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Functional gene-based discovery of phenazines from the actinobacteria associated with marine sponges in the South China Sea

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Abstract Phenazines represent a large group of nitrogen-containing heterocyclic compounds produced by the diverse group of bacteria including actinobacteria. In this study, a total of 197 actinobacterial strains were isolated from seven different marine sponge species in the South China Sea using five different culture media. Eighty-seven morphologically different actinobacterial strains were selected and grouped into 13 genera, including *Actinoalloteichus*, *Kocuria*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardiopsis*, *Prauserella*, *Rhodococcus*, *Saccharopolyspora*, *Salinispora*, *Serinicoccus*, and *Streptomyces* by the phylogenetic analysis of 16S rRNA gene. Based on the screening of *phz*E genes, ten strains, including five *Streptomyces*, two *Nocardiopsis*, one *Salinispora*, one *Micrococcus*, and one *Serinicoccus* were found to be potential for phenazine production. The level of *phzE* gene expression was highly expressed in *Nocardiopsis* sp. 13-33-15, 13-12-13, and *Serinicoccus* sp. 13-12-4 on the fifth day of fermentation. Finally, 1,6-dihydroxy phenazine (**1**) from *Nocardiopsis* sp. 13-33-15 and 13-12-13, and 1,6-dimethoxy phenazine (**2**) from *Nocardiopsis* sp. 13-33-15 were isolated and identified successfully based on ESI-MS and NMR analysis. The compounds **1** and **2** showed antibacterial activity against *Bacillus mycoides* SJ14, *Staphylococcus aureus* SJ51, *Escherichia coli* SJ42, and *Micrococcus luteus* SJ47. This study suggests that the integrated approach of gene screening and chemical analysis is an effective strategy to find the target compounds and lays the basis for the production of phenazine from the sponge-associated actinobacteria.

Keywords Actinobacteria · Sponges · Diversity · Phenazine

Introduction

Marine sponges (phylum *Porifera*), which are evolutionarily olden metazoans, multicellular invertebrate stalkless filter-feeders, represent a major component in benthic populations of the global oceans. Marine sponges are rich sources of novel compounds, which have multiple bioactivities with medicinal and pharmaceutical applications (Blunt et al. 2012). Sponges are an important resource for the marine drug development. Presently, five compounds or semisynthetic analogues produced from the sponges have been concluded as medicines, and 13 compounds are in clinical trials for several purposes, mostly as anticancer drugs, and 100 compounds are included in the assessment of preclinical trials (Mayer et al. 2010).

The interior of sponge species contains abundant microbial populations together with archaea, bacteria, fungi, and viruses (Fuerst 2014). Culture-dependent and culture-independent molecular approaches have demonstrated that at least 47 bacterial phyla and candidate phyla are associated with the marine sponges (Reveillaud et al. 2014). The majority of the phyla belong to Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira, Cyanobacteria, Bacteriodetes, Gemmatimonadetes, Planctomycetes, Spirochaetes, and Proteobacteria (Webster and Taylor 2012). The microbes associated with marine sponges have the number of responsibilities such as nutrient acquirement, processing of metabolic waste, and the production of secondary metabolites. These

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microbes also mediate nutrient cycles such as carbon, nitrogen, and sulfur within the sponge tissues (Taylor et al. 2007). Additionally, the microbial mediated chemical defenses have also been detected within the sponges (Hochmuth et al. 2010). In a few instances, the chemicals produced by the sponge-associated microbes have been identified to be formerly produced by the host sponge (Konig et al. 2006).

Actinobacteria derived from the marine organisms are believed to be good sources for isolating new bioactive compounds (Zotchev 2012; Valliappan et al. 2014). The actinobacteria associated with marine sponges have attracted more attention due to their biotechnological potential (Montalvo et al. 2005; Hentschel et al. 2006; Piel 2006). The sponge-associated actinobacteria hold the pharmic activities such as antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant, and anticancer, the active compounds are categorized into the chemical groups such as polyketides, peptides, alkaloids, isoprenoids, phenazines, indolocarbazoles, fatty acids, sterols, and terpenes (Abdelmohsen et al. 2014a; Valliappan et al. 2014).

Phenazines represent a large group of structurally diverse nitrogen-containing heterocyclic compounds with biological activities (Laursen and Nielsen 2004; Pierson and Pierson 2010; Gao et al. 2012; Mavrodi et al. 2013), such as antibiotic (Abken et al. 1998), antimalarial (Makgatho et al. 2000; de Andrade-Neto et al. 2004), antibacterial (Laursen and Nielsen 2004; Liu et al. 2007), and anticancer activities (Abdelfattah et al. 2011a, b; Gao et al. 2012). Additionally, phenazine is a small molecule, and it can invade easily into the tissues and organs and influence the multiple targets (Gao et al. 2012). Therefore, phenazine might be a potential compound for chemoprevention and has a possible role in both clinical and industrial processes.

Marine actinobacteria are known to produce phenazines with medicinal applications (Gao et al. 2012). The structurally different types of phenazines have been identified from the sponge-associated actinobacteria, including phenzine 1,6-dicarboxilic acid, streptophenazine A-H, and JBIR 46-48 (Khan et al. 2010; Izumikawa et al. 2010; Mitova et al. 2008; Schneemann et al. 2011). Therefore, it is the great value to isolate actinobacteria from the sponges to produce phenazines with biological activity. Since, only a few percentage of the actinobacteria are able to produce phenazine under the fermentation condition, the selection of positive phenazine producer based on the chemical screening is difficult and time consuming (Izumikawa et al. 2010). Thus, genetic screening of actinobacteria using a primer targeting the fragment of *phz*E gene (Schneemann et al. 2011) is a promising strategy to identify the phenazine-producing actinobacteria. Herein, we reported genetic approach at both DNA and RNA levels to detect the potential phenazine producers from the South China Sea sponge-associated actinobacteria, and finally test their ability for the production of phenazine by fermentation and chemical analysis under the guidance of *phzE* gene sequencing.

Materials and methods

Sample collection

Seven visually healthy sponges (*Theonella swinhoei*, *Dysidea arenaria*, *Agelas sceptrum*, *Ircinia* sp., *Haliclona simulans*, *Smenospongia aurea*, and *Iotrochota* sp.) were collected by SCUBA diving within a 15-m radius at ca. 10-m depths near Yongxing Island (112° 20′ E, 16° 50′ N) of the South China Sea (112° 20′ E, 16° 50′ N) in August 2013 and identified based on the 28S ribosomal ribonucleic acid sequencing (four species of sponges) and sponge morphology (three species of sponges) (Hooper and Van Soest 2002). Sponges were kept in plastic bags containing seawater, transported to the laboratory on ice, and immediately stored at −80 °C.

