1.6	8163.2 \pm 2.5	43.604
CTX-M-15+CTX+STR	180.363 \pm 1.8	10389.6 \pm 0.4	57.603
CTX-M-15+CTX+FOX	304.45 \pm 0.9	10389.6 \pm 2.2	34.12
CTX-M-15+STR+FOX+CTX	103.589 \pm 0.6	5197.74 \pm 1.3	50.176
CTX-M-15+CTX+STR+FOX	149.419 \pm 0.2	6014.971 \pm 0.7	40.255
CTX-M-15+CTX+FOX+STR	129.165 \pm 2.1	6349.142 \pm 1.5	49.155

CTX, cefotaxime; STR, streptomycin; FOX, cefoxitin.

Kinetic constants are reported as the average of \pm standard error from three independent experiment.

catalytic efficiency (Kcat/Km) of enzyme for all streptomycin and cephalosporin combinations except for cefotaxime alone. Catalytic activity (Kcat) of the enzyme with nitrocefin was found to decrease in all combinations except in presence of cefotaxime, cefotaxime along with streptomycin and cefoxitin and streptomycin along with cefoxitin.

DISCUSSION

Fluorescence quenching phenomenon occurs due to various molecular interactions such as reaction in the excited state when the electrons in the higher energy level is returned back to lower energy level, energy transferring, molecular rearrangements and static and dynamic quenching. It is being carried out to elucidate the mechanism of interaction of enzyme with drug molecules (Eftink and Ghiron, 1976; Lakowicz, 1988). Fluorescence quenching spectra showed the linear dependence of quenching with different drug combinations which implies that only one type of quenching mechanism either static or dynamic dominated in the process (Rehman et al., 2014). There is significant interaction between CTX-M-15 and drugs (cefotaxime, cefoxitin, and streptomycin), which was responsible for the quenching mechanism, inferred by Ksv values ($\sim 10^4 \text{ M}^{-1}$) as shown in Table 1. The kq values in all the cases were of the order of 10^{12} – $10^{13} \text{ M}^{-1}\text{s}^{-1}$ which were found considerably larger than the maximum dynamic quenching constant $\sim 10^{10} \text{ M}^{-1}\text{s}^{-1}$, indicating that the drug-induced quenching of CTX-M-15 fluorescence was due to complex formation. The microenvironment around the binding site is becoming less hydrophobic upon binding of drug by exposing more residues for interaction as seen by increased values of binding sites.

Greater interaction of CTX-M-15 with cefotaxime than streptomycin was observed with higher Ksv (stern volmer constant), kq (binding constant) and Ka (association constant). Decrease in Ksv and kq was observed in all the combinations

of drug binding with respect to cefotaxime, suggesting that the quenching of CTX-M-15 fluorescence was initiated by complex formation in the ground state rather than by dynamic quenching. However, increase in the value (n) in all the cases except when CTX-M-15 interacted with cephalosporins or aminoglycoside, shows that the microenvironment around the binding site is becoming less hydrophobic upon binding of drug. It is probably due to exposure of more binding site residues for interaction. Maximum drug interaction was observed in all the three triple combinations of drug and combination of streptomycin and cefotaxime with CTX-M-15 with the increase in the values of binding constant comparing to CTX-M-15 with cefotaxime alone. It indicates that streptomycin induces structural changes in CTX-M-15 thereby making conditions unfavorable for proper binding of cefotaxime on its active site for hydrolysis. Hence decrease in stern volmer and quenching constant was observed.

Far-UV CD spectra showed remarkable change in secondary structure of CTX-M-15 enzyme at 208 and 222 nm with respect to native enzyme in all the drug treated combinations. At 208 nm all the combinations showed at least 3.7% decrease in alpha helix peak. Whereas, on combination with cefotaxime and streptomycin, 22.2% increase in the peak was observed. At 222 nm all the combinations showed 24.11% increase in the peak. While, cefotaxime and streptomycin in combination showed maximum rise in the peak (47.48%) as shown in Figures 3, 4. Hence the CD-spectral analysis showed disruption in the overall conformation of CTX-M-15 enzyme upon both single and combined binding of cephalosporin and aminoglycoside. Moreover, conformational changes upon binding of cefotaxime favors hydrolysis, whereas structural changes occurred due to binding in combination, leading to reduced hydrolysis of cefotaxime.

Steady-state kinetics data showed the decrease in the affinity of the CTX-M-15 enzyme toward nitrocefin in the presence of streptomycin alone and with all other synergistic drug combinations. Also synergistic drug treatment (in 1:10, 1:10:10, 1:10:10:10 molar ratio of CTXM-15 and drug) decreased the catalytic efficiency of enzyme to about 27% in all the cases except in the presence of cefotaxime alone. Enzyme treated with cefotaxime alone was found 33% more efficient, when combined along with streptomycin and cefoxitin, the efficiency was reduced to about 53.4%. Whereas, enzyme treated with streptomycin alone caused to reduce its efficiency by 25.8%, however when combined with cefotaxime, its efficiency was decreased by 27.2% with cooperative sigmoidal binding curve (allosteric behavior). If enzyme combined with cefoxitin, its efficiency was decreased to about 49.5% while in combination of cefoxitin and cefotaxime, it was reduced by 41.9%. Hence, the study shows that the enzyme incubated first with streptomycin then with cefotaxime in 1:10:10 molar ratio, shows allosteric behavior. It indicates that streptomycin binding on enzyme promotes cooperative binding of cefotaxime. Moreover, upon streptomycin incubation as single and in cefotaxime/cefoxitin/streptomycin combinations, catalytic efficiency of enzyme was decreased significantly which supports synergistic effect of two drugs. While single drug was not effective against multidrug resistant strain carrying CTX-M-15 enzyme. This can be explained as binding

