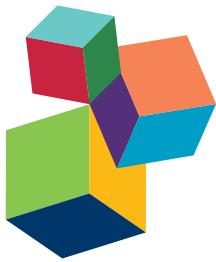


THE GLOBAL CHALLENGE POSED BY THE MULTIRESISTANT INTERNATIONAL CLONES OF BACTERIAL PATHOGENS

EDITED BY: Miklos Fuzi and Margaret Ip

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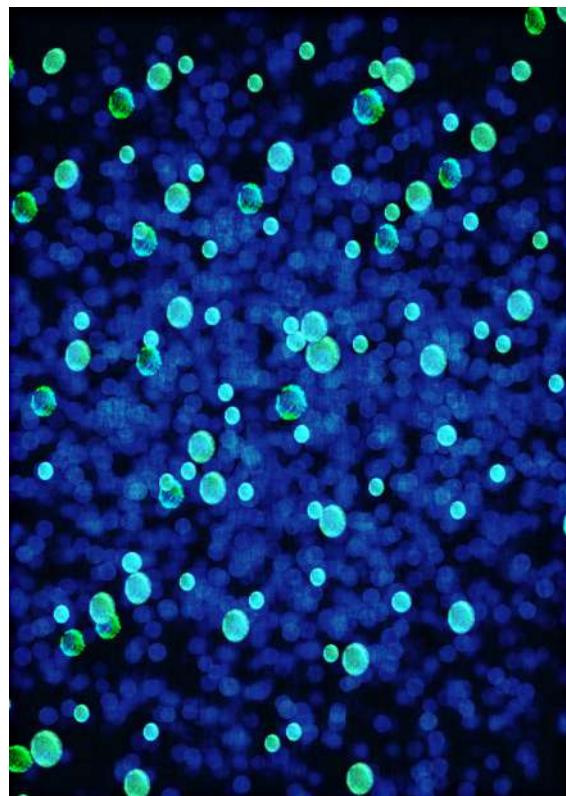
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THE GLOBAL CHALLENGE POSED BY THE MULTIRESISTANT INTERNATIONAL CLONES OF BACTERIAL PATHOGENS

Topic Editors:

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Various species of bacteria

Image by Gerd Altmann, available at Pixabay, <https://pixabay.com/hu/bakt%C3%A9riumok-bakt%C3%A9riumfajok-ut%C3%A1nz%C3%A1ls-808751/>

Multiresistant bacterial pathogens pose a serious problem worldwide making the appropriate treatment of patients with healthcare-associated infections a challenge. The spread of antibiotic resistance is either mediated by mobile genetic elements (MGEs) or the dissemination of genetically-related groups of pathogens, “high-risk clonal complexes”.

Interestingly most multiresistant healthcare-associated bacteria command just a few dominant international clonal complexes causing infections in various geographical areas. It is of utmost importance to identify the determinants associated with and promoting the spread of antibiotic resistance and the dissemination of these multiresistant pathogens. The Topic comprises mostly of population and epidemiological studies investigating antibiotic resistance mechanisms, MGEs and the impact of antibiotic resistance, and the production of virulence factors on the clonal dynamics of a diverse range of bacterial species.

Though, the exploration of the mechanisms governing clonal dynamics and the dissemination of antibiotic resistance will remain a salient issue for a considerable time to come we believe that the papers published in the Topic have usefully contributed to the better understanding of some of the processes involved and supplement papers investigating the “non-bacterial” constituents of clonal mobility, like proper medical practice and compliance with hygienic standards.

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Editorial: The Global Challenge Posed by the Multiresistant International Clones of Bacterial Pathogens

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Keywords: multidrug-resistant pathogen, clone, dissemination, fitness, virulence factor

Editorial on the Research Topic

The Global Challenge Posed by the Multiresistant International Clones of Bacterial Pathogens

Multiresistant bacterial pathogens pose a serious problem worldwide making the appropriate treatment of patients with healthcare-associated infections a challenge (www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf).

The spread of antibiotic resistance is either mediated by mobile genetic elements (MGEs) or the dissemination of genetically-related groups of pathogens, “high-risk clonal complexes” (Cantón et al., 2003; Willems et al., 2011; Woodford et al., 2011; Baquero et al., 2015).

Interestingly most multiresistant healthcare-associated bacteria command just a few dominant international clonal complexes causing infections in various geographical areas (Woodford et al., 2011; Willems et al., 2011).

All articles in this Frontiers special issue investigate bacterial species included in the commination list of the Center for Disease Control as pathogens constituting a serious current antibiotic resistance threat (www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf).

It is of utmost importance to identify the determinants associated with and promoting the spread of antibiotic resistance and the dissemination of these multiresistant pathogens. The Topic comprises mostly of population and epidemiological studies investigating antibiotic resistance mechanisms, MGEs and the impact of antibiotic resistance, and the production of virulence factors on the clonal dynamics of a diverse range of bacterial species.

Some papers investigate the production of carbapenemases in *Enterobacteriaceae*.

Lee et al. review the available data on the global spread of KPC-, NDM-, and OXA-48 type carbapenemase-producing *Klebsiella pneumoniae* showing also how major clones disseminated on various continents. The gravity of the situation is emphasized and the remaining therapeutical options explored.

Wang et al. report on the dissemination of the NDM-1 gene in gram-negative pathogens and Sarkar et al. characterized a plasmid carrying *bla*_{NDM-1} from *Salmonella enterica* Serovar *Senftenberg* a potential vehicle for the spread of the carbapenemase gene.

A number of papers explore clonal dynamics and resistance determinants in non-fermenting bacteria.

Chang et al. showed how carbapenem resistant strains of *Acinetobacter baumannii* clonal complex 92 (international clone II) producing OXA-23 type carbapenemase spread in China at the expense of local isolates.

Farshadzadeh et al. analyzed the clonality of *A. baumannii* strains from burn patients in Iran and genetically characterized the isolates.

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Lean et al. compared the genomes of two carbapenem resistant ST195 *A. baumannii* strains showing different susceptibility profiles to polymyxin.

Bahador et al. characterized a novel OXA-23-like gene in *A. baumannii*.

Liu et al. demonstrated that genetic changes and subsequent higher level of expression of OXA-23 β -lactamase resulted in high-level resistance to some β -lactam antibiotics in a strain of international clone II (IC II) *A. baumannii*.

Wong et al. modified the Hodge-test to more reliably detect carbapenemase production in *Pseudomonas aeruginosa* and *A. baumannii*.

Kittinger et al. report on the antibiotic resistance of *Pseudomonas* spp. isolated from various reaches of the river Danube and demonstrate the persistence of a high resistance rate for meropenem.

One paper investigates clonality in *Campylobacter*. Klein-Jöbstl et al. provide data on the clonal affiliation and antibiotic resistance of *Campylobacter jejuni* isolates from Austrian calves.

Some papers report novel findings on the clonality and related resistance mechanisms in *S. aureus*.

Abdulgader et al. provided the first comprehensive review of the clonal division of methicillin-resistant *S. aureus* (MRSA) in Africa.

Shittu et al. report relevant data on the clonal distribution of MRSA in Nigeria. In addition significant associations between various toxin genes and individual clonal complexes are demonstrated.

Sassmannshausen et al. studied the risk factors associated with the carriage of MRSA in healthcare workers in the German part of the Dutch-German EUREGIO and determined the clonal division of the isolates.

Furi et al. investigated how resistance to triclosan develops in *Staphylococci*. Authors elucidated the mechanism of integration for IS1272 which is novel and includes targeting DNA secondary structures. The mechanism and that mediated by related transposons allow for both the clonal and horizontal dissemination of triclosan resistance in *Staphylococci* because it is associated with low fitness cost (Oggioni et al., 2012).

Casarez-Dominguez et al. made a valuable addition to an area still under intense scrutiny: how sub-inhibitory concentration of vancomycin influences the expression of various virulence factors in *S. aureus*.

Two papers investigate the clonal dynamics and related antibiotic resistance mechanisms in *Enterococci*.