Isolation of actinobacteria

To remove the loosely attached bacteria from the surrounding seawater, sponge samples were thoroughly rinsed with sterile artificial seawater (ASW) (Sun et al. 2010) for at least three times, followed by 1 g of sponge material sliced into pieces about 1 cm^3 and then homogenized in sterile mortars. Homogenates were pretreated by incubating in a water bath at 55 °C for 5 min, serially diluted and plated in triplicate on agar plates. Five different types of media including M1 (Mincer et al. 2002), M2 (Zhang et al. 2013), M5 (Zhang et al. 2013), Kusters (Poongodi et al. 2014), and arginine glycerin agar (Zhang et al. 2014) were used to isolate actinobacteria. All media were prepared in ASW and supplemented with nalidixic acid (25/mg) and nystatin (50/mg). The inoculated plates were incubated at 28 °C for 2–6 weeks. Colonies were counted and representative of distinct colonies were picked and restreaked on yeast extract-malt extract agar (ISP 2) (Shirling and Gottlieb 1966). *Nocardiopsis* sp. 13-33-15, *Nocardiopsis* sp. 13-12-13, and *Serinicoccus* sp. 13-12-4 were deposited in China Center for Type Culture Collection under the number CCTCC AA 2014034, CCTCC AA 2014033, and CCTCC AB 2014234, respectively.

Molecular identification and phylogenetic analysis

Eighty-seven actinobacterial strains were selected based on their source sponge, colony morphology, aerial and substrate mycelia for the molecular identification and phylogenetic analysis. Isolation of genomic DNA, PCR amplification, and sequencing of the 16S ribosomal RNA (rRNA) genes were performed according to Sun et al. (2010). The resulting 16S rRNA gene sequences were evaluated by comparing with those sequences previously submitted in the public databases using NCBI (http://www.ncbi.nlm.nih.gov/) Basic Local Alignment Search Tool (BLAST). The phylogenetic trees were constructed with related sequences retrieved from the

public databases using the maximum likelihood algorithm with bootstrap values based on 1000 replications in MEGA version 5.1.

Amplification and identification of the phenazine gene fragments

The *phz*E gene fragment of phenazine pathway was PCR-amplified using the degenerated primers, *phz*Ef (5′-GAAG GCGCCAACTTCGTYATCAA-3′) and *phz*Er (5′-GCCY TCGATGAAGTACTCGGTGTG-3′) (Schneemann et al. 2011). The PCR amplification was carried out in a 50-μl reactions containing 20 ng of DNA, 2× master mix, 5 % (*v*/*v*) dimethyl sulfoxide, and 0.2-μM concentrations of both primers. The PCR conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 36 cycles of denaturation at 94 °C for 30 s, primer annealing at 54.7 °C for 60 s, and primer extensions at 72 °C for 120 s, followed by a final extension at 72 °C for 420 s was performed. The amplified products were recovered and purified using Agarose Gel DNA extraction kit (CWBio gel extraction kit) following the manufacturer's instructions.

The PCR products were cloned using the TOPO TA cloning kit (TransGen) according to the manufacturer's protocol. The positive clones were selected and sequenced using the M13F primer on ABI 3730xl capillary sequencers (Applied Biosystems). Sequences were analyzed using the NCBI BLAST.

Reverse transcription PCR

Total RNA was isolated from the culture of *phz*E-positive strains, incubated at different days in 28 °C using the CWBio RNA pure plant kit. The reverse transcriptase reactions were carried out at 50 °C for 30 min using 0.2 μg of the total RNA template using CWBio HiFi-Script cDNA kit. The *phz*E gene from cDNA was amplified according to the method given above.

Fermentation and chemical analysis

Actinobacteria with the expression of *phz*E genes were inoculated into 20 mL of ISP2 medium in 250-mL Erlenmeyer flasks and incubated at 28 °C on rotary shakers (280 rpm) for 36 h. Each seed culture was aseptically transferred to 1-L Erlenmeyer flasks containing 400 mL of the GYM medium and incubated at 28 °C on rotary shakers (280 rpm) for 7 days. After fermentation, pH of the medium (20 L) was reduced to pH 4 using concentrated HCl and extracted with equal volumes of ethyl acetate for three times. The organic phase was concentrated under reduced pressure to give a crude extract. The crude extract was subjected to semipreparative RP-C18 (X Aqua-C_{18} 5 μm, 10×250 mm) HPLC (Agilent Technologies, USA) and eluted with 50 % methanol at a flow rate of 2 mL/min to afford phenazines from 13-12-13 (**1**) (tR= 30.7 min) and 13-33-15 (**1**, **2**) (tR=33.2 min; 34.7 min), respectively. Further, the fractions of F1 and F2 were repeatedly applied to semipreparative RP-C18 (Eclipse XDB-C_{18} 5 μm, 4.6×150 mm) HPLC and eluted with a linear gradient from 10 to 100 % aqueous CH_3CN over the course of 20 min and gave compounds **1** (tR 6.7 min) and **2** (tR 6.17 min).

For LC-QTOF-MS analysis, the methanol solution of compounds **1** and **2** was detected on an ultra-performance liquid and quadrupole time of flight mass spectroscopy (UPLC-QTOF-MS Premier, Waters Corporation, USA). The compounds **1** and **2** were separated on a C_{18} RP-column (ACQUITY BEH-C_{18} 1.7 μm, 2.1×100 mm, Waters Co.), with the linear gradient elution from H_2O to 100 % MeCN. Total ion chromatography (TIC) and mass spectrum of selected ion were acquired in positive electrospray ionization mass spectrum (ESI-MS) mode.

In the case of NMR analysis, the compounds **1** and **2** were dissolved in CD_3OD and $CDCl_3$, respectively. Proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) spectrum was recorded on an ADVANCE III 400 spectrometer (400 MHz, Bruker, and 100 MHz, Bruker, respectively).

Antimicrobial tests of phenazines

To test antimicrobial activity, compounds 1 and 2 were suspended in DMSO (1 μg/μL) and 20 μL of the each sample was applied to a paper disk (d=6 mm). The disks were then placed onto an agar plate inoculated with *Bacillus mycoides* SJ14, *Staphylococcus aureus* SJ51, *Escherichia coli* SJ42, *Micrococcus luteus* SJ47, *Saccharomyces cerevisiae* SJ32, and *Rhodotorula* sp. SJ24. Ampicillin (1 μg/μL) and kanamycin (1 μg/μL) were used as positive controls against the bacteria and yeast.

Nucleotide sequence accession numbers

The GenBank accession numbers obtained for 16S rRNA genes and *phz*E genes were KM886124 - KM886210 and KM923774 - KM923783, respectively. The GenBank accession numbers obtained for the 28S rRNA gene of sponges were as follows: *Theonella swinhoei* (13-2, JF506040), *Dysidea arenaria* (13-3, KJ675585), *Ircinia* sp. (13-17, KC774023), and *Iotrochota* sp. (13-36, KC762714).