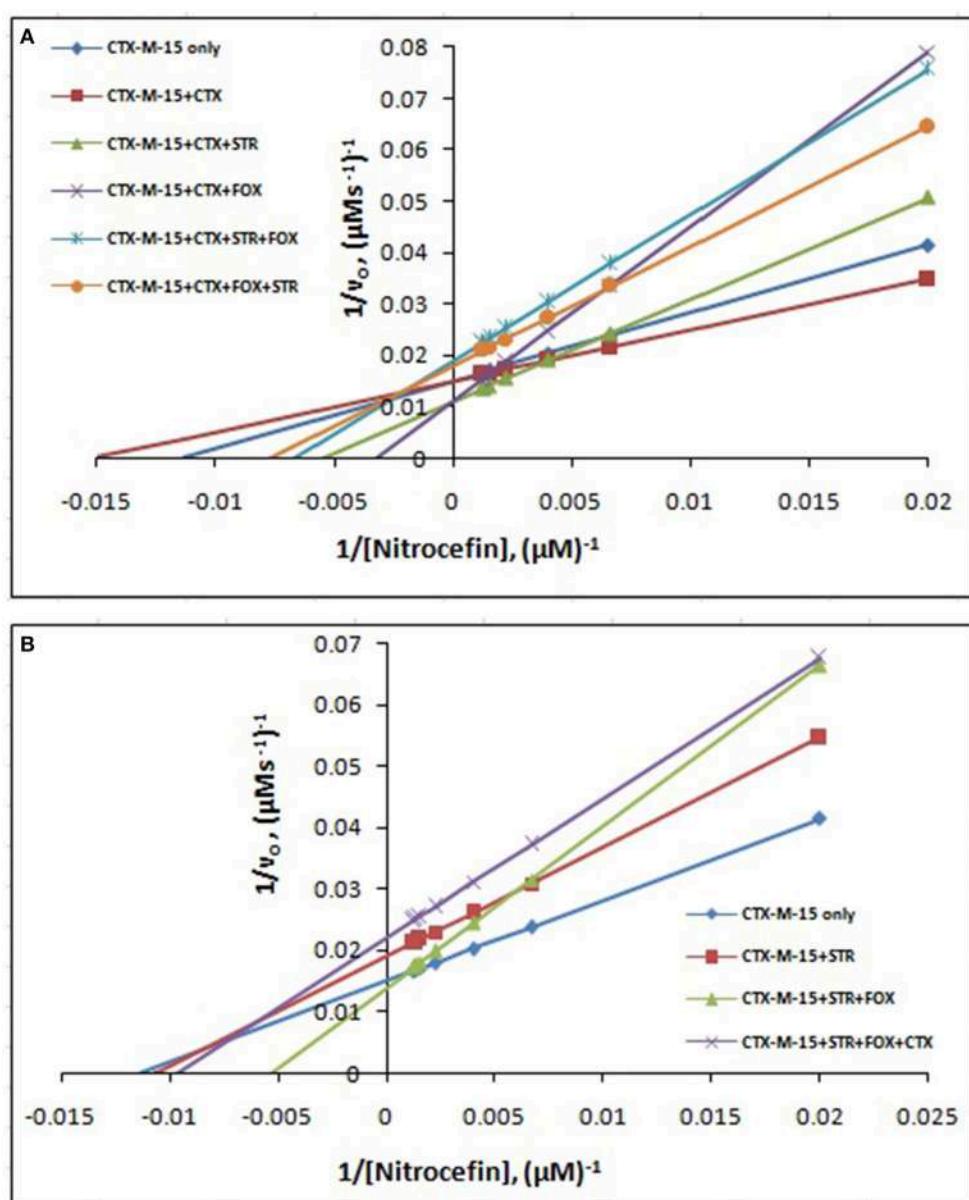


FIGURE 6 | Lineweaver-Burk plot. Steady-state kinetics of Nitrocefin hydrolysis by CTX-M-15 in the absence and presence of different combinations of **(A)** Cefotaxime/Streptomycin/Cefoxitin and **(B)** Streptomycin/ Cefotaxime/ Cefoxitin at 298 K was carried out. The concentration of CTX-M-15 was 8.75 nM while the concentration of drugs were 87.5 nM in 50 mM sodium phosphate buffer, pH 7.4.

of streptomycin may induce structural changes in CTX-M-15, hence cefotaxime was not hydrolyzed and available to act on its target in bacterial cells to kill. This is the first time we have demonstrated a possible mechanism of synergistic effect of cefoxitin, streptomycin and cefotaxime against multi-drug resistant strains. In this mechanism based study, streptomycin induced conformational changes in CTX-M-15 leading to reduced binding of cefotaxime on its active site of hydrolysis which in turn decreases its hydrolysis. Consequently, cefotaxime is available for target site inhibition in bacterial cells (Figure 7).

CONCLUSION

Our study demonstrated first time a possible molecular mechanism of synergistic effect of combination therapy of streptomycin and cefotaxime against CTX-M-15 producing multi-drug resistant strain. The study revealed that binding of streptomycin induces conformational changes in CTX-M-15 leading to reduced binding of cefotaxime which in turn reduces its hydrolysis. Consequently, cefotaxime is available for target site inhibition in bacterial cells.