Guzman Prieto et al. provide an excellent review of the available data on the emergence of the human clones of *E. faecalis* and *E. faecium* emphasizing the differences between the two species.

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Novais et al. used comparative genomics to demonstrate the extensive horizontal dissemination of the PBP5C-type genes conferring reduced susceptibility to ampicillin in *E. faecium* in various sequence types. The work also highlights the co-diversification of PBP5 with the species reflecting the ancestral presence in this background.

Two additional papers investigate relevant antibiotic resistance mechanisms related to clonality in other gram-positive species.

Huang et al. present the first comparative genomic analysis of the conjugative and integrative elements family ICESa2603 in *Streptococci*. The elements are capable of transmitting antibiotic resistance genes to various species and lineages of *Streptococci* since their acquisition does not seem to be associated with any fitness cost.

Kroeger et al. genetically characterized the BC3310 efflux pump—a member of the “unknown major facilitator family 2” (UMF 2)—in *B. cereus*.

The robust impact exerted by fluoroquinolone resistance on the clonal dynamics of various pathogens is explored by two papers.

Chattaway et al. report on the dissemination of fluoroquinolone-resistant non-typhoidal *Salmonella*, *E. coli*, and *Vibrio cholerae* lineages in Sub-Saharan Africa.

Fuzi demonstrates—referring to data published earlier by his group and by other laboratories—that diverse fitness cost associated with resistance to fluoroquinolones could have substantially contributed to the selection of the major international clones of MRSA, ESBL-producing *K. pneumoniae*, ESBL-producing *E. coli*, and *Clostridium difficile*.

Though, the exploration of the mechanisms governing clonal dynamics and the dissemination of antibiotic resistance will remain a salient issue for a considerable time to come. We believe that the papers published in the Topic have usefully contributed to the better understanding of some of the processes involved and supplement papers investigating the “non-bacterial” constituents of clonal mobility, like proper medical practice and compliance with hygienic standards.

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The author confirms being the sole contributor of this work and approved it for publication.

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Cantón, R., Coque, T. M., and Baquero, F. (2003). Multi-resistant Gram-negative bacilli: from epidemics to endemics. *Curr. Opin. Infect. Dis.* 16, 315–325. doi: 10.1097/00132980-200310000-00013

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Multilocus Sequence Typing and Antimicrobial Resistance of *Campylobacter jejuni* Isolated from Dairy Calves in Austria

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Human campylobacteriosis is primarily associated with poultry but also cattle. In this study, 55 *Campylobacter jejuni* strains isolated from 382 dairy calves' feces were differentiated by multilocus sequence typing and tested for antimicrobial resistance. The most prevalent sequence type (ST) was ST883 (20.0%), followed by ST48 (14.5%), and ST50 (9.1%). In contrast to ST48 and ST50, ST883 has rarely been described in cattle previously. Furthermore, risk factor analysis was performed for the presence of the most prevalent STs in these calves. Multiple regression analysis revealed that the type of farm (organic vs. conventional) and calf housing (place, and individual vs. group) were identified as significantly ($p < 0.05$) associated with the presence of ST883 in calves, and ST50 was associated with calf diarrhea. Antimicrobial resistance was detected in 58.2% of the isolates. Most of the resistant isolates (81.3%) were resistant to more than one antimicrobial. Most frequently, resistance to ciprofloxacin (49.1%), followed by nalidixic acid (42.8%), and tetracycline (14.5%) was observed. The results of the present study support the hypothesis that dairy calves may serve as a potential reservoir for *C. jejuni* and pose a risk for transmission, including antimicrobial resistant isolates to the environment and to humans.

Keywords: *Campylobacter jejuni*, dairy calf, MLST, antimicrobial resistance

INTRODUCTION

Campylobacter jejuni is one of the most common causes of bacterial gastro-enteritis in humans and is of significant public health concern worldwide. Human campylobacteriosis is primarily associated with poultry, followed by cattle (French et al., 2009; de Haan et al., 2010b; Mughini Gras et al., 2012). Main risk factors are consumption of contaminated food, particularly poultry meat, raw milk, and water, as well as close contact to animals (Schildt et al., 2006; Heuvelink et al., 2009; Mughini Gras et al., 2012).

To distinguish between different *Campylobacter* strains, various methods have been applied, whereby multilocus sequence typing (MLST) has been identified as one of the best methods for application in epidemiological studies (Dingle et al., 2001; Korczak et al., 2009). The most

commonly identified *C. jejuni* clonal complexes (CC) in bovines are CC21 and CC61. Sequence types (ST) of CC21 are not only typical for bovines, but also for other ruminants (sheep), poultry, and humans. In contrast, CC61 STs have been described as cattle associated (Kärenlampi et al., 2007; French et al., 2009; Grove-White et al., 2010; Bianchini et al., 2014), but are also frequently identified in humans, but not in poultry, suggesting that cattle may be an important source of human infection by contamination of food and water (French et al., 2009; Grove-White et al., 2010; Bianchini et al., 2014). Furthermore, CC42, CC45, CC48, and CC403 are frequently detected in cattle. Most of these CCs (CC42, CC45, and CC48) are also frequently identified in humans (French et al., 2009). These findings underline the importance of cattle in the epidemiology of human campylobacteriosis.

Campylobacter has been classified by the European Union as a zoonotic pathogen to be screened for antimicrobial resistance (Council directive, 2003/99/EC). However, this screening is limited to chicken and turkey isolates and does not include isolates from cattle. Key reasons for this are missing extensive European wide information on the risk of cattle isolates for human disease and low rates in antimicrobial resistance reported for cattle isolates (Aarestrup et al., 1997).

The aim of the present study was to evaluate different genotypes of *C. jejuni* in feces of preweaned calves in Austrian dairy herds by use of MLST and to investigate their antimicrobial resistance.

MATERIALS AND METHODS

Samples

Fecal samples were collected from preweaned calves on 100 dairy farms in two Austrian regions (Lower Austria and Styria) during 2009 and 2010. This study was part of a study designed to examine differences between calves and farms with and without diarrhea (Klein et al., 2013; Klein-Jöbstl et al., 2014). For selection of farms, local veterinarians were asked to provide lists with dairy farms with a documented problem of calf diarrhea during the last year. A farm with diarrhea problems was defined as a farm with multiple treatments for calf diarrhea by the veterinarian. Out of these lists, farms were randomly chosen. Additionally, farms from the same geographical region and of similar structure, with no history of calf diarrhea problems and no diarrheic calf at the time of sampling, were examined. In herds with five or less preweaned calves (which was the case on 62 of all farms), all calves were tested. In herds with more than five preweaned calves, five animals were randomly chosen. On the assumption of an inter-herd prevalence of over 40% (Ellis-Iversen et al., 2009) five samples were required from each herd to detect one positive calf with 95% confidence (calculation by use of Win Episcope 2.0¹). Samples were taken directly from the rectum. Feces were placed in sterile plastic tubes and transported to the laboratory in coolers. Farm management characteristics were evaluated by a face to face interview by use of a questionnaire during

the farm visit (Table 1; Klein et al., 2013). All sampled calves were examined clinically according to the clinical examination of ruminants (Radostits et al., 2007). Feces was evaluated as described by Larson et al. (1977), where score 3 and 4 were categorized as diarrheic. Furthermore, the calf rearing areas were inspected and hygiene was estimated by evaluation of calf housings (bedding and pen walls) and the calves themselves according to Lundborg et al. (2005).

Results regarding risk factors for the presence of *C. jejuni* in calves were published elsewhere (Klein et al., 2013).

This study was discussed and approved by the institutional ethics committee of the University of Veterinary Medicine Vienna in accordance with Good Scientific Practice and national legislation.

Laboratory Procedures

All fecal samples were processed within one day, held chilled until processing. Samples were prepared for detection and isolation of thermophilic *Campylobacter* according to standards described by ISO-10272-(2002). Briefly, after enrichment in Bolton Broth (Oxoid, Basingstoke, England) at 42°C for 48 h under microaerophilic conditions (10% CO₂, 5% O₂, and 85% N₂), the samples were plated on two selective agars, modified CCDA (charcoal cefoperazone deoxycholate; Oxoid, Basingstoke, England) and CampyFoodAgar (Bio Merieux, Marcy l'Etoile, France) and incubated at 42°C for 48 h under microaerophilic conditions. Additionally, all fecal samples were directly streaked onto the two selective agars without prior enrichment. One morphological typical colony per sample was differentiated by aerobic incubation, PCR (Linton et al., 1997) and 16S-rRNA-gene sequencing on selected strains.