Results

Diversity of culturable actinobacteria associated with sponges

Totally, 197 actinobacterial strains were isolated on a range of selective media (M1, M2, M5, Kusters, and AGA) from the

seven sponges. Highest numbers of actinobacterial isolates (16 isolates) were recovered from the M2 for *Ircinia* sp., whereas only one isolate was recovered from AGA for *Haliclona simulans*. The actinobacteria isolated from the seven sponge species were significantly different. *Ircinia* sp. yielded the highest number of isolates (61), followed by *Theonella swinhoei* (34), *Dysidea arenaria* (32), *Smenospongia aurea* (29), *Agelas sceptrum* (27), *Haliclona simulans* (15), and *Iotrochota* sp. (7) (Fig. 1).

According to the actinobacterial colony morphology and the source sponge, 87 strains were selected for 16S rRNA gene sequencing. Based on the BLAST analysis, 56 representative sequences were used to construct the phylogenetic trees. Sequence comparison with the previously submitted sequences in NCBI GenBank database revealed that these strains were affiliated with eight families and 13 genera. The highest numbers of isolates were found to be identified as genus *Salinispora*, which is a marine obligate actinobacterium (26), followed by *Streptomyces* (25), *Nocardiopsis* (7), *Serinicoccus* (6), *Nocardia* (5), *Saccharopolyspora* (4), *Kocuria* (4), *Micrococcus* (4), *Rhodococcus* (2), *Prauserella* (1), *Micromonospora* (1), *Mycobacterium* (1), and *Actinoalloteichus* (1) (Fig. 2). The sponge *Dysidea arenaria* provided the maximum of six genera, including *Streptomyces*, *Salinispora*, *Saccharopolyspora*, *Kocuria*, *Prauserella*, and *Micromonospora* in the medium M2.

Interestingly, 17 strains showed only 94–98 % sequence similarities with previously described species (Table S1), which was also supported by phylogenetic analysis (Figs. 3, 4, and 5). These strains were closely related to the genera *Nocardia*, *Salinispora*, *Streptomyces*, *Micrococcus*, and *Saccharopolyspora*. The phylogenetic relationships among the 56 actinobacterial strains are shown in Figs. 3, 4, and 5 along with their nearest NCBI (BLASTn) matches and type strains. The 19 *Streptomyces* strains were assembled into 16 clusters (Fig. 3). The isolate 13-18-23 showed 97 % similarity with *Streptomyces* sp. NCL 716 (FJ919811), which might be a new species within the genus *Streptomyces*. Similarly, *Micrococcus* sp.13-18-21 from *Ircinia* sp. also exhibited a distinct clade within *Micrococcus* (Fig. 4). Interestingly, phylogenetic analysis of the strains belonging to the family *Micromonosporaceae*, *Pseudonocardiaceae*, *Mycobacteriaceae*, and *Nocardiaceae* (Fig. 5) revealed that ten isolates, 13-18-9, 13-18-31, 13-18-20, 13-18-25, 13-18-24, 13-18-17, 13-18-8, 13-18-32, 13-18-33, and 13-3-44, belong to the genera *Salinispora*, which forms a different clade and the lowest sequence similarity, further proposing that these isolates might belong to a novel species within *Salinispora*. Similarly, the isolates *Saccharopolyspora* sp. 13-18-42 and *Nocardia* sp. 13-2-4 formed a separate clade indicating that it might be also a new species within the genera *Saccharopolyspora* and *Nocardia*, respectively.

Screening of actinobacteria with *phz*E gene

The PCR amplification of *phz*E gene fragments showed that 10 out of 87 strains were positive for the presence of phenazines. *phz*E gene fragments were detected in the strains

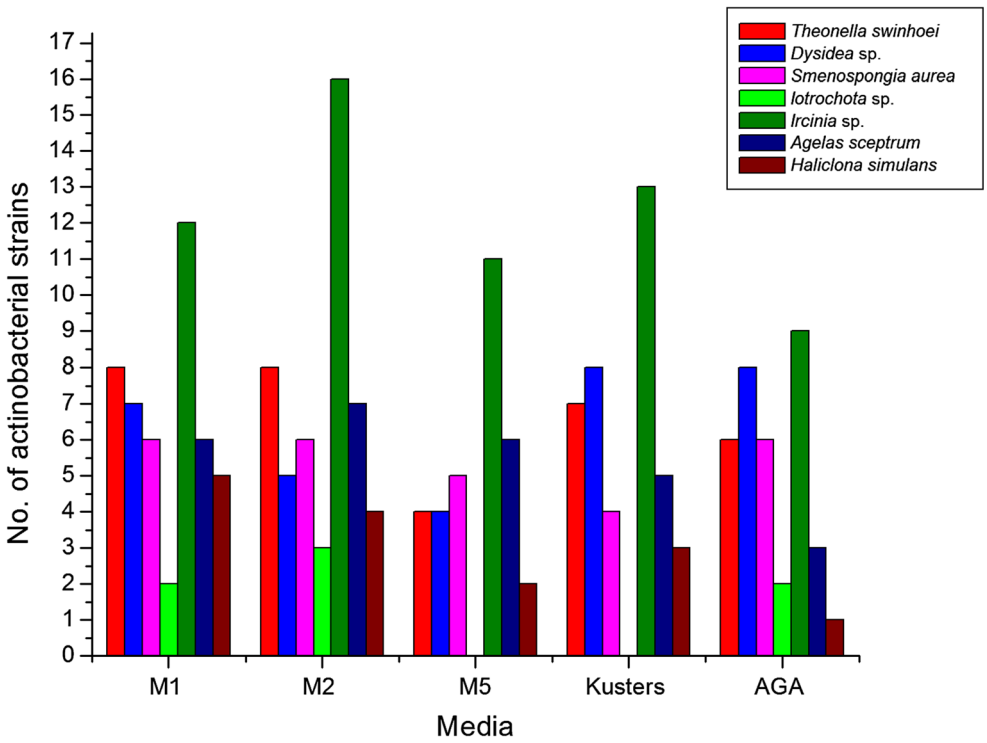

Fig. 1 Total number of actinobacterial colonies recorded on five different selective media for seven species of sponges