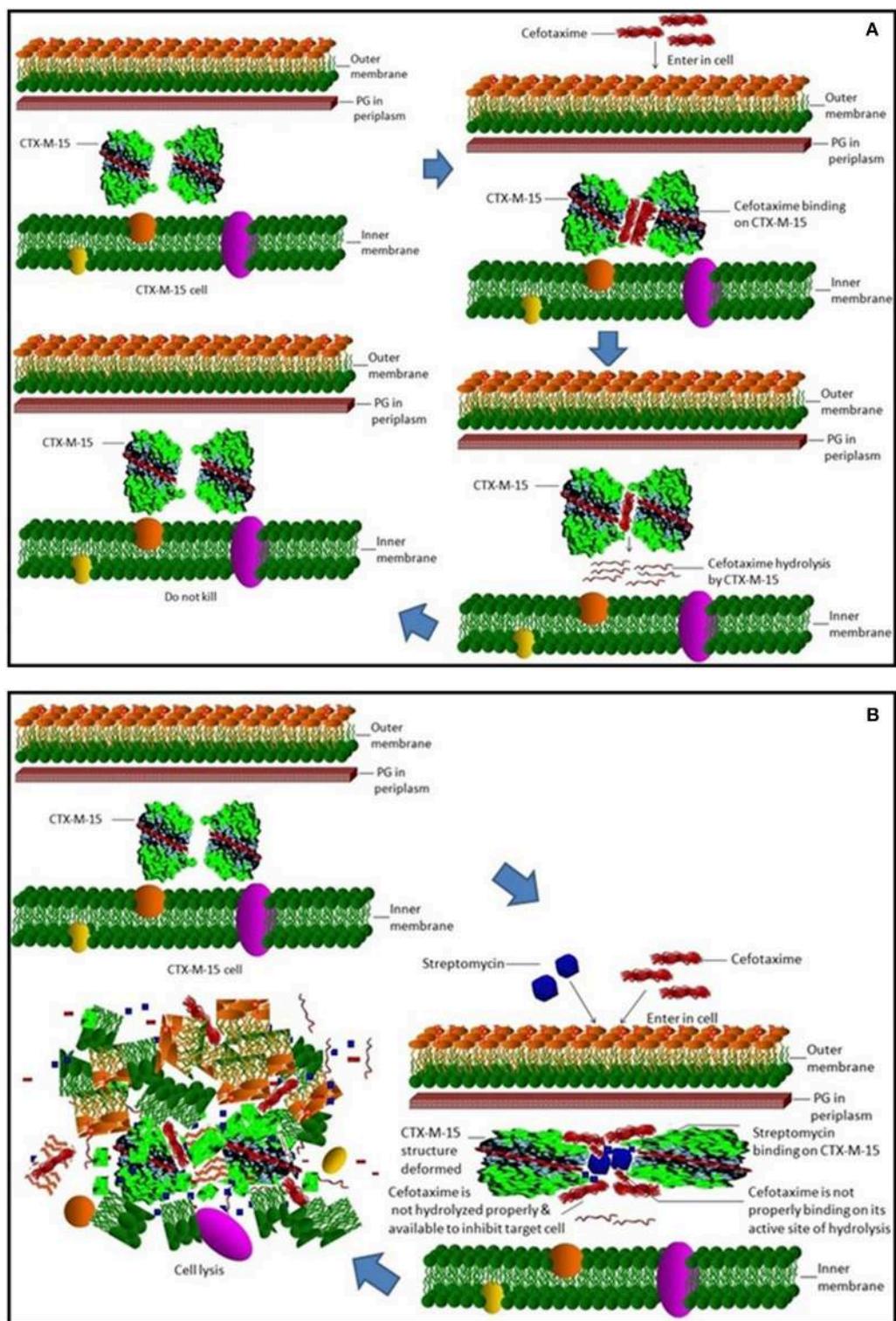


FIGURE 7 | Synergistic effect of streptomycin and cefotaxime on CTX-M-15. **(A)** shows inability of cell killing by cefotaxime due to its hydrolysis by CTX-M-15 enzyme. **(B)** shows cellular lyses by available un-hydrolyzed cefotaxime due to streptomycin which leads to reduced binding of cefotaxime on its active site of hydrolysis.

AUTHOR CONTRIBUTIONS

LM: performed experiment and written first draft. AK: designed problem, interpret data, provide reagents, checked manuscript.

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The Potential of *Streptomyces* as Biocontrol Agents against the Rice Blast Fungus, *Magnaporthe oryzae* (*Pyricularia oryzae*)

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Rice is a staple food source for more than three billion people worldwide. However, rice is vulnerable to diseases, the most destructive among them being rice blast, which is caused by the fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). This fungus attacks rice plants at all stages of development, causing annual losses of approximately 10–30% in various rice producing regions. Synthetic fungicides are often able to effectively control plant diseases, but some fungicides result in serious environmental and health problems. Therefore, there is growing interest in discovering and developing new, improved fungicides based on natural products as well as introducing alternative measures such as biocontrol agents to manage plant diseases. *Streptomyces* bacteria appear to be promising biocontrol agents against a wide range of phytopathogenic fungi, which is not surprising given their ability to produce various bioactive compounds. This review provides insight into the biocontrol potential of *Streptomyces* against the rice blast fungus, *M. oryzae*. The ability of various *Streptomyces* spp. to act as biocontrol agents of rice blast disease has been studied by researchers under both laboratory and greenhouse/growth chamber conditions. Laboratory studies have shown that *Streptomyces* exhibit inhibitory activity against *M. oryzae*. In greenhouse studies, infected rice seedlings treated with *Streptomyces* resulted in up to 88.3% disease reduction of rice blast. Studies clearly show that *Streptomyces* spp. have the potential to be used as highly effective biocontrol agents against rice blast disease; however, the efficacy of any biocontrol agent may be affected by several factors including environmental conditions and methods of application. In order to fully exploit their potential, further studies on the isolation, formulation and application methods of *Streptomyces* along with field experiments are required to establish them as effective biocontrol agents.

Keywords: *Streptomyces*, biocontrol, antifungal, rice, disease

INTRODUCTION

Rice (*Oryza sativa*) is an important food crop and it is the staple diet of over three billion people around the world, particularly in Asia¹ (Abdullah et al., 2006; Skamnioti and Gurr, 2009; Hosseyni-Moghaddam and Soltani, 2013). Nearly half of Asia's population depends on rice as their main food source, making Asia the region with the highest rice consumption—more than 110 kg per capita annually (Hosseyni-Moghaddam and Soltani, 2013; Muthayya et al., 2014). Rice is grown in more than a 100 countries across a wide range of climatic conditions, ranging from rivers deltas to mountainous regions (Kyndt et al., 2014). Asian countries including China, India, Indonesia, Thailand, Philippines, Vietnam, Bangladesh, and Myanmar account for approximately 90% of the world's total rice production (Khush, 2005; Abdullah et al., 2006; Muthayya et al., 2014). The other rice-producing countries include Egypt, Brazil, sub-Saharan countries, and the USA (Kyndt et al., 2014). Wherever it is grown, however, rice is susceptible to diseases with far reaching economic implications. Infection with phytopathogenic fungi are among the most worrying of these diseases as it may result in significant crop yield losses, and additionally, some of the fungi produce compounds which are potentially toxic upon consumption (Chaiharn et al., 2009; Suprapta, 2012). For instance, several *Fusarium*, *Aspergillus*, and *Penicillium* species are capable of producing mycotoxins (e.g., aflatoxins, citrinin, fumonisins, ochratoxin A, and zearalenone) which can be harmful to human beings if they are ingested via consumption of contaminated rice (Almaguer et al., 2012; Ferre, 2016). Given that the present methods of preventing rice diseases are not entirely satisfactory from several angles, it is imperative to seek new and effective methods of prevention in order to produce rice that is safe for consumption as well as to reduce crop yield losses.