MLST

The MLST analysis was carried out as described by Dingle et al. (2001). Genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands). The seven housekeeping

TABLE 1 | Variables surveyed on farm.

Area of interest	Variable
Farm characteristics	Region; production (organic vs. conventional); number of cattle and cows on farm; other farm animals than cattle on farm; if yes, which other farm animals, contact to other farm animals; workers on farm; other animals (companion animals) with access to the cows' and calves' stable; water source
Housing	Housing of cows; pasture; calving area; calf housing (location, type, bedding)
Calf feeding	Colostrum management; milk feeding; feeding of hay, and concentrates; water
Hygiene	Cleaning and disinfection of different areas and barns; feed hygiene; cleaning of feeding equipment; milking hygiene
Miscellaneous	Dry off regime; dry period length
Individually sampled calves	Age (days); housing; feeding; diseases; treatments; treatment with antimicrobials; feeding of non-saleable milk

¹<http://www.winepi.net/uk/index.html>

loci defined by Dingle et al. (2001) as are *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA* were amplified using primers and protocols as described (Dingle et al., 2001). Sequencing was carried out by BigDye Terminator v3.1 cycle sequencing kit and an Applied Biosystems 310 ABI Prism genetic analyser. Sequence data were analyzed for MLST Types using the Campylobacter Multi Locus Sequence Typing website² developed by Jolley and Maiden (2010) and funded by the Wellcome Trust.

Antimicrobial Resistance Testing

Antimicrobial resistance was determined using CLSI M45-A³ for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Isolates were tested using disk susceptibility and the minimal inhibitory concentration was determined by antimicrobial dilution against ampicillin, amoxicillin/clavulanate, chloramphenicol, ciprofloxacin, colistin, erythromycin, gentamicin, nalidixic acid, neomycin, streptomycin, and tetracycline. As clinical breakpoints for *C. jejuni* are documented only for ciprofloxacin, erythromycin, and tetracycline (EUCAST) epidemiological cut-off-values, which have been determined by the European Committee on Antimicrobial Susceptibility Testing⁴, were applied on all *C. jejuni* isolates. For colistin and neomycin, no epidemiological cut-off-values have been determined. For these two antimicrobial substances cut-off were evaluated by comparing against values given in the literature and according to the distribution of our isolates were 16 mg/l for colistin and 4 mg/l for neomycin (El-Adawy et al., 2012; Ghimire et al., 2014).

Detection of *gyrA* Mutations

In all quinolone resistant isolates the quinolone resistance-determining region (QRDR) of the *gyrA* gene a 220Bp PCR product was amplified with primers GyrA-for 5'-gctatgcaaaatgtgaggc-3' and GyrA-rev 5'-cagttataacgcattcg cagcgg-3' to detect the responsible point mutation at codon 86. Genomic DNA used for amplification was extracted as described above (MLST).

Statistical Analysis

Data were statistically analyzed using PASW, version 20.0 (IBM Cooperation, New York, NY, USA).

The presence of each MLST ST was summarized in a binary variable. The presence of each ST was given as an individual variable, where presence of the given ST was categorized as one and not present as zero. Similarly, resistances against antimicrobials were categorized as either resistant (=1) or not resistant (=0). Different STs, resistances as well as farm characteristics or management factors were compared with the most prevalent STs and resistances against antimicrobials. Depending on the independent variable either Fisher's exact test, Chi square test, binary logistic regression, or *t*-test was calculated. Correlation between *C. jejuni* MLST-types and resistance against

antimicrobials were tested by Spearman correlation coefficient. The level of significance was set at a *p*-value of <0.05.

The presence of the most prevalent STs was associated with farm characteristics and management in a two-step process. First, the presence of the ST was compared to the different independent variables as described above by either Fisher's exact test, Chi square test, binary logistic regression or *t*-test. All variables were tested for correlation among each other by Spearman correlation coefficient before entering the model. If a correlation between two variables >0.60 was given, one of the covariates was discarded taking biological plausibility into account. In a second step, variables with a *p*-value ≤0.20 were entered in a multiple logistic regression model. Confounding was monitored by the change in the coefficient of a variable after removing another variable (Dohoo et al., 2009). If the change of the estimates was ≥25% the removed variable was considered to have a potential confounding effect and was consequently forced into the model. Model fit was evaluated with the Hosmer–Lemeshow test for 10 groups.

RESULTS

In total, 382 calves were sampled on 100 farms. Mean herd size was 40 ± 29 dairy cows (varying between 5 and 223 cows). Mean herd size did not differ between *C. jejuni* positive and negative farms (*p* = 0.67).

The median age of the sampled animals was 17 days [25 and 75% interquartile range (IR) 10–28]. *C. jejuni* positive calves were as young as 3 days and up to 67 days (median 18, IR 11–36). From 382 fecal samples, 55 (14.4%) were positive for *C. jejuni*. Another four samples (1.0%) were positive for *C. coli*. On farm level, on 30 of the 100 farms at least one animal shed *C. jejuni*, whereas only on five of these 30 farms all sampled animals were positive.

MLST of *C. jejuni* Isolates from Calves

The 55 *C. jejuni* isolates yielded 19 STs of which two were previously unreported. The isolates were assigned to eight clonal complexes (CC), dominated by three CCs (CC21, CC48, and CC206) that accounted for 74.5%. Half of the isolates (50.9%) belonged to CC21. The most prevalent STs were ST883 (20.0%), followed by ST48 (14.5%), and ST50 (9.1%; Table 2).

On 24 of the 30 *C. jejuni* positive farms (80%) one ST was present. On five farms two and on one farm three different types were isolated.

MLST Types and Risk Factors

The presence of *C. jejuni* ST883 in calves compared to the presence of other STs in calves was significantly associated with season, the presence of calf diarrhea on farm, the type of farm (organic vs. conventional), workers on farm, the feeding of waste milk, the separation of the calf from its dam after birth, calf feeding, and calf housing (individual versus group, and within cows' barn versus outside the barn). These variables were entered in the multiple logistic regression model. As farm had a confounding effect, this variable was forced into the model. In the final model, the type of farm and calf housing pointed out to be significantly associated with the presence of ST883 in calves

²<http://pubmlst.org/campylobacter/>

³www.clsi.org

⁴www.eucast.org

TABLE 2 | *Campylobacter jejuni* MLST types among 55 isolates from prewean dairy calves.

CC	ST	N positive samples	N positive farms
21	21	4	4
	47	1	1
	50	5	4
	864	4	2
	883	11	6
	1943	3	1
22	22	1	1
	2497	3	2
42	42	2	1
	2580	1	1
45	45	1	1
48	48	8	6
206	122	2	1
	572	2	1
	6021	1	1
353	356	1	1
354	4899	3	2
	Unknown	2	2

(Table 3). On conventional farms, the risk for calves to shed ST883 was lower compared with organic farms. Housing calves in groups inside the cows' barn was identified as a risk for shedding ST883.

No significant associations were found with regard to the presence of ST48.

The presence of ST50 strains compared to other STs were associated with farm, type of farm (organic vs. conventional), farm size (number of cows on farm), the presence of poultry on farm, diarrhea, calf feeding, and antibiotic treatment in the calf. Farm was left in the multiple logistic model as it had a confounding effect. Finally, only one variable stayed significant in the final model. Calves suffering from diarrhea at the time of sampling had a higher risk to be ST50 positive than calves not suffering from diarrhea (OR 23.21, 95%CI 23.21-248.87, $p = 0.01$).