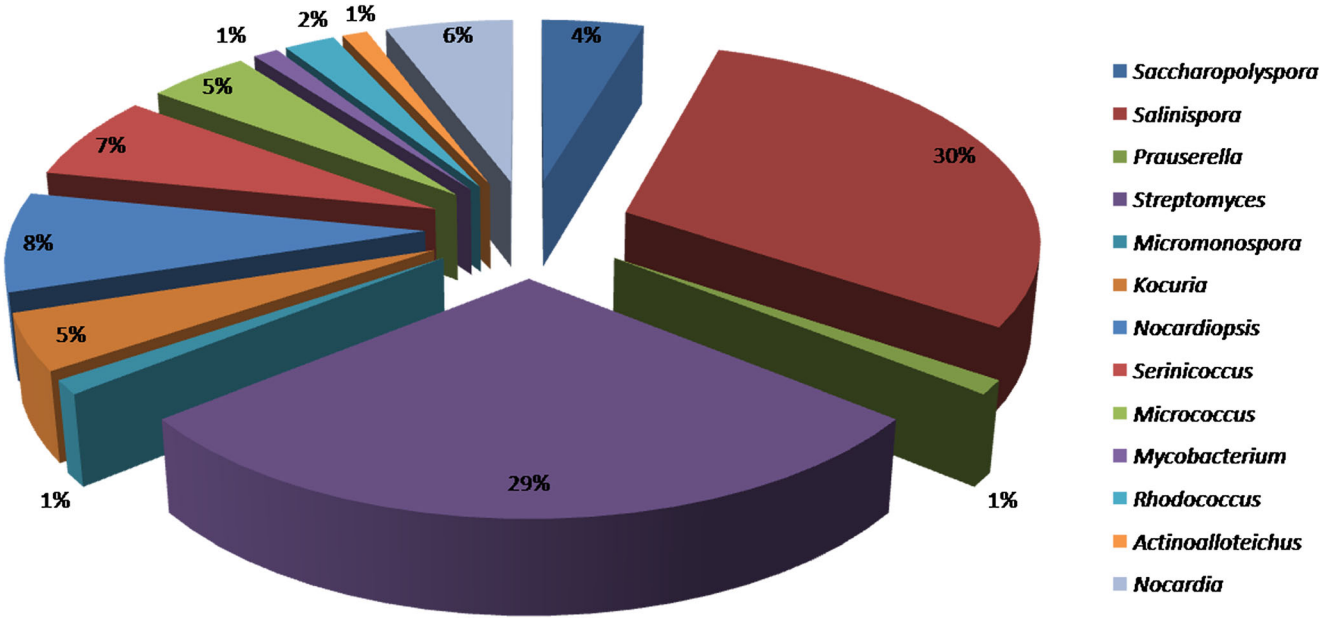

Fig. 2 Diversity of actinobacteria associated with sponges in the South China Sea

representing *Streptomyces* (5), *Nocardiopsis* (2), *Micrococcus* (1), *Salinispora* (1), and *Serinicoccus* (1). The sequence length of *phz*E gene fragments of these strains ranged from 423 to 444 bp. All these sequences showed the similarity in the range of 76 to 84 % with the phenazine gene cluster and *phz*E gene fragments related to *Pseudomonas* and *Streptomyces*. The summary of the BLAST searches of the sequence analysis is given in Table 1. Furthermore, we confirmed the expression of the *phz*E gene using reverse transcription PCR. As a result, the *phz*E gene was highly expressed in *Serinicoccus* sp. 13-12-4 (CCTCC AB 2014234), *Nocardiopsis* sp. 13-33-15 (CCTCC AA 2014034), and *Nocardiopsis* sp. 13-12-13 (CCTCC AA 2014033) on the fifth day of fermentation. Particularly, phzE gene was not expressed in strains *Salinispora* sp. 13-3-2 and *Micrococcus* sp. 13-18-21 during the incubation period, whereas the remaining five strains were poorly expressed on the fifth day of fermentation. Subsequently, their expression levels were gradiently decreased until the 12th day of fermentation (Fig. 6).

Preparation of phenazine by actinobateria with *phz*E gene expression

Based on the *phz*E gene screening, reverse transcription PCR, UV absorption, and RP-HPLC separation, phenazines were isolated successfully from the *Nocardiopsis* sp. 13-33-15 and *Nocardiopsis* sp. 13-12-13. However, phenazine was not found in the fermentation broth of *Serinicoccus* sp. 13-12-4.

1,6-Dihydroxy phenazine (**1**) (Fig. 7) was isolated from *Nocardiopsis* sp. 13-33-15 and *Nocardiopsis* sp. 13-12-13 as yellow powder. ESI-MS showed the molecular ion clusters for [M+H] in positive mode with the molecular formula $C_{12}H_9N_2O_2$ and molecular ion peak at *m/z* 213.06 (Fig. S1). Consequently, the UV spectrum showed absorption at 270 nm. The ^{1}H-NMR (400 MHz, Cd_3Od) showed δ 7.20 (2H, m, 2, 7-H) and δ 7.71~7.78 (4H, m, 3,4,8,9 - H) (Fig. S2). ^{13}C NMR (100 MHz, Cd_3Od) showed δ 111.03 (C-2,7), 119.65 (C-4,9), 131.79 (C-3,8), 136.15 (C-5a, 10a), 142.55 (C-4a, 9a), 153.85 (C-1,6) (Fig. S3).

1,6-Dimethoxy phenazine (**2**) (Fig. 7) was isolated as yellow needles from *Nocardiopsis* sp. 13-33-15. The UV absorption spectrum was observed at 270 nm. The molecular formula was identified as $C_{14}H_{12}O_2N_2$ in ESI-MS, and molecular ion peak was observed at *m/z* 241.09 for [M+H] (Fig. S4). The ^{1}H-NMR (400 MHz, $CDCl_3$) displayed the methoxy protons (δ 4.21) and aromatic protons (δ 7.13–8.03) (Fig. S5). The ^{13}C NMR (400 MHz, $CDCl_3$) displayed seven spectrums among them six are aromatic carbons (δ 107.08 (C-2,7), 122.25 (C-4, 9), 130.35 (C-3,8), 137.1 (C-5a, 10a), 143.23 (C-4a, 9a), 155.12 (C-1,6)), and one is methoxy carbon (56.7) (Fig. S6). The presence of phenazine and methoxy group was confirmed based on both molecular formula and NMR spectrum.

Antimicrobial activity of phenazines produced by actinobateria associated with sponges

The purified compounds **1**and **2** (1 μg/μL) effectively inhibited the growth of *Bacillus mycoides* SJ14, *Staphylococcus aureus* SJ51, *Escherichia coli* SJ42, and *Micrococcus luteus* SJ47 with the inhibition zone ranging from 8 to 25 mm against 14 to 26 mm caused by ampicillin, and kanamycin in the above concentrations. The antibacterial activities of compounds **1** and **2** against *Bacillus mycoides* SJ14,