Currently, *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) is regarded as one of the most important phytopathogenic fungi as it is the causal agent of rice blast—the most destructive disease of rice (Dean et al., 2012). *M. oryzae* B. Couch (anamorph *Pyricularia oryzae* Cav.), also known as *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc.), is a haploid filamentous ascomycete fungus (Bussaban et al., 2005). *M. oryzae* is defined as a new species distinct from *M. grisea* based on multilocus gene genealogy and laboratory mating experiments by Couch and Kohn (2002). On the basis of phylogenetic analysis, *Magnaporthe* is now separated into two distinct clades—one clade associated with *Digitaria* (crabgrass) maintains the name *M. grisea*, while the other clade associated with rice and other cultivated grasses was characterized as a novel species and given the name *M. oryzae*. Given the phylogenetic differences, however, there are no morphological differences between the isolates from these two clades. As a result, the names *M. oryzae* and *M. grisea* are still used interchangeably by scientists for the fungal isolates that infect rice (Besi et al., 2009; Wilson and Talbot, 2009).

Magnaporthe oryzae infects the aerial parts of the rice plant—including leaves, nodes, stems, and panicles—at all stages of development (Wilson and Talbot, 2009). Infection results

in rice blast symptoms such as leaf blast, node blast, collar rot, neck rot, and panicle blast; this generally manifests as purplish/grayish/brownish/whitish spots or lesions as well as withering of leaves (Kato, 2001). This fungus was later discovered to also have the ability to infect the roots of the rice plant; and infection of the root may eventually spread to the aerial tissues, causing rice blast disease (Dufresne and Osbourn, 2001; Sesma and Osbourn, 2004). However, the exact nature and sequence of the process by which rice blast infects the roots of the rice plant has yet to be fully elucidated (Sesma and Osbourn, 2004). Rice blast disease has been reported in approximately 85 countries, mainly in Asia, Africa, and Latin America (Kato, 2001; Besi et al., 2009). Yield loss due to rice blast ranges from approximately 10–30% annually in the various rice producing countries and can reach up to 50% during disease epidemics (Skamnioti and Gurr, 2009; Ashkani et al., 2015).

Efforts have been made by researchers to identify and analyze the avirulence (AVR) genes of *M. oryzae*. This would serve as the basis of understanding fungal mechanisms of pathogenesis and clarifying the mechanisms responsible for the coevolution of fungal effectors and their host targets (Yoshida et al., 2009). Besides this, development of cultivar-specific resistance involving gene-for-gene system can be achieved by conditioning resistance through high-yielding rice cultivars carrying single dominant disease resistance (*R*) genes to a single corresponding dominant AVR gene in a particular pathogen strain (Skamnioti and Gurr, 2009). To date, four AVR genes have been isolated from *M. oryzae*: *PWL1* and *PWL2* genes which encode Gly-rich hydrophobic proteins with secretion signal sequences, *AVR-Pita* gene which encodes a putative secreted protein with similarity to metalloproteases, and *ACE1* gene which encodes a putative hybrid protein of a polyketide synthase and a peptide synthase (Skamnioti and Gurr, 2009; Yoshida et al., 2009). In contrast, more than 25 resistance (*R*) genes encoding proteins that recognize *M. oryzae* AVRs have been mapped on the rice genome (Yoshida et al., 2009).

Besides breeding of cultivar-specific resistance, several control methods have been attempted to manage plant diseases. Among these, chemical control is the most commonly used method yielding effective management of plant diseases (Hirooka and Ishii, 2013). With reference to control of rice blast, chemical control involves the use of pesticides, specifically fungicides (Skamnioti and Gurr, 2009). A number of fungicides have been used against this disease, for instance, azoxystrobin, benomyl, carbendazim, carpropamid, dithiocarbamate, edifenphos, fenoxanil, tiadinil, tricyclazole, pyroquilon, probenazole, iprobenfos, isoprothiolane, metominostrobin, and propiconazole (Kato, 2001; Skamnioti and Gurr, 2009; Pooja and Katoch, 2014). Generally, the effectiveness of fungicides depends on several factors: the compound itself, the timing and method of application, the level of disease present, the efficiency of disease forecasting systems, and the rate of emergence of fungicide resistant strains (Kato, 2001; Skamnioti and Gurr, 2009). Although they are effective at controlling the fungal infections in rice, there are growing public concerns over the use of synthetic fungicides. Misuse and excessive use of synthetic pesticides (e.g., fungicides, insecticides, and herbicides) might

¹<http://ricepedia.org/rice-as-food/the-global-staple-rice-consumers>

cause environmental pollution, residual toxicity, development of pesticide resistance, reduce soil quality, and damage to natural ecosystems (Pimentel et al., 1991; Komárek et al., 2010; Suprapta, 2012; Yoon et al., 2013). Furthermore, human exposure to pesticides may cause poisoning and harmful side-effects to organs and/or biological processes (Fattah et al., 2015). Pesticide poisoning is a significant occupational health issue in developing countries, likely due to insufficient or poor occupational safety practices. Appropriate work practices are essential to ensure the safety of workers, particularly in the case of agricultural workers who are often exposed to pesticides during application and handling operations such as mixing, cleaning, loading spray equipment, and disposing of empty containers (Kesavachandran et al., 2009). Side effects and symptoms caused by exposure to pesticides have been reported in several developing countries. For instance, farmers in Vietnam reported symptoms such as skin irritation, headache, dizziness, eye irritation, shortness of breath, and acetyl cholinesterase inhibition due to the exposure to various pesticides during mixing and spraying (Dasgupta et al., 2007).

New and improved fungicides with minimal side effects are required in order to prevent these concerns. Nowadays, natural products which are safe for the environment and have low toxicity to living organisms are gaining interest as important sources for the development of fungicides, and these may serve as effective substitutes for synthetic fungicides (Martínez, 2012; Yoon et al., 2013). Moreover, another alternative approach to the use of fungicides is the use of microbial antagonists as biocontrol agents (Suprapta, 2012). Biocontrol agents are microorganisms that suppress plant pathogens (Pal and Gardener, 2006); they can achieve biological control through competition, antibiosis, and hyperparasitism (Montesinos, 2003). Biological control of plant diseases is known to be more cost effective, safe and environmentally friendly as compared to the use of fungicides. *Streptomyces* bacteria are among the microbial antagonists that have been exploited for the biological control of plant diseases. This review aims to encapsulate the current body of knowledge of the biocontrol potential of *Streptomyces* against the rice blast fungus, *M. oryzae*.