Antimicrobial Resistance in *C. jejuni*

Of the 55 *C. jejuni* strains, 32 (58.2%) were resistant to at least one of the tested antimicrobials. Strains were resistant to ampicillin, ciprofloxacin, nalidixic acid, neomycin, streptomycin, and tetracycline (Figure 1). Twenty-six of the isolates (47.3%) were resistant to at least two of the tested antimicrobials. Seven of these isolates were resistant against three to five antimicrobials. Most frequently, resistance to ciprofloxacin was observed (49.1%), followed by nalidixic acid with 42.8%, and tetracycline (14.5%). No resistance was observed against amoxicillin/clavulanate, chloramphenicol, colistin, erythromycin, and gentamicin.

Associations could be observed in resistance against ciprofloxacin and nalidixic acid ($p = 0.01$), as well as against ciprofloxacin and ampicillin ($p = 0.02$). All isolates that were resistant against nalidixic acid or ampicillin were also resistant against ciprofloxacin.

Genetic Identification of Quinolone Resistant Isolates

As almost half of the isolates were identified to be quinolone resistant in all of these isolates the QRDR of the *gyrA* gene was sequenced to detect the responsible point mutation at codon 86 the most important mechanism in *C. jejuni* for quinolone resistance. In all but one of the 27 isolates the point mutation at codon 86 (ACA to ATA) resulting in a substitution of isoleucine for threonine in gyrase A has been detected. One sensitive isolate sequenced confirmed the original sequence (ACA) as seen in sensitive isolates. Another mutation detected in three of the isolates at location codon 110 did not change the amino acid composition of the enzyme. One isolate resistant to ciprofloxacin and nalidixic acid did not have any mutation in the sequenced region.

Antimicrobial Resistance and MLST-Types

Of the 11 STs that were detected repeatedly, in four STs (ST122, ST572, ST883, and ST4899) all isolates were ciprofloxacin resistant, whereas in ST883 this association was significant ($p = 0.01$). In ST42, ST864, ST1943, and ST2497, no resistance against ciprofloxacin were observed. Similar findings were obtained for nalidixic acid resistances, see Table 4.

Testing for correlation between *C. jejuni* MLST-types and antimicrobial resistance revealed significant associations between ST48 and ciprofloxacin resistance (coefficient = -0.33; $p = 0.01$), between ST572 and resistance against ampicillin (coefficient = -0.61; $p < 0.01$), between ST864 and ciprofloxacin (coefficient = -0.28; $p = 0.04$) and tetracycline (coefficient = 0.28; $p = 0.04$) resistance, and between ST883 and ciprofloxacin and nalidixic acid, respectively (coefficient = 0.51; $p < 0.01$).

Only two of the 55 *C. jejuni* positive calves were previously treated with antimicrobials. Both of these calves shed antimicrobial resistant *C. jejuni* (ST50). Both strains were

TABLE 3 | Variables significantly associated with the presence of *C. jejuni* sequence type (ST) 883 in the final multiple logistic regression with farm forced into the model as a confounder.

Variable	ST				
	ST883	Others	OR ¹	95% CI ²	p
Farm					0.45
Type of farm	Organic	6	6	1	
	Conventional	38	5	0.62	0.01-0.64
Calf housing	Individual	40	4	1	
	Group	4	7	23.16	2.08-257.43
	Within cows' barn	15	9	1	
	Outside cows' barn	29	2	0.78	0.01-0.93
					0.04

¹OR = odds ratio²CI = confidence intervalHosmer-Lemeshow for the model $p = 0.77$.

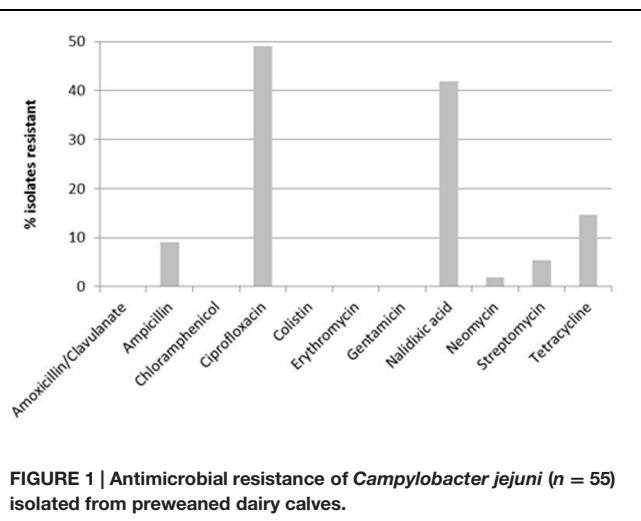


FIGURE 1 | Antimicrobial resistance of *Campylobacter jejuni* ($n = 55$) isolated from preweaned dairy calves.

resistant to ciprofloxacin and nalidixic acid, one additionally against tetracycline.

There was no association between the evaluated use of antimicrobials on farm and resistances.

DISCUSSION

To the knowledge of the authors studies examining *C. jejuni* MLST types in cattle have been limited to quinolone-resistant *C. jejuni* in Austria (Kovac et al., 2015) and studies in dairy calves are sparse.

Campylobacter jejuni isolates detected in the calves of our study were dominated by CC21, a CC regularly associated with cattle (Manning et al., 2003; Kwan et al., 2008; Ragimbeau et al., 2008; de Haan et al., 2010b; Bianchini et al., 2014; Jonas et al., 2015). Ten of the 19 *C. jejuni* STs detected were previously described in cattle. ST 883, the most common ST in our study, has only been described in cattle sporadically (de Haan et al., 2010b; Bianchini et al., 2014), and was furthermore sporadically associated with poultry and human campylobacteriosis (Wirz et al., 2010; Kittl et al., 2013b). In the study by Kovac et al. (2015) where ciprofloxacin-resistant *C. jejuni* of 17 cattle from Austria were examined, only one was ST883. In contrast, ST48 and ST50, the second and third most prevalent STs in the present study, were commonly associated with bovines, including Austria (Kwan et al., 2008; Ragimbeau et al., 2008; Rapp et al., 2014; Kovac et al., 2015). This ST was also associated with humans and other species, e.g., poultry (Ragimbeau et al., 2008; de Haan et al., 2010a). In the PubMLST database so far 32 human isolates and 33 chicken isolates from Austria have been downloaded between 2008 and 2014. Interestingly CC21 the CC most prevalent in calves in our study was also dominant in human isolates in Austria (21% of all isolates) whereas the clonal complex CC464, CC353, and CC354 dominated in chicken isolates.

Different studies emphasized the role of cattle in human campylobacteriosis. Following poultry, bovines were frequently

associated with human infections (Wilson et al., 2009; de Haan et al., 2010a; Mughini Gras et al., 2012). Risk factors that have been described are direct contact to cattle and to cattle feces, as well as consumption of raw milk (Eberhart-Phillips et al., 1997; Smith et al., 2004; Schildt et al., 2006). Direct contact and consumption of raw milk is given not only for farmers but also, e.g., during farm vacation which is popular in Austria with nearly 10,000 farms offering this service (Grüner Bericht, n.d.). Furthermore, contaminated food or water can play a role in human infection (Clark et al., 2005; Levesque et al., 2008; Mughini Gras et al., 2012). The three dominating CCs (CC21, CC48, and CC206) and STs (ST883, ST48, and ST50) of our study were also recovered from infected humans (Ragimbeau et al., 2008; Mullner et al., 2009; Sheppard et al., 2009; de Haan et al., 2010a). This result indicates that calves may be a potential source of human infection, but this cannot be proven by this study. Gilpin et al. (2008) found indistinguishable *C. jejuni* genotypes in dairy calves and humans, using Penner serotyping and pulsed field gel electrophoreses, and came to the same conclusion, that calves may be a source of human campylobacteriosis.

TABLE 4 | Antimicrobial resistance of the 55 *C. jejuni* MLST types obtained from dairy preweaned calves.