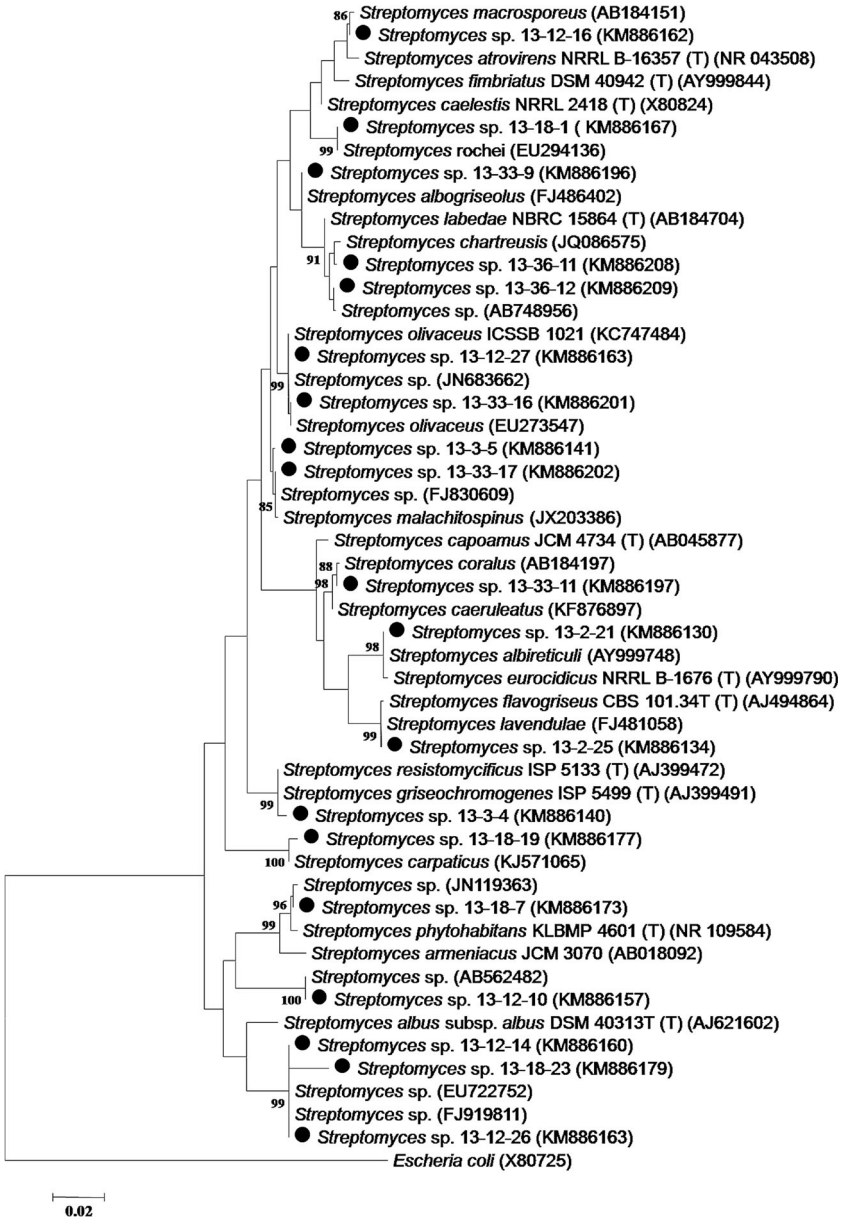

Fig. 3 Maximum likelihood phylogenetic tree of culturable *Streptomycetaceae* isolated from the South China Sea sponges and their NCBI (BLASTn) relatives based on the 16S rRNA gene sequences. Sequences obtained in this study are marked (●). Bootstrap values (1000 resamples) are given in percent at the nodes of the tree (>80). The outgroup is *Escherichia coli* ATCC 11775 T. The *scale bar* indicates 0.02 nucleotide substitution per nucleotide position

Staphylococcus aureus SJ51, and *Micrococcus luteus* SJ47 were slightly higher than the kanamycin. No inhibition zone was observed against *Saccharomyces cerevisiae* SJ32 and *Rhodotorula* sp. SJ24 (Table 2).

Discussion

To date, more than 50 sponge species have been reported to be hosts for actinobacteria. One hundred twelve genera within 39 families of actinobacteria have been detected within different sponges based on culture-independent methods, and 44 genera of the culturable actinobacteria have been isolated within the marine sponges (Valliappan et al. 2014). Relatively, the actinobacteria showed to be randomly associated with sponges (Abdelmohsen et al. 2014a, b). However, it is obvious that actinobacteria are well adapted to survive within the marine sponges (Webster and Taylor 2012). Most of them are the major producers of bioactive compounds with medicinal application (Zotchev 2012; Valliappan et al. 2014).

In this study, for the first time, actinobacteria were isolated from the sponges *Theonella swinhoei*, *Smenospongia aurea*, and *Ircinia* sp. in the South China Sea. Totally, 13 genera, including *Salinispora*, *Streptomyces*, *Nocardiopsis*, *Serinicoccus*, *Nocardia*, *Saccharopolyspora*, *Kocuria*, *Micrococcus*, *Rhodococcus*, *Prauserella*, *Micromonospora*, *Mycobacterium*, and *Actinoalloteichus* were identified to be associated with these sponges. The total numbers of actinobacterial isolates varied between different sponges, e.g., *Ircinia* sp. harbored the highest number of actinobacterial isolates, while *Dysidea arenaria* had