Streptomyces* spp. AS BIOCONTROL AGENTS AGAINST *M. oryzae

Streptomyces is the largest genus of the phylum *Actinobacteria* and was first proposed by Waksman and Henrici (1943). The genus *Streptomyces* consists of a group of Gram-positive, aerobic, non-motile, catalase positive, and non-acid-fast bacteria with a filamentous form that resembles fungi (Flärdh and Buttner, 2009; Hasani et al., 2014). Currently, over 700 species of *Streptomyces* have been identified² and these bacteria have relatively large genomes of approximately 8–9 Mbp in size with a high GC content of more than 70% (Wu et al., 2005; Hasani et al., 2014; Ser et al., 2015c).

²<http://www.bacterio.net/>

The members of *Streptomyces* are well-known for their ability to produce a variety of bioactive compounds with different bioactivities such as antibacterial (Schumacher et al., 2003; Ramesh and Mathivanan, 2009; de Lima Procópio et al., 2012; Kumar et al., 2014; Lee et al., 2014a,b; Ser et al., 2016a), antifungal (Lam, 2006), antiviral (Ara et al., 2014), immunosuppressive (Kino et al., 1987), anticancer, and antioxidant properties (Ser et al., 2015a; Tan et al., 2015, 2016). These bioactive compounds have important applications in various fields. For example, approximately 75% of commercially useful antibiotics were derived from the genus *Streptomyces* and they are thus the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Pelaez, 2006; Lee et al., 2014b; Kashif et al., 2016). *Streptomyces* strains also have important applications in the agricultural field through their biological control potential against phytopathogens, particularly phytopathogenic fungi (Gonzalez-Franco and Robles-Hernandez, 2009). In line with this, research on the biological control of rice blast disease using different *Streptomyces* species has been conducted under laboratory conditions, as well as in greenhouse or growth chamber conditions.

***In vitro* Experiments**

Studies have reported *in vitro* antagonism of different *Streptomyces* spp. against *M. oryzae* which were tested using the dual culture method. Dual culture method has been widely used for preliminary screening of biocontrol agents including fungi and bacteria. This method allows the biocontrol agent and pathogen to interact on a solid medium in a Petri dish, under optimal conditions for both organisms. The degree of inhibition is recorded by observing the inhibition zone produced or the overgrowth of the pathogen by the biocontrol agent (Desai et al., 2002).

In a study conducted by Ningthoujam et al. (2009), *S. vinaceusdrappus* isolated from Loktak lake sediment was first reported to exhibit antagonistic activity against *M. oryzae* (*P. oryzae* MTCC 1477). *S. vinaceusdrappus* also showed maximal antagonistic activity against most of the rice fungal pathogens tested, which include *Curvularia oryzae*, *Bipolaris oryzae*, and *Fusarium oxysporum*. *S. vinaceusdrappus* inhibited the mycelial growth of *P. oryzae* by 53.5%, which was relatively good since more than 50% of the mycelial growth was inhibited. Boukaew and Prasertsan (2014) reported that *Streptomyces philanthi* RM-1-138 isolated from rhizosphere soil of chili pepper in Southern Thailand exhibited significant antifungal activity against *M. oryzae* (*P. oryzae* PTRRC-18), with 88.73% inhibition of mycelial growth of the rice blast fungus. This suggests that *S. philanthi* RM-1-138 has greater inhibition against the rice blast fungus as compared to *S. vinaceusdrappus*.

The *in vitro* assay conducted by Li et al. (2011) showed that *Streptomyces globisporus* JK-1 demonstrated the most pronounced inhibitory effects against *M. oryzae* as compared to other phytopathogenic fungi tested in the study. *S. globisporus* JK-1 inhibited mycelial growth of *M. oryzae* with an inhibition zone of 15 mm out of 35 mm. A study also showed that endophytic *Streptomyces* from rice cultivars in China demonstrate antagonism against rice fungal pathogens,

particularly 54.5% of *Streptomyces griseofuscus* and 21.8% of *Streptomyces hygroscopicus* were the most active among the studied population of antagonistic endophytic *Streptomyces* which exhibited strong antagonism against *M. oryzae* (Tian et al., 2004). *Streptomyces sindeneusis* isolate 263 and *Streptomyces* isolate 339 obtained from agricultural soils of Kerman in Iran were also found to inhibit *M. oryzae* (Zarandi et al., 2009, 2013). In addition, Khalil et al. (2014) reported that *Streptomyces flavotricini* isolated from Egyptian rice field soils showed the strongest antifungal activity against *M. oryzae*; the antifungal compound produced by *S. flavotricini* was successfully purified and identified as dihydroxy viridiofungin ($C_{37}H_{58}N_2O_{10}$).

Based on these findings, *S. vinaceusdrappus*, *S. philanthi* RM-1-138, *S. griseofuscus*, *S. hygroscopicus*, *Streptomyces* isolate 339, and *S. flavotricini* are potential candidates for use as biocontrol agents against rice blast as they possess inhibitory activity against *M. oryzae* (Table 1). Based on the percentage of mycelial growth inhibition, *S. philanthi* RM-1-138 appears to be one of the most promising agents for the inhibition of *M. oryzae*. However, studies involving greenhouse or field experiments are still required to more definitively evaluate the biocontrol potential of these *Streptomyces* spp. against *M. oryzae*.

Greenhouse/Growth Chamber Experiments

The limitation of laboratory experiments is that they only prove the antagonistic activity of *Streptomyces* spp. against *M. oryzae* under certain conditions. The antagonism exhibited by *Streptomyces* in laboratory experiments might not necessarily reflect antagonism under greenhouse or field experiments. Greenhouse or growth chamber experiments are conducted for the purpose of further evaluating the efficacy of *Streptomyces* strains as biocontrol agents. For *S. sindeneusis* isolate 263 and *S. globisporus* JK-1, studies on their biocontrol potential against the fungus under greenhouse conditions were conducted by Zarandi et al. (2009) and Li et al. (2011) respectively. Zarandi et al. (2009) reported that typical blast symptoms were observed when rice plants at the three leaf-stage of vegetative phase were treated with *M. oryzae*. The percentage of diseased leaf area was evaluated according to the method developed by the International Rice Research Institute (IRRI). It was found that the rice plants receiving treatment with *S. sindeneusis* isolate 263 showed significantly reduced lesion development. The diseased leaf area was 8% for rice plants treated with *M. oryzae* alone, while it was only 0.5% for rice plants treated with *S. sindeneusis* isolate 263 in combination with *M. oryzae*. This result indicates that *S. sindeneusis* isolate 263 acted as an antagonist.