CC	ST	N isolates	N resistances	Antimicrobial resistance
21	21	2	2	CIP, NAL
	21	1	3	AMP, CIP, NAL
	21	1	1	STREP
	47	1	1	TET
	50	2	3	CIP, NAL, TET
	50	2	2	CIP, NAL
	50	1	0	
	864	2	1	TET
	864	2	0	
	883	9	2	CIP, NAL
22	883	1	3	CIP, NAL, STREP
	883	1	3	AMP, CIP, NAL
	1943	3	0	
	22	1	0	
	2497	3	0	
	42	2	0	
	45	1	0	
	48	7	0	
	48	1	2	CIP, NAL
	206	122	2	CIP, NAL
353	572	1	2	AMP, CIP
	572	1	3	AMP, CIP, NAL
	6021	1	5	AMP, CIP, NEO, STREP, TET
	356	1	0	
	354	2	2	CIP, NAL
	4899	1	1	CIP
	Unknown	1	0	
	Unknown	1	1	TET

CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; AMP, ampicillin; STREP, streptomycin; NEO, neomycin.

Early campylobacter infection in calves might be due to a high level of environmental contamination, as well as direct contact with feces and ingestion of milk (Bianchini et al., 2014). Contamination of calf housings represents a risk for early infection with *C. jejuni* ST883. Calves housed within the cows' barn were at higher risk to shed ST883 than calves housed in a special barn for calves and young animals or outside the barn. Additionally, grouping of animals was a risk factor for shedding ST883. These findings suggest that close contact to adult as well as to other young cattle lead to higher infection pressure and mutual infection between animals. Furthermore, grouping can be a stress factor and consequently lead to a higher rate of campylobacter shedding and infection (Rapp et al., 2014).

Furthermore, ST883 was associated with the type of farm. On conventional farms, the risk for preweaned calves to be ST883 positive was lower than on organic farms, a finding that can hardly be explained. Possibly a certain clone is circulating on these farms and has been established.

The presence of ST50 was associated with diarrhea at the time of sampling in calves. Although some authors (Al-Mashat and Taylor, 1983; Diker et al., 1990; Schulze, 1992) suggested a possible role of *Campylobacter* in calf diarrhea, in other studies no association between *C. jejuni* and disease was given (De Rycke et al., 1986; Acha et al., 2004), as was also true for the calves of the present study (Klein et al., 2013) and more likely other pathogens generally associated with calf diarrhea may be the cause of disease.

More than half of the calves (55%) originated from farms where also poultry was kept. Most of the STs detected in the calves of our study were also described in poultry. In the present study, specific types like ST50, frequently detected in poultry (de Haan et al., 2010b; Griekspoor et al., 2010; Kovanen et al., 2014), were associated with the presence of poultry on farm, suggesting cross-contamination between the two species. In contrast, other STs (e.g., ST21, ST48, and ST883) also regularly detected in poultry (Wirz et al., 2010; Kittl et al., 2013b) appeared in calves independently of the presence of poultry.

Antimicrobial resistance, particularly multidrug resistance is of public health concern. In the present study, 58.2% of the *C. jejuni* isolates were resistant to at least one and 47.3% against at least two of the tested antimicrobials. Because only two of the 55 *C. jejuni* positive calves were previously treated with antibiotics, no valid conclusion can be drawn if resistance to STs in this study was associated with previous antibiotic treatment. Nevertheless, these two treated animals shed *C. jejuni* resistant to two and three of the tested antimicrobials, respectively.

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In the present study, antimicrobial resistance to quinolones was detected most often. This has also been described for isolates originating from other sources (Oporto et al., 2009; Kittl et al., 2013a), explained by the fact that a single mutation is sufficient to cause resistance (Wang et al., 1993). This was confirmed in our study as all but one resistant isolates harbored a point mutation in codon 86 of the *gyrA* gene. Quinolone resistance has been described to be associated with specific ST types as detected in our study (Kittl et al., 2013a; Kovac et al., 2015). A survey performed in Austria revealed that quinolones are frequently used by Austrian veterinarians for treatment of cattle (Mayrhofer et al., 2006). This might explain a high level of quinolone resistance in *C. jejuni* isolates from calves.

CONCLUSION

The results of the present study support the hypothesis that cattle including dairy calves may be a reservoir for *C. jejuni* and represent a risk for transmission of these bacteria to the environment and to humans. Cattle have not been recognized as an important source for antimicrobial resistant *Campylobacter* sp. or other bacteria, yet. Nevertheless, high resistance rates found in this and other recent studies point out that screening for antimicrobial resistance in cattle is necessary to better understand the epidemiology of resistance and its spread.

AUTHOR CONTRIBUTIONS

DK-J designed the study, took all samples, performed statistical analysis, and drafted the manuscript. DS performed laboratory work and analysis. MI advised statistical analysis and interpretation, and reviewed the manuscript. MD supported the statistical analysis and reviewed the manuscript. FH designed the study together with DK-J and supervised the study, supervised, and performed laboratory work and analysis, and provided valuable references and suggestions during the preparation of the manuscript.

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Molecular epidemiology of Methicillin-resistant *Staphylococcus aureus* in Africa: a systematic review

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a serious global problem, with considerable impact on patients and substantial health care costs. This systematic review provides an overview on the clonal diversity of MRSA, as well as the prevalence of Panton-Valentine leukocidin (PVL)-positive MRSA in Africa. A search on the molecular characterization of MRSA in Africa was conducted by two authors using predefined terms. We screened for articles published in English and French through to October 2014 from five electronic databases. A total of 57 eligible studies were identified. Thirty-four reports from 15 countries provided adequate genotyping data. CC5 is the predominant clonal complex in the healthcare setting in Africa. The hospital-associated MRSA ST239/ST241-III [3A] was identified in nine African countries. This clone was also described with SCCmec type IV [2B] in Algeria and Nigeria, and type V [5C] in Niger. In Africa, the European ST80-IV [2B] clone was limited to Algeria, Egypt and Tunisia. The clonal types ST22-IV [2B], ST36-II [2A], and ST612-IV [2B] were only reported in South Africa. No clear distinctions were observed between MRSA responsible for hospital and community infections. The community clones ST8-IV [2B] and ST88-IV [2B] were reported both in the hospital and community settings in Angola, Cameroon, Gabon, Ghana, Madagascar, Nigeria, and São Tomé and Príncipe. The proportion of PVL-positive MRSA carriage and/or infections ranged from 0.3 to 100% in humans. A number of pandemic clones were identified in Africa. Moreover, some MRSA clones are limited to specific countries or regions. We strongly advocate for more surveillance studies on MRSA in Africa.

Keywords: *Staphylococcus aureus*, MRSA, molecular epidemiology, Africa, systematic review

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major public health concern and is responsible for both hospital- and community-associated infections worldwide (De Kraker et al., 2011; CDC, 2013; Falagas et al., 2013; Garza-González and Dowzicky, 2013; Lee et al., 2013; Chen and Huang, 2014). It is estimated that MRSA infections within the health care setting alone affected more than 150,000 patients annually in the European Union, with an additional cost of 380 million Euros (Köck et al., 2010). In the United States of America, 80,461 invasive MRSA infections and 11,285 related deaths occurred in 2011, and an estimated annual burden of between \$1.4 billion and

13.8 billion was attributed to community-acquired MRSA (CDC, 2013; Lee et al., 2013). Besides, MRSA has been established as a pathogen for domestic animals and linked with livestock-associated infections (Verkade and Kluytmans, 2013).

Methicillin resistance is usually due to the *mecA* gene, borne on the staphylococcal cassette chromosome *mec* (SCC*mec*) that codes for a 78-kDa penicillin binding protein (PBP2a), with decreased affinity to methicillin and all beta-lactam antibiotics (Chambers, 1997). To date, eleven SCC*mec* types have been identified (IWG-SCC, 2009). Some cassettes, for example, SCC*mec* II (53 kb) and SCC*mec* III (67 kb), are large and possess mobile genetic elements (MGE), such as integrated plasmids (pUB110, pI258, and pT181) and transposons (e.g., Tn554) (Ito et al., 2001), and are frequently associated with hospital-acquired MRSA (Ma et al., 2002; Ito et al., 2004). In contrast, SCC*mec* IV (21–24 kb) and V (27 kb) are shorter elements, generally susceptible to non-beta-lactam antibiotics, and linked with community MRSA (Chambers and Deleo, 2010). However, the spread of various MRSA clones between the hospital and community settings has made the dichotomous ranking difficult (Deurenberg and Stobberingh, 2008). Recently, a variant *mecA* gene (named *mecC*) which is situated on an SCC*mec* XI element has been described (Shore et al., 2011). It has a higher relative affinity for oxacillin as compared with cefoxitin (Kim et al., 2012), and exhibits only 69% sequence similarity at the nucleotide level and 63% amino-acid identity to *meca*/PBP2a (Paterson et al., 2014b). Furthermore, based on whole genome sequencing, mutations of the endogenous penicillin-binding proteins (PBP) 1, 2, and 3 in *mecA* and *mecC* negative strains have been postulated as a possible alternative mechanism for beta-lactam resistance in MRSA (Ba et al., 2014).