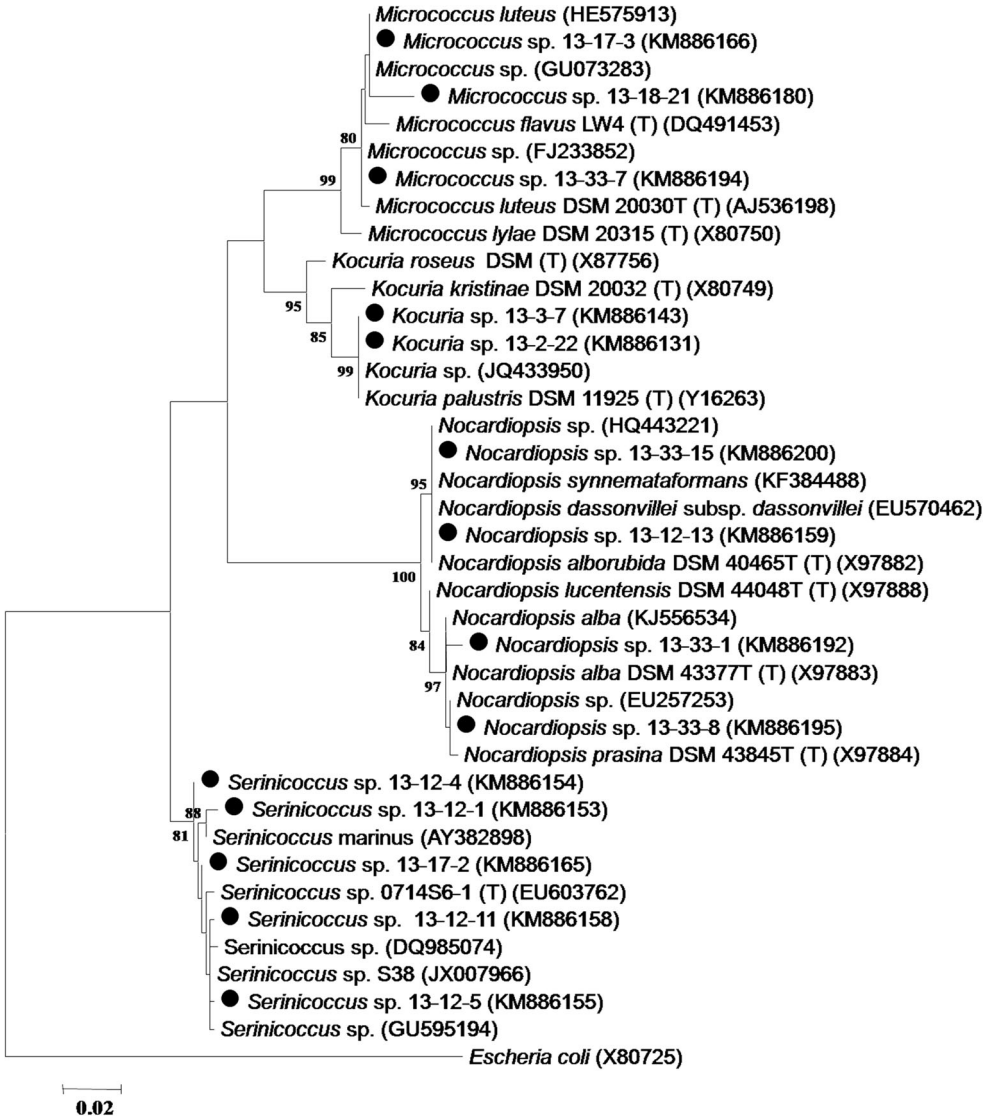

Fig. 4 Maximum likelihood phylogenetic tree of culturable *Nocardiopsaceae*, *Micrococcaceae*, and *Intrasporangiacea* isolated from the South China Sea sponges and their NCBI (BLASTn) relatives based on the 16S rRNA gene sequences. Sequences obtained in this study are marked (●). Bootstrap values (1000 resamples) are given in percent at the nodes of the tree (>80). The outgroup is *Escherichia coli* ATCC 11775 T. The *scale bar* indicates 0.02 nucleotide substitution per nucleotide position

the highest number of actinobacterial genera. Among these sponges, *Iotrochota* sp. yielded only three representative isolates and each belongs to different genera, including *Mycobacterium*, *Serinicoccus*, and *Micrococcus*, which was not previously isolated from the *Iotrochota* sp. (*Cellulosimicrobium*, *Nocardiopsis*, and *Streptomyces*) (Jiang et al. 2008). This supported the fact that the number of actinobacteria and its genera varied among sponge species (Abdelmohsen et al. 2014a, b). Some studies also proved that the similar species of sponges from distinct geographic regions had different actinobacterial assemblages (Webster and Taylor 2012). Selvin et al. (2009) reported that *Micromonospora*, *Saccharomonospora*, and *Streptomyces* were the major groups in the marine sponge *Dendrillanigra*, whereas Xi et al. (2012) reported that *Micromonospora* and *Streptomyces* were the major groups of culturable actinobacteria associated with marine sponges. Similarly, abundant *Streptomyces* (Selvin et al. 2009; Xi et al. 2012; Valliappan et al. 2014) was isolated from the seven species of sponges tested in this study. Particularly, for the first time, genus *Salinispora* was found to be dominant in the sponge *Ircinia* sp. with lowest sequence similarity, which indicates that it might be a novel genus/species within the family *Micromonosporaceae*.

Based on the investigation of culture-independent strategy, 39 different genera have been detected from sponges (Khan et al. 2014; Han et al. 2012; Jackson et al. 2012; Simister et al. 2012; Li et al. 2011; Sun et al. 2010). However, 18 actinobacterial genera, i.e., *Actinomyces*, *Aeromicrobium*, *Amycolatopsis*, *Dermacoccus*, *Dermatophilus*, *Gordonia*, *Iamia*, *Illumatobacter*, *Isoptericola*, *Leucobacter*, *Microlunatus*, *Millisia*, *Nitriliruptor*, *Nocardioides*, *Salinibacterium*, *Terrabacter*, *Tsukamurella*, and *Williamsia*, detected by the culture-independent methods have not been isolated from the marine sponges. Therefore, it is essential to optimize the isolation strategies to improve the actinobacterial cultivation from the marine sponges.