Li et al. (2011) compared the control of the rice blast using *Streptomyces* and fungicide by infecting rice plants at the five leaf-stage during the vegetative growth phase with *M. oryzae*, followed by treatment with culture filtrates of *S. globisporus* JK-1 and tricyclazole respectively. Tricyclazole is one of the commonly used fungicides for the control of rice blast disease with several advantages over other fungicides, for instance, it is systemic in rice for blast control and has long residual effectiveness (Froyd et al., 1976). The results showed that control efficacy

for *S. globisporus* JK-1 treatment was 88.3% and for tricyclazole was 79.4%, compared to the inoculated control. This suggests that *S. globisporus* is as efficient and possibly even superior to tricyclazole, with the additional benefits of biocontrol agents as compared to synthetic agents described earlier.

Additionally, a novel *Streptomyces* strain, BG2-53, with 96% homology to *S. lipmanii* based on analysis of 16S rDNA sequences, exhibited potent antifungal activity against *M. oryzae* under growth chamber conditions (Lee et al., 2002). The strain showed the highest degree of fungal control in comparison to fungicides such as Blasticidin-S and Tricyclazole. However, the evaluation on the extent of rice disease infection was solely based on visual estimation, unlike other studies where the Standard Evaluation System of IRRI was applied.

Overall, the results of greenhouse and growth chamber experiments strongly suggest that several *Streptomyces* spp. possess antagonistic activities against *M. oryzae*, and therefore have the potential to effectively control rice blast. Field experiments are still required to more definitively estimate the efficacy of *Streptomyces* spp. as biocontrol agents under real life conditions as environmental factors greatly affect their performance.

Bioactive Compounds from *Streptomyces* spp. with Antifungal Activity against *M. oryzae*

The suppression of rice blast in the greenhouse by certain *Streptomyces* might indicate the presence of bioactive compound(s) with antifungal activity against *M. oryzae* in the culture filtrates. *Streptomyces* are prolific producers of bioactive compounds. Some well-known antibiotics produced by *Streptomyces* have been used as fungicides for the control of rice blast, for instance, Blasticidin-S and Kasugamycin. Blasticidin-S, isolated from *S. griseochromogenes* was the first antibiotic commercially introduced for the control of rice blast in Japan (Fukunaga et al., 1955; Takeuchi et al., 1958; Tapadar and Jha, 2013). Kasugamycin was discovered soon after; it was first isolated from *S. kasugaensis* by Umezawa et al. (1965). Kasugamycin has been safely used to protect rice plants against blast disease; it has relatively low mammalian toxicity and no phytotoxicity toward rice plants and most crops (Yamaguchi, 1982; Coping and Duke, 2007). Recently, the significance of Blasticidin-S as a fungicide has decreased as it has been replaced by new pathogen-specific fungicides with lower toxicity (Copping and Duke, 2007). Kasugamycin is currently still on the market and is sold in several different formulations such as wettable powder, granule, and soluble liquid under the trade names Kasumin and Kasumin-Bordeaux from Hokko Chemical Industry, Co., Ltd (Copping and Duke, 2007; Hokko, 2015).

Several studies have performed testing of the various compounds produced by *Streptomyces* on *M. oryzae*. These studies have shown that several compounds produced by *Streptomyces* spp. exhibited antifungal activity against *M. oryzae* (Appendix 1). The antibiotic Oligomycin A was first isolated from *S. diastatochromogenes* and was found to be active against several other phytopathogenic fungi in addition to *M. oryzae* such

TABLE 1 | Summary of studies applying different *Streptomyces* strains as biocontrol agents for the control of rice blast disease caused by *Magnaporthe oryzae*.

Biocontrol agent	Type of experiment	Application method	Formulation	Results (percentage inhibition of <i>M. oryzae</i>)	Reference
<i>Streptomyces</i> strain BG2-53	Growth chamber experiment	Foliar spraying	Liquid (broth)	Streptomyces BG2-53 showed highest fungal control (98%) than Blasticidin-S (86%) and Tricyclazole (95%)	Lee et al., 2002
Endophytic <i>Streptomyces</i> (<i>S. griseofuscus</i> , <i>S. hygroscopicus</i> , <i>S. globisporus</i> , <i>S. aureus</i> , <i>S. albosporus</i>)	<i>In vitro</i> experiment using dual culture method	Not available	Not available	<i>Streptomyces griseofuscus</i> and <i>Streptomyces hygroscopicus</i> exhibited strongest antagonism against <i>M. oryzae</i>	Tian et al., 2004
<i>S. vinaceoruber</i>	<i>In vitro</i> experiment using dual culture method	Not available	Not available	Mycelial growth inhibition of <i>M. oryzae</i> (53.5%)	Ningthoujam et al., 2009
<i>S. sindeneus</i> isolate 263	<i>In vitro</i> experiment using dual culture method	Not available	Not available	Antifungal activity against <i>M. oryzae</i>	Zarandi et al., 2009
	Greenhouse experiment	Foliar spraying	Liquid (culture filtrates)	Rice plants treated with <i>M. oryzae</i> alone showed typical blast symptoms and 8% diseased leaf area; rice plants treated with <i>M. oryzae</i> plus <i>Streptomyces sindeneus</i> isolate 263 showed 0.5% diseased leaf area	
<i>S. globisporus</i> JK-1	<i>In vitro</i> experiment using dual culture method	Not available	Not available	Mycelial growth inhibition of <i>M. oryzae</i> (42.9%)	Li et al., 2011
	Greenhouse experiment	Foliar spraying	Liquid (culture filtrates)	<i>Streptomyces globisporus</i> JK-1 treatment (88.3%) showed highest fungal control than Tricyclazole (79.4%), as compared to the inoculated control	
				Antifungal activity against <i>M. oryzae</i>	Zarandi et al., 2013
<i>Streptomyces</i> isolate 339	<i>In vitro</i> experiment using dual culture method	Not available	Not available		
<i>S. philanthi</i> RM-1-138	<i>In vitro</i> experiment using dual culture method	Not available	Not available	Mycelial growth inhibition of <i>M. oryzae</i> (88.73%)	Boukaew and Prasertsan, 2014
<i>S. flavotrichii</i>	<i>In vitro</i> experiment using dual culture method	Not available	Not available	Antifungal activity against <i>M. oryzae</i> (40 mm inhibition zone)*	Khalil et al., 2014

*The percentage of inhibition of *M. oryzae* cannot be estimated due to insufficient information reported in the study.

as *Botrytis cinerea*, *Cladosporium cucumerinum*, *Colletotrichum lagenarium*, *Phytophthora capsici*, *Alternaria alternata*, and *Aspergillus niger* (Smith et al., 1954; Kim et al., 1999; Yang et al., 2010). Oligomycin A's ability to control the development of rice blast was evaluated in the greenhouse and the results showed that rice plants treated with Oligomycin A (50 µg/mL) had reduced lesions. When the concentration of Oligomycin A was increased up to 500 µg/mL, the rice plants did not show any rice blast disease symptoms (Kim et al., 1999).