There is great interest in tracking, identifying and understanding the diversity of MRSA in various settings. Currently, the most widely used molecular techniques include *Staphylococcus* protein A gene typing (*spa*) and multilocus sequence typing (MLST). Studies (particularly using MLST) have provided evidence that a small set of lineages, clonal complex (CC)5, CC8, CC22, CC30, and CC45, are associated with most of the MRSA infections in hospitals (Stefani et al., 2012). Besides, a number of different geographically distinct lineages, CC1, CC8, CC30, and CC80, have also been associated with community MRSA infections (Chatterjee and Otto, 2013), while CC8 and CC30 have been identified as pandemic lineages both in the hospital and community setting (Chatterjee and Otto, 2013). Furthermore, regional clones have been described in Australia (sequence type [ST] 93) (Coombs et al., 2009), India (ST772) (D’Souza et al., 2010; Shambat et al., 2012), South Korea (ST72) (Kim et al., 2007), Taiwan and China (ST59) (Chen and Huang, 2014).

The distribution of MRSA clones in Africa is not well-described. Understanding the molecular epidemiology of MRSA in Africa is important as a recent review indicated that since the year 2000, the prevalence of MRSA appears to be increasing in many African countries and pose a visible threat to the continent (Falagas et al., 2013). Furthermore, there is evidence of the replacement of existing MRSA clones with different and new clonal types in a number of countries (Conceição et al., 2007; Aires-de-Sousa et al., 2008; Albrecht et al., 2011; Espadinha

et al., 2013; Lim et al., 2013; Nimmo et al., 2013) but information on this trend is lacking in Africa. The occurrence and changes in clonal identities, and their geographic spread is important to understand the spread and evolution of MRSA.

The Panton-Valentine Leukocidin (PVL) is a two-component pore-forming toxin with cytolytic activity on defined cells of the immune system (neutrophils, macrophages and monocytes) (Löffler et al., 2010; Yoong and Torres, 2013). It is encoded by the *lukS-PV* and *lukF-PV* genes (Boakes et al., 2011), and PVL-producing *S. aureus* exhibit a propensity for causing mainly severe and often recurrent skin and soft tissue infections (Shallcross et al., 2013). In addition, PVL-positive MRSA are associated with community onset-pneumonia (Vandenesch et al., 2003). Although the PVL genes are mainly carried by community-associated MRSA (CA-MRSA) (Vandenesch et al., 2003), data from West and Central Africa showed that at least 40% of clinical methicillin-susceptible *S. aureus* (MSSA) isolates in this region are PVL-positive (Breurec et al., 2011a; Schaumburg et al., 2011; Shittu et al., 2011; Egyir et al., 2014a). Therefore, the acquisition of the *mecA* gene by PVL-positive MSSA and the possible dissemination of PVL-positive CA-MRSA could present a significant challenge in disease management and infection control in resource-limited countries in Africa.

This systematic review examined published literature on the molecular epidemiology of MRSA in Africa. By summarizing currently available data on the continent, our objective was to describe the distribution of MRSA clones, the prevalence of PVL-positive MRSA, and to highlight the need to develop more comprehensive surveillance and reporting systems for multidrug-resistant organisms such as MRSA in Africa.

Methods

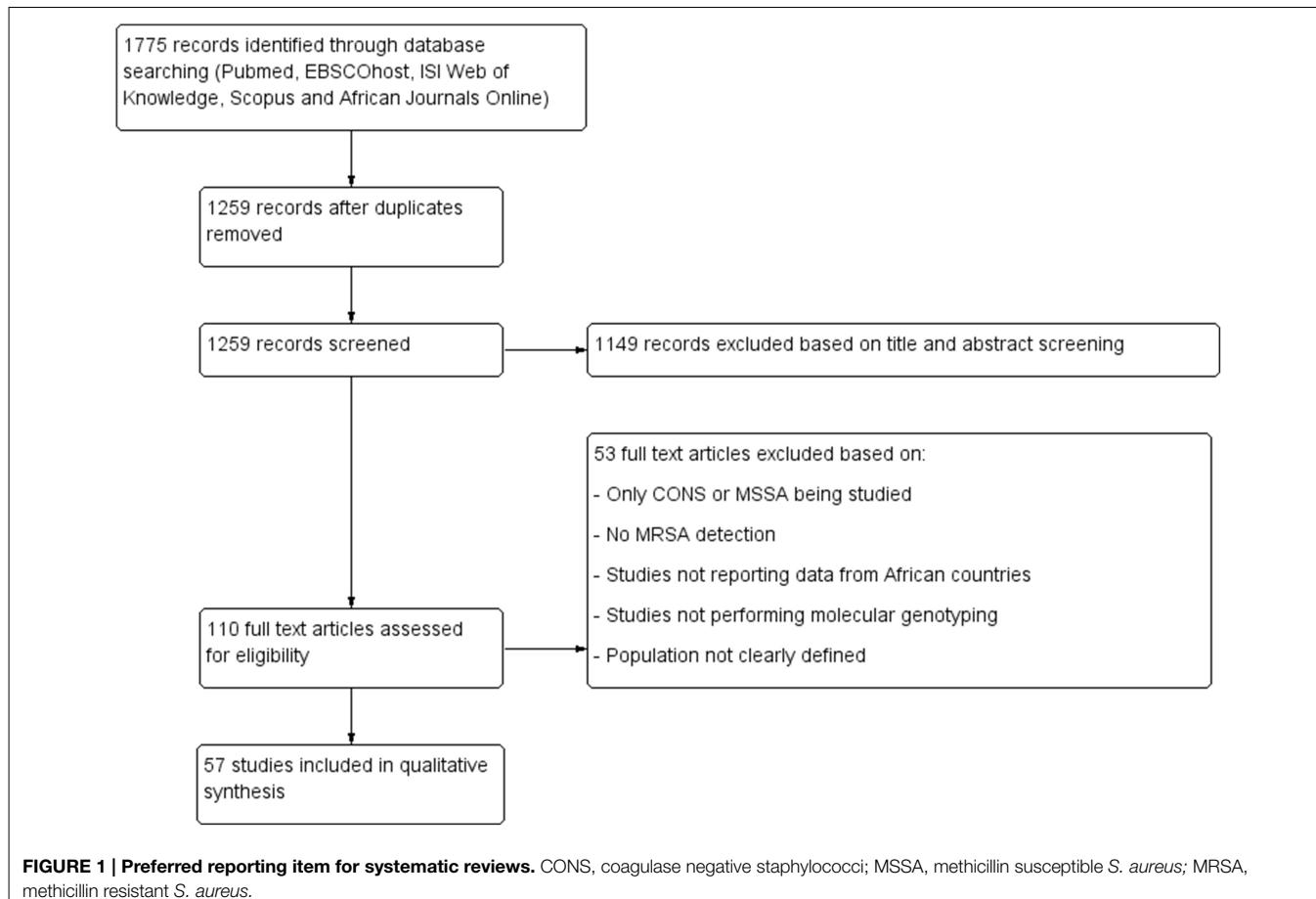
This systematic review was conducted in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (Moher et al., 2009).

Literature Search Strategy

The relevant English and French articles available in five electronic databases (MEDLINE, EBSCHost, ISI Web of knowledge, Scopus, and African Journals Online) were retrieved by two authors using predefined search terms (Table S1). The literature search was conducted until 31 October 2014.