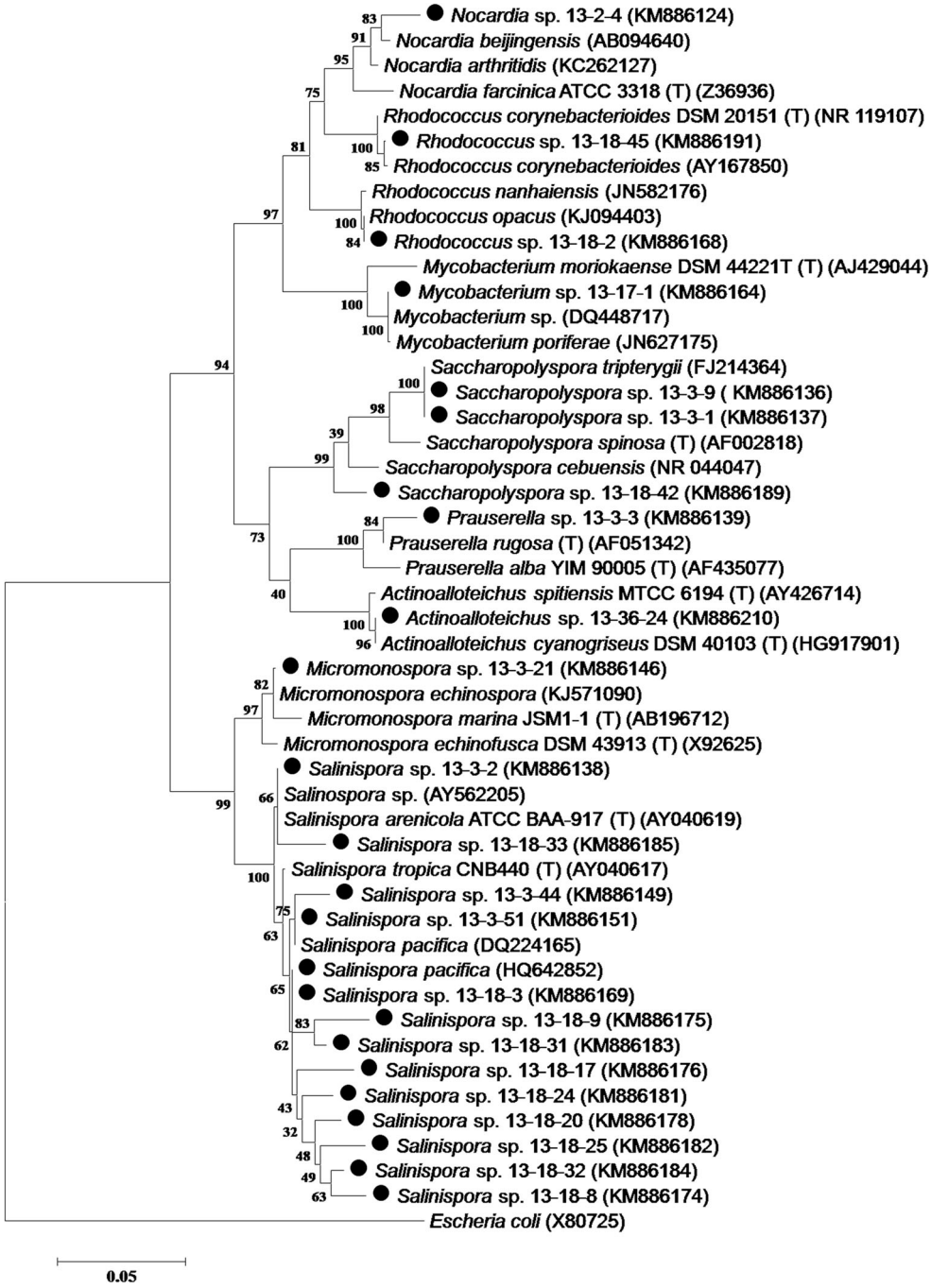

Fig. 5 Maximum likelihood phylogenetic tree of culturable *Micromonosporaceae*, *Pseudonocardiaceae*, *Mycobacteriaceae*, and *Nocardiaceae* isolated from the South China Sea sponges and their NCBI (BLASTn) relatives based on the 16S rRNA gene sequences. Sequences obtained in this study are marked (●). Bootstrap values (1000 resamples) are given in percent at the nodes of the tree (>80). The outgroup is *Escherichia coli* ATCC 11775 T. The *scale bar* indicates 0.05 nucleotide substitution per nucleotide position

In the present study, heat pretreatment and the serial dilution methods were applied successfully to isolate the actinobacteria from seven sponge samples using five different media. The intention of the heat treatment is to decrease the amount of bacteria and to enrich the actinobacteria (Jensen et al. 1991). This strategy has been used successfully to isolate rare actnobacteria (Mincer et al. 2002; Kim et al. 2005; Jensen et al. 2005; Öner et al. 2014). The results showed that media composed of rich nutrients, particularly the presence of yeast extract and peptone yield the highest number of culturable actinobacteria, which was consistent with previous results (Öner et al. 2014; Vicente et al. 2013; Abdelmohsen et al. 2014b). The isolation and consequent molecular identification of actinobacteria illustrated that the isolation rate and diversity of actinobacteria varied on different media. Therefore, the usages of different media are required to attain the maximum isolation of sponge-associated actinobacteria. Several rare and phylogenitically distinct actinobacteria were isolated in this study, e.g., *Salinispora*, *Serinicoccus*, *Nocardia*, *Kocuria*, *Prauserella*, and *Actinoalloteichus*. Meanwhile, strain 13-3-

Table 1 Phenazine biosynthesis genes and sequence similarity analysis based on BLAST

Strain no.	Accession no.	Related phenazine gene	Related producer	Closest accession no.	Gene similarity (%)
Micrococcus sp. 13-18-21	KM923774	Putative phenazine gene cluster	*Streptomyces* sp. SpC080624SC-11	KF808339	81
Nocardiopsis sp. 13-12-13	KM923778	Streptonigrin biosynthetic gene	*Streptomyces flocculus* strain CGMCC 4.1223	JQ414024	76
Nocardiopsis sp. 13-33-15	KM923783	SgpC (sgpC) gene, partial cds; SgpD (sgpD) gene, complete cds; and SgpE	*Streptomyces griseoluteus* strain P510	HM363127	81
Salinispora sp. 13-3-2	KM923776	SgpC (sgpC) gene, partial cds; SgpD (sgpD) gene, complete cds; and SgpE	*Streptomyces griseoluteus* strain P510	HM363127	81
Serinicoccus sp. 13-12-4	KM923779	Putative 2-amino-2-desoxy-isochorismate synthase (*phz*E) gene	*Streptomyces* sp. LB114	HM460710	79
Streptomyces sp. 13-3-4	KM923775	Pyocyanine biosynthesis operon	*Pseudomonas aeruginosa*	AF005404	76
Streptomyces sp. 13-12-10	KM923777	Pyocyanine biosynthesis operon	*Pseudomonas aeruginosa*	AF005404	84
Streptomyces sp. 13-2-25	KM923780	Anthranilate synthase (*phz*E) gene	*Streptomyces lomondensis* strain S015	KF144612	79
Streptomyces sp. 13-2-21	KM923781	Putative 2-amino-2-desoxy-isochorismate synthase (*phz*E) gene	*Streptomyces* sp. LB129	HM460711	85
Streptomyces sp. 13-33-9	KM923782	Furanonaphthoquinone I operon (fnq) and phenazine operon (ephz)	*Streptomyces cinnamonensis* DSM 1042	AM384985	84

3 assigned to the genus *Prauserella* was isolated first time from the marine sponges, which was more closely related to the type strain of *Prauserella aidingensis* YIM 90636 isolated from the salt lake in Yunnan Province, China (Li et al. 2009). Even though, the new approaches of isolation such as encapsulation of cells in gel microdroplets (Zengler et al. 2002) or the employment of diffusion chambers (Lewis et al. 2010), microbial traps (Sizova et al. 2012), and isolation chips (Pahlow et al. 2013) can be used in the future to isolate the uncultivable actinobacteria from the marine sponges.

Mavrodi et al. (2010) designed four different pairs of primers targeting *phz*F gene to study the phenazine- producing bacteria from the plant and soil. Moreover, Schneemann et al. (2011) designed the universal primer targeting *phz*E gene to investigate the different groups of bacteria, including actinobacteria and *Pseudomonas* without any prior information on phylogenetic classification. Schneemann et al. (2011) identified 22 known phenazines from the actinobacteria based on the *phz*E gene detection. Hence, the results suggested that actinobacteria with *phz*E genes were possible to produce phenazines. In this study, the PCR screening of 87 marine sponge-associated actinobacteria revealed that the *phz*E genes were distributed unevenly among different taxa. The detection of *phz*E gene in the genera *Micrococcus* and *Serinicoccus* recommended that these less studied genera signify an unexplored source for natural products. Even though the actinobacteria contain *phz*E gene, it might not have the ability to produce phenazine in the laboratory conditions. Hence, the expressions of these genes using reverse transcription PCR are required to identify the highly active phenazine-producing strains.