Rapamycin—also known as Sirolimus—was initially isolated from *S. hygroscopicus* (Sehgal et al., 1975; Sehgal, 1998). Rapamycin is a potent antifungal agent found to be effective against *Candida albicans*, *Microsporum gypseum*, and *Trichophyton granulosum* (Sehgal et al., 1975). Bastidas et al. (2012) reported that Rapamycin inhibited the growth of *Mucor circinelloides* and *Rhizopus oryzae*, both of which are post-harvest fruit pathogens (Johnson et al., 1990; Kwon et al., 2011).

Other compounds such as Pyrroles (Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-) are commonly found in various *Streptomyces* species (Robertson and Stevens, 2014; Ser et al., 2015b, 2016b,c; Tan et al., 2015; Awla et al., 2016). Furthermore, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) was found to be able to protect plants from phytopathogenic fungi (Zhou et al., 2015).

Bioactive compounds derived from *Streptomyces* can be used for the management of fungal plant diseases as an alternative to synthetic fungicides (Prabavathy et al., 2006). These natural compounds are biologically synthesized and often biodegradable. Thus, they may be useful for the development of agricultural fungicides that are more pathogen-specific with minimal side-effects toward the environment (Varma and Dubey, 1999; Prabavathy et al., 2006). In order to formally establish any organism as a commercially viable biocontrol agent, studies involving the application of the microbial antagonist in question on a certain plant for the control of a particular pathogen are required to investigate their effectiveness. Although it is too early to draw any definite conclusions, the *Streptomyces* spp. listed in **Table 1** could be considered potential biocontrol agents, as they are capable of producing compounds with antifungal activity against *M. oryzae*.

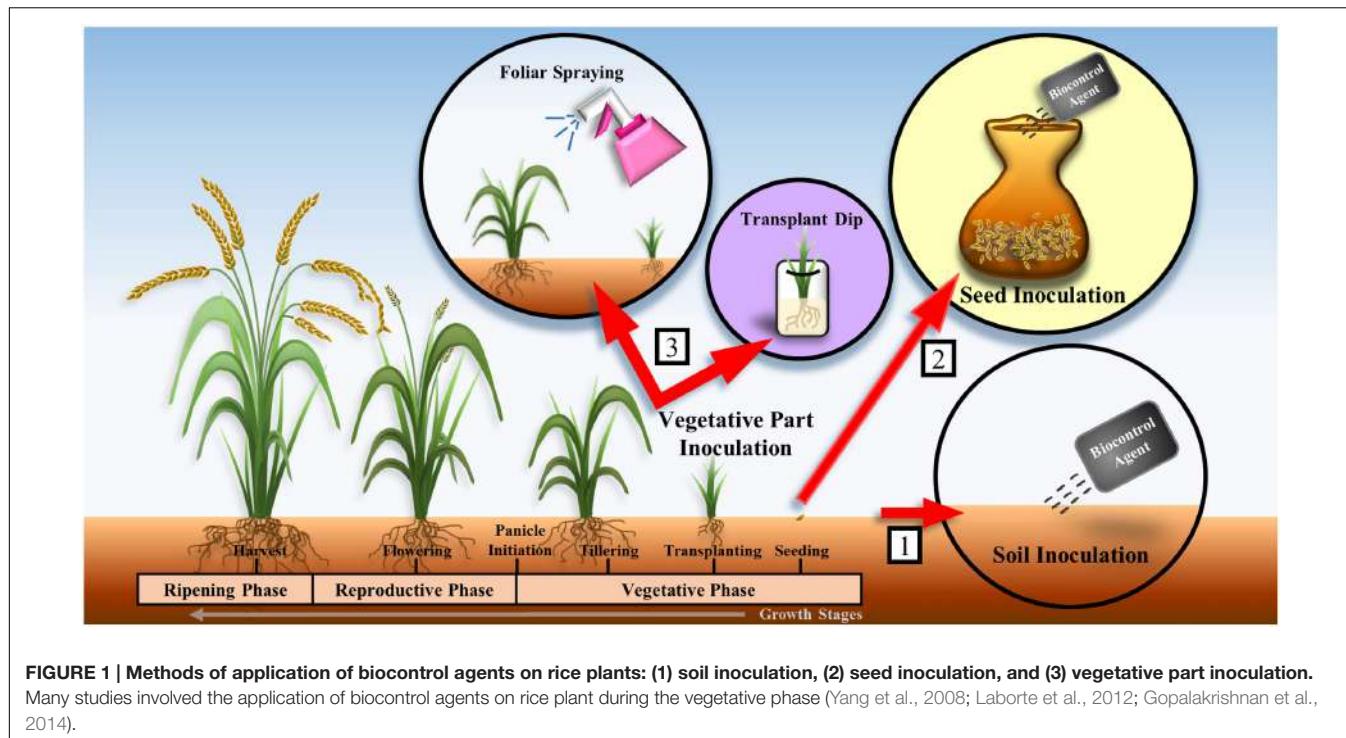
ANTAGONISTIC MECHANISMS OF BIOCONTROL AGENTS

It is known that antagonistic activities of bacteria against fungal pathogens can be achieved through three main mechanisms: competition for nutrients and space, antibiosis, and parasitism (Gonzalez-Franco and Robles-Hernandez, 2009; Boukaew and Prasertsan, 2014). The advantages of *Streptomyces* spp. include their ability to colonize plant root surfaces, survive in various types of soil and also produce spores which allow them to survive longer and in various extreme conditions (Gonzalez-Franco and Robles-Hernandez, 2009; Ningthoujam et al., 2009). Antibiosis happens when the antagonist present in the plant produces metabolites such as antibiotics or antifungals which can inhibit or kill the pathogen. *Streptomyces* spp. used as