Eligible Article Identification

Figure 1 summarizes the study selection process. All duplicate articles were removed and data on MSSA as well as *in-vitro* studies were also excluded. The eligibility of published reports in this review was based primarily on polymerase chain reaction (PCR) detection of the *mecA* gene, and the use of at least one molecular tool for genotyping of MRSA strains (Table 1). In addition, worldwide surveys that covered African countries were also included. An MRSA clone was defined based on the combination of MLST sequence type (ST) and SCC*mec* typing data as previously reported (Okuma et al., 2002). The nomenclature of the SCC*mec* types was as proposed by the International Working



Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC, 2009). SCC_{mec} elements that could not be classified were indicated as non-typeable (SCC_{mec}-NT). In this study, we categorized MRSA into various CCs according to the current eBURST scheme, Version 3 (accessed 30 October 2014) (eBURST, www.mlst.net, V3)¹.

Data Extraction and Synthesis

The relevant data were extracted from each of the articles as stated in **Table 1**. Separate articles that analyzed the same *S. aureus* isolates but answered different questions were considered as a single study.

eBURST Analysis

The relationship between the MRSA STs described in this review and other lineages reported world-wide was analyzed using the eBURST scheme. The allelic profiles were downloaded from the MLST website (<http://saureus.mlst.net/>) which included the African MRSA STs as well as 223 representative and randomly selected STs (from each CC) based on the differences in their allelic profiles. The minimum spanning tree was constructed by the goeBURST algorithm using the Phyloviz software v1.1 (<http://www.phyloviz.net/>).

¹eBURST [Online]. Available online at: <http://www.mlst.net> version 3.

Results

Literature Search

The systematic search of the five electronic databases yielded 1775 articles (**Figure 1**). No additional studies were identified from AJOL. After the removal of duplicate studies and assessment of titles and abstracts, 110 full-text articles were screened, of which 57 studies were considered eligible for the qualitative analysis according to our inclusion criteria.

Characteristics of the Studies Included in the Systematic Review

Most of the data analyzed were obtained from single center studies conducted mainly in five countries; Tunisia ($n = 13$), Nigeria ($n = 9$), South Africa ($n = 7$), Algeria ($n = 5$), and Egypt ($n = 3$) (**Table 1**). Multicenter studies were only reported in two articles (Goering et al., 2008; Breurec et al., 2011b), including a survey which comprised five African countries, Cameroon, Madagascar, Morocco, Niger and Senegal (Breurec et al., 2011b), and an inter-continental multicenter study, which included South Africa (Goering et al., 2008). Only three studies investigated the detection of MRSA in animals (**Table 1**).

In most of the reports included in this study, *S. aureus* was identified by phenotypic and culture characteristics, while molecular identification (16S rRNA, detection of the thermonuclease

TABLE 1 | Characteristics of eligible articles that studied Methicillin resistant *Staphylococcus aureus*.

Country	Study period type	Study population (sample)	No. of <i>S. aureus</i> isolates	No. of <i>S. aureus</i> molecular identification	No. of MRSA ^a	Setting (no.)	Genotyping tools				PVL	Detection of genes	References		
							SCCmec	coa	agr	spa typing	MLST	PFGE			
STUDIES CONDUCTED IN HUMANS															
Algeria	2003–2004	Clinical samples from hospitals and community	614	–	204	HA (40)/ CA (21)	✓	–	✓	–	✓	✓	–	Ramdanibouguesse et al., 2006	
	2004–2007	Human infections (in-and-out patients)	65	–	23	NR	✓	–	✓	–	✓	–	–	Belkhoucha et al., 2009	
	2005–2007	From military hospital (Pus, venous catheter, tracheal aspirates, lumbar puncture fluid, blood culture and urine)	NR	–	64	HA (50)/ CA (14)	✓	–	–	–	✓	–	✓	Ouchenane et al., 2011, 2013	
	2006–2007	Healthy and hospitalized individuals	221 ^a 52 ^b	GyrA PCR	99 ^a 23 ^b	HA (65)/ CA (84)	✓	–	✓	✓	NR	✓	–	✓	Antri et al., 2011
	2010–2011	Children and neonates (SSTI, bacteraemia, otitis and bone/joint infections)	129	–	25	HA (15)/ CA (10)	✓	–	–	✓	✓	✓	–	Djoudi et al., 2013	
Angola	2012	Nasal swabs from inpatients and HCW	117	–	68	NR	✓	–	✓	✓	✓	✓	–	Conceição et al., 2014	
Egypt	2007–2008	Pus, sputum, wounds, abscess, blood, urine, and discharge	NR	–	21	CA (4)	✓	✓	✓	✓	NR	✓	–	Enany et al., 2010	
NR		SSTI and nasal swabs	38	–	18	CA (18)	✓	–	–	–	✓	–	–	Sobhy et al., 2012	
NR		Septic wounds, UTI and RTI (nasal swabs)	10	–	7	–	–	✓	–	✓	–	–	–	El-Jakee et al., 2011*	
Gabon	2008–2010	asymptomatic carriers (nares, axillae, inguinal swabs) and patients (abscess, wound, blood and others)	217	nuc and 16S rRNA PCR	12	HA (6)/ CA (6)	✓	–	✓	✓	✓	–	✓	Schaumburg et al., 2011; Ateba Ngoa et al., 2012	
	2010–2013	swabs from <i>S. aureus</i> <td>460</td> <td>–</td> <td>9</td> <td>NR</td> <td>✓</td> <td>–</td> <td>✓</td> <td>✓</td> <td>–</td> <td>✓</td> <td>–</td> <td>Schaumburg et al., 2014</td>	460	–	9	NR	✓	–	✓	✓	–	✓	–	Schaumburg et al., 2014	
NR		Blood culture of one patient	1	–	1	NR	–	–	✓	✓	–	✓	–	Huson et al., 2014	

(Continued)

TABLE 1 | Continued

Country	Study period	Study population (sample type)	No. of <i>S. aureus</i> isolates	<i>S. aureus</i> molecular identification	No. of MRSA ^{II}	Setting (no.)	Genotyping tools			PVL	Detection of genes	References
							SCCmec	coa	agr spa typing	MLST	PFGE	
Ghana	2011–2012	In-patients and hospital staff	105	spa gene PCR	6	NR	✓	–	✓	✓	–	Egyir et al., 2013
	2010–2012	SSTI and blood samples from six hospitals	308	–	9	NR	✓	–	✓	✓	–	Egyir et al., 2014a
	2011–2012	Nasal swabs from apparently healthy carriers	124	–	2	HA (2)	✓	–	✓	✓	–	Egyir et al., 2014b
Kenya	2005–2007	In and out-patients with SSTI boil, abscess, cellulitis and ulcer	84	–	69	NR	✓	–	–	✓	–	Maina et al., 2013
	2011	Nasal and axillary skin swabs from hospitalized patients	85	–	6	NR	✓	–	✓	✓	✓	Aiken et al., 2014
Libya	2009–2010	Nasal swabs from in-patient children, their mothers, out-patient children and HCW	758	–	70	HA (12) CA (6)	–	–	–	✓	–	Al-haddad et al., 2014
Mali	2005	Asymptomatic nasal carriers	88	–	1	CA (1)	–	–	✓	–	–	Ruijy et al., 2008
Mozambique	2010–2011	Post-operative, burn wound infections, skin and soft tissue abscesses	99	–	9	HA (8), CA (1)	–	–	✓	–	–	Van der Meeren et al., 2014
Nigeria	1998–2002	Wounds, aspirate, amniotic fluid	276	–	4	NR	✓	–	–	✓	–	Adesida et al., 2005
	2002–2004	Wound samples, blood cultures, urine, otitis media and ocular related infections	200	–	3	NR	✓	–	–	✓	–	Shittu and Lin, 2006b
	2007–2012	Clinical specimen	150	–	12	NR	✓	–	–	✓	–	✓
	2007	Surgical and pediatric patients wound samples, corneal, conjunctival, auricular, genital and nasal swabs	346	–	70	HA (42), CA (28)	✓	–	✓	✓	–	Ghebremedhin et al., 2009
	2008–2010	HIV-positive and healthy individuals (nasal swabs)	202	–	26	NR	–	✓	–	✓	–	Olaelekan et al., 2012