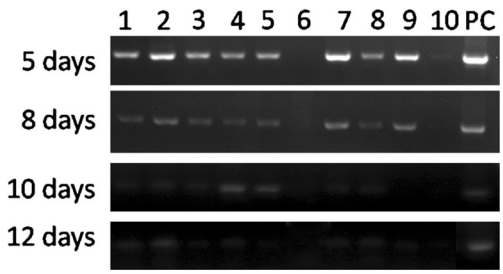

Fig. 6 Expression of the *phz*E gene on different days of fermentation. (**1**) *Streptomyces* sp. 13-33-9, (**2**) *Nocardiopsis* sp. 13-33-15, (**3**) *Streptomyces* sp. 13-2-21, (**4**) *Streptomyces* sp. 13-2-25, (**5**) *Streptomyces* sp. 13-3-4,(**6**) *Salinispora* sp. 13-3-2, (**7**) *Serinicoccus* sp. 13-12-4, (**8**) *Serinicoccus* sp. 13-12-10, (**9**) *Nocardiopsis* sp. 13-12-13, (**10**) *Micrococcus* sp. 13-18-21, and (**PC**) DNA of *Nocardiopsis* sp. 13-12-13

The lowest sequence similarity with previously published phenazine biosynthetic genes revealed that the production of

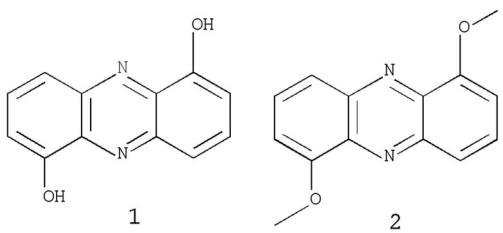

Fig. 7 Structures of compounds 1 and 2

Table 2 Antibacterial activities of phenazines isolated from *Nocardiopsis* sp. 13-12-13 and *Nocardiopsis* sp. 13-33-15

Test organisms	1,6-Dihydroxy phenazine (1)	1,6-Dimethoxy phenzine (2)	Ampicillin	Kanamycin
Bacillus mycoides SJ14	16±0.5	20±0.4	25±0.5	15±0.5
Staphylococcus aureus SJ51	25±0.6	21±0.1	26±0.2	18±0.7
Escherichia coli SJ42	8±0.4	10±0.6	16±0.6	14±0.4
Micrococcus luteus SJ47	18±0.9	23±0.5	25±0.8	15±0.5
Saccharomyces cerevisiae SJ32	0	0	9±0.3	4±0.7
Rhodotorula sp. SJ24	0	0	7±0.4	3±0.3

the compounds using this gene sequence might not be accurately predicted, and subsequently, it should be required to be confirmed by chemical analysis. Even though the genetic screening at both DNA and RNA levels of the phenazine is easy to detect the phenazine-producing actinobacteria, it is difficult to isolate phenazines from the fermentation broth e.g. *Serinicoccus* sp. 13-12-4 due to the difficulty in the detection of phenazine by UV detector and purifying the correct fractions using RP-HPLC. Further, the isolation and purification of phenazine in pure state is most important and time-consuming. Hence, the application of new discriminatory methods from extraction to purification is required to reduce the time and enhance the level of purity for identification.

Using the strategy above, three strains, i.e., *Nocardiopsis* sp. 13-33-15, *Nocardiopsis* sp. 13-12-13, and *Serinicoccus* sp. 13-12-4, were found to be having *phz*E gene activity. Consequently, two compounds 1,6-dihydroxy phenazine and 1, 6-dimethoxy phenazine were successfully obtained from *Nocardiopsis* sp. 13-33-15 and *Nocardiopsis* sp. 13-12-13. It might be considered that 1,6-dihydroxy phenazine was originated from two decarboxylative hydroxylations of phenazine-1,6-dicarboxylic catalyzed by *Mpz*9, which was indicated by the gene cluster of the strain *Streptomyces* sp. SpC080624SC-11 (Zeyhle et al. 2014) and the analogue to the *Phz*S of *Pseudomonas aeruginosa* (Greenhagen et al. 2008). Interestingly, 1,6-dihydroxy phenazine was identified in both *Nocardiopsis* sp. 13-33-15 and 13-12-13, indicating that 1,6-dihydroxy phenazine might act as the substrate for the synthesis of 1,6-dimethoxy phenazine in *Nocardiopsis* sp. 13-33-15 and also for other phenazines in *Nocardiopsis* sp. 13-12-13. In the future, genome-based approach will be helpful to identify the putative gene cluster and the biosynthetic pathway of these compounds (Heine et al. 2014; Zeyhle et al. 2014).

Streptomyces genome sequencing revealed that every strain has several genes to produce >20 possible secondary metabolites (Bentley et al. 2002; Ikeda et al. 2003; Ohnishi et al. 2008). However, only a part of them are expressed during fermentation. Likewise, phenazine biosynthetic gene cluster discovered from the *Streptomyces tendae* Tu1028 was inactive and it was activated by introducing a constitutive promoter in the upstream of the phenazine biosynthetic genes, which led to the synthesis of phenazine-1-carboxylic acid (PCA) and a new derivative of phenazine (Saleh et al. 2012). The results of *phz*E gene expression analysis revealed that the strains from *Salinispora* and *Micrococcus* were not active, while the strains *Streptomyces* sp. 13-3-4, *Streptomyces* sp. 13-12-10, *Streptomyces* sp. 13-2-25, *Streptomyces* sp. 13-2-21, and *Streptomyces* sp. 13-33-9 were weekly expressed in the fermentation medium. It indicated that these isolates were inactive or less active to produce phenazines in the fermentation medium. In theory, the related pathway could be activated by the suitable fermentation condition to produce phenazine. For example, the methods such as rifampcin resistance (rpoB) mutations, ribosome engineering, *dasR–N*-acetylglucosamine system, LAL regulatory system, metabolic engineering, and cell-to-cell interactions might be used to induce or enhance the expression of phenazine biosynthetic genes (Ochi and Hosaka 2013; Hosaka et al. 2009; van Wezel and McDowall 2011; Craney et al. 2012; Onaka et al. 2011).

In conclusion, seven species of South China Sea sponges were found to host abundant and diverse culturable actinobacteria. Comparatively, different sponges had considerably different actinobacterial species. Totally, 13 different genera and 17 putatively novel isolates were isolated from these sponges. To our knowledge, this study is the first report on the isolation of *Prauserella* from the marine sponges. The genetic screening of *phz*E gene revealed that ten actinobacterial strains have the potential to produce phenazines. Further, the gene expression at RNA level and chemical analysis showed that only two strains have the ability to produce phenazine in the fermentation medium. Finally, 1,6-dihydroxy phenazine and 1,6-dimethoxy phenazine were purified from *Nocardiopsis* sp. 13-33-15 and *Nocardiopsis* sp. 13-12-13. The antimicrobial test proved that these two phenazines were active against bacteria. These results suggested that the integrated approach of gene screening at both DNA and RNA levels, and chemical analysis is a valuable approach to guide the preparation of target compounds, and highlighted the potential of South China Sea sponges as a resource of novel actinobacteria for marine drugs development.

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