biocontrol agents produced antibiotics such as geldanamycin and nigericin for the control of plant diseases, which were proven by the presence of antibiotics in soil (Rothrock and Gottlieb, 1984; Trejo-Estrada et al., 1998). Likewise, hyperparasitism may occur due to the release of extracellular lytic enzymes such as chitinases and glucanases from the biocontrol agent (Gonzalez-Franco and Robles-Hernandez, 2009; Palaniyandi et al., 2013). It has also been shown that *Streptomyces* spp. are capable of producing chitinases and glucanases which play important roles in destruction of fungal cell walls (Mahadevan and Crawford, 1997; El-Tarably et al., 2000; Gonzalez-Franco and Robles-Hernandez, 2009). The colonization ability and competitive traits of *Streptomyces* could result in successful competition against phytopathogenic fungi and suppression of their growth. It can be presumed that the suppression of rice blast by *Streptomyces* spp. might be due to these mechanisms, but further studies are required to provide evidence regarding the actual biocontrol mechanisms of *Streptomyces* against *M. oryzae*.

CHALLENGES FOR SUCCESSFUL APPLICATION OF BIOCONTROL AGENTS

One of the major challenges encountered during the selection of biocontrol agents is that biocontrol agents that appear efficacious based on *in vitro* experiments might not be effective in controlling plant diseases in greenhouse or field conditions. The efficacy of biocontrol agents is affected by organic matter, pH, nutrient level, and moisture level of the soil. Owing to the variations in environmental conditions in different locations, biocontrol agents that perform well in *in vitro* conditions might fail in greenhouse or field experiments. Therefore, the environmental factors at the location where biocontrol agents will be applied should be taken into consideration during the selection of appropriate biocontrol agents. Ideally, the biocontrol agents should be isolated from and applied to locations with similar environmental factors in order to achieve successful biological control (Suprapta, 2012). Furthermore, the formulation (e.g., powder, liquid, or granule) and the method of application of biocontrol agents such as soil inoculation, seed inoculation, and vegetative part inoculation should be examined (**Figure 1**) as they are important in determining the outcomes of field experiments (Ou, 1980; Dubey, 1993). Soil inoculation involves mixing of the biocontrol agent with soil or spreading the biocontrol agent in sowing furrows by drip systems (Vasudevan et al., 2002). Seed inoculation involves dipping seeds in a culture of the biocontrol agent or mixing the seeds with the inoculant using appropriate wetting agents (Dubey, 1993; Vasudevan et al., 2002; Yang et al., 2008). Vegetative part inoculation involves aerial/foliar spraying of the biocontrol agent or seedling treatment by dipping the roots of the seedlings into a solution containing the biocontrol agent prior to transplantation (Vasudevan et al., 2002; Gopalakrishnan et al., 2014) (**Figure 1**). The appropriate application method is likely to contribute significantly to the success of the biocontrol agents in the field trials (Suprapta, 2012).



EXAMPLES OF COMMERCIAL *Streptomyces* BIOCONTROL AGENTS

Some *Streptomyces* spp. have been successfully developed into commercial biocontrol agents and tested for the control of other plant diseases. For example, *Streptomyces griseoviridis* strain K61 (Mycostop®), which has been tested for the control of *Ceratocystis radicicola* that causes black scorch on date palm and soilborne pathogens of tomato such as *Fusarium oxysporum* f.sp. *lycopersici* and *Verticillium dahliae* (Suleman et al., 2002; Minuto et al., 2006). Other commercial *Streptomyces* biocontrol agents include *S. lydicus* WYEC108 (Actinovate®, Micro108®, Action Iron®), and *S. saraceanicus* KH400 (YAN TEN *S. saraceanicus*) (Elliott et al., 2009; Palaniyandi et al., 2013). Biocontrol agents are relatively safe toward humans as no adverse effects in users and other workers have been reported following exposure to these commercial products (Pest Management Regulatory Agency, 2003; U. S. Environmental Protection Agency, 2005). However, hypersensitivity may occur in certain individuals on exposure to biological dust produced during handling of *S. griseoviridis* strain K61 (Mycostop®) dry end-product (Pest Management Regulatory Agency, 2003). Hence, wearing appropriate safety equipment is required when handling these agents. With reference to the control of rice blast disease, however, commercial *Streptomyces* biocontrol agents have yet to be developed.

CONCLUSION

Rice blast, the result of infection by *M. oryzae*, is the most destructive disease of rice, leading to crop yield losses and

economic damage. While chemical control has been the mainstay of controlling this infection, biological control has now been introduced as an alternative for the management of rice blast disease. Biological control of plant diseases is typically inexpensive, long lasting, and safe toward the environment and living organisms; however, biological control can be a slow process and the search for suitable biocontrol agents requires considerable time and effort. *Streptomyces* spp. certainly demonstrate the potential to be developed as biocontrol agents due to their various beneficial properties. Based on current research findings, *S. vinaceorubrum*, *S. philanthi* RM-1-138, *S. griseofuscus*, *S. hygroscopicus*, *Streptomyces* isolate 339, and *S. flavotrichini* showed antifungal activity against *M. oryzae* under *in vitro* conditions. *S. sindensis* isolate 263 and *S. globisporus* JK-1 demonstrated *in vitro* antifungal activity against *M. oryzae* as well as successful biocontrol of rice blast in greenhouse experiments. *Streptomyces* strain BG2-53, which appears to be a novel strain, showed antifungal activity against *M. oryzae* under growth chamber conditions. These *Streptomyces* spp. possess antagonistic activities against *M. oryzae*, with *S. globisporus* JK-1 showing high control efficacy of up to 88.3%. Furthermore, studies have revealed that *Streptomyces* produces various compounds with antifungal activity against *M. oryzae*. Therefore, they are excellent candidates as biocontrol agents for the biological control of this devastating rice blast disease. In order to establish *Streptomyces* as biocontrol agents, more field experiments should be conducted to determine their control efficacy under different environmental conditions. Additionally, more work is needed to optimize isolation, formulation and application methods of *Streptomyces* in order to fully maximize their potential as effective agents to control rice blast.

AUTHOR CONTRIBUTIONS

The literature review and manuscript writing were performed by JL and H-LS, while TK, L-HC, PP, K-GC, B-HG, and L-HL provided vital guidance and insight for the writing. The research topic was conceptualized by L-HL and B-HG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmib.2017.00003/full#supplementary-material>

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