(Continued)

TABLE 1 | Continued

Country	Study period	Study population (sample type)	No. of <i>S. aureus</i> isolates	<i>S. aureus</i> molecular identification	No. of MRSA [¶] (no.)	Setting (no.)	Genotyping tools			PF/L	Detection of genes	References	
							SCCmec	coa	agr	spa typing	MLST	PFGE	
	2009	Wound infections, semen, UTI, chronic ulcer, conjunctivitis, throat infections	68	—	11	NR	✓	—	✓	✓	—	✓	Shittu et al., 2011
	2009–2011	Patients and carriers	62	—	22	NR	✓	—	—	✓	—	✓	Raij et al., 2013
	2010	Clinical samples from patients with burns, septicemia, wound infections, osteomyelitis, bronchitis and GIT	51	<i>tuf</i> gene PCR	15	NR	✓	—	✓	✓	—	✓	Shittu et al., 2012
NR	Urine, blood and aspirates, wound, eye and ear, urethral and endocervical swab	116	—	48	HA (40), CA (8)	✓	—	—	—	✓	—	✓	Terry Ali et al., 2012
South Africa	2001–2003	Wound samples, sputum, otitis media and blood culture	227	<i>nuc</i> gene PCR	61	NR	✓	✓	—	✓	—	✓	Shittu and Lin, 2006a; Shittu et al., 2007
	2001–2003	Isolates from 16 laboratories in KZN	241	—	24	NR	✓	—	—	✓	—	—	Essa et al., 2009
	2001–2003	Wounds, sputum, otitis media, urine and blood culture	NR	—	61	NR	✓	—	✓	✓	✓	—	Shittu et al., 2009
	2005–2006	Bacteremia, SSTI, urine, catheter tip, cerebrospinal and drainage fluids	NR	—	320	HA	✓	—	✓	✓	✓	✓	Moodley et al., 2010
	2006–2007	Clinical samples	NR	—	97	HA (79), CA (4)	✓	—	✓	✓	✓	✓	Makgotho et al., 2009
	2007–2008	Pus and pus swabs, urine, blood, RTS and CVCT	NR	—	100	CA (10)	✓	—	✓	✓	✓	—	Jansen van Rensburg et al., 2011, 2012
	2009–2010	A wide range of clinical specimens mostly SSTI	367	—	56	NR	✓	—	✓	✓	✓	—	Oosthuysen et al., 2014
São Tome and Príncipe	2010–2012	Patients and healthy carriers	52	—	14	NR	✓	—	✓	✓	✓	✓	Conceição et al., 2013

(Continued)

TABLE 1 | Continued

Country	Study period	Study population (sample type)	No. of <i>S. aureus</i> isolates	No. of <i>S. aureus</i> molecular identification	No. of MRSA [¶] (no.)	Setting (no.)	Genotyping tools				PVL	Detection of genes	References
							SCCmec	coa	agr	spa typing	MLST	PFGE	
Tanzania	2008	Wound, nasal swab and pus	160	—	24	HA	—	—	✓	✓	—	✓	—
2010	Apparently healthy children under 5 years (nasal swabs)	114	nuC gene PCR	12	CA	—	—	—	✓	—	—	—	Moremi et al., 2012
Tunisia	1998–2007	Clinical specimens from neutropenic patients	72	nuC gene PCR	13	HA (13)	✓	—	—	✓	—	—	Bouchami et al., 2009
2002	Patients who developed MRSA infections	NR	—	6	HA (6)	—	—	—	✓	—	—	—	Ben Ayed et al., 2010
2003–2004	Pus, blood, pleural fluid, venous catheter	NR	—	72	NR	✓	—	✓	—	—	✓	—	✓ Ben Nejma et al., 2006
2003–2004	Pathological samples from different wards	147	—	19	NR	—	—	—	✓	—	—	—	Ben Saïda et al., 2005
2003–2005	Pus and associated with cutaneous infections	NR	—	64	CA (64)	✓	—	✓	✓	✓	—	✓	Ben Nejma et al., 2013
2004	Cutaneous pus, blood cultures, urine and puncture fluids	NR	—	34	HA (32), CA (2)	✓	—	—	—	—	—	—	Ben Jomaa-Jemili et al., 2006
2004–2005	Cutaneous pus, RTS, urine, blood culture,	475	—	57	NR	—	—	✓	—	—	—	—	Ben Ayed et al., 2006
2004–2008	Samples from hospitals and community	NR	—	69	HA (41), CA (28)	✓	—	✓	✓	—	✓	—	Ben Jomaa-Jemili et al., 2013
2006–2008	Children with CA invasive infections bacteraemia and osteomyelitis	36	—	8	CA (8)	✓	—	✓	✓	✓	—	—	Kechrid et al., 2011
2007	Pus and skin infections	NR	—	11	CA (11)	✓	—	✓	✓	✓	✓	—	Ben Nejma et al., 2009
2008	Pus and blood culture (case report)	2	—	2	NR	✓	—	✓	✓	—	✓	—	Zribi et al., 2011
2008–2009	Humans in contact with animals	55	—	1	CA (1)	✓	—	✓	✓	—	✓	✓	Ben Slama et al., 2011

(Continued)

TABLE 1 | Continued

Country	Study period	Study population (sample type)	No. of <i>S. aureus</i> isolates	<i>S. aureus</i> molecular identification	No. of MRSA [¶]	Setting (no.)	Genotyping tools				PVL	Detection of genes	References
							SCCmec	coa	agr	spa typing	MLST	PFGE	
Uganda	2009–2010	Swabs from patients, HCW and from hospital environment (sinks, door handles, surgical trays, bed and table surfaces)	41	—	41	NR	✓	—	—	—	✓	✓	Kateete et al., 2011
	2011–2012	SSI	64	nuc gene PCR	24	NR	✓	—	✓	—	✓	—	Seni et al., 2013
Multicenter [#]	2007–2008	SSTI, bacteraemia/septicaemia, urine, wounds osteomyelitis and myositis	NR	—	86	CA (9), HA (77)	✓	—	✓	✓	—	✓	Breurec et al., 2011b
Multicentre ^x	2004–2005	Uncomplicated skin infections	292	—	105	HA (3)	✓	—	—	✓	✓	—	Goering et al., 2008
STUDIES CONDUCTED IN ANIMALS													
Egypt	NR	Cows and buffaloes milk, cattle septic wounds	9	—	5	NR	—	✓	—	✓	—	—	El-Jakee et al., 2011*
Senegal	2009–2011	Pigs (nasal swabs)	73	—	6	NR	✓	—	✓	—	✓	—	Fall et al., 2012
Tunisia	2010	Healthy sheep (nasal swabs)	73	—	5	CA (6)	✓	—	✓	✓	✓	—	Gharsa et al., 2012

agr, Accessory gene regulator; CA, Community-acquired methicillin-resistant *S. aureus*; coa, Coagulase gene; CVCT, Central venous catheter tips; GIT, Genital tract infections; gna, DNA gyrase gene; HA, Hospital-acquired methicillin-resistant *S. aureus*; HCW, Health care workers; HIV, Human immunodeficiency virus; KZN, KwaZulu-Natal province; MLST, Multilocus locus sequence typing; MRSA, Methicillin-resistant *Staphylococcus aureus*; No., Number of isolates; NR, Not reported; nuc, Threonine/leucine gene; PCR, Polymerase chain reaction; PFGE, Pulsed-field gel electrophoresis; PVL, Panor-Valentine-Lukicidin genes; SCCmec, Staphylococcal chromosomal cassette mec element; RTI, Respiratory tract infections; rRNA, Ribosomal ribonucleic acid; RTs, Respiratory tract specimens; spa, *Staphylococcus aureus* protein A gene; SSTI, Skin and soft tissue infections; SSJ, Surgical site infections; UTI, Urinary tract infections.

[¶]MRSA as confirmed by meca PCR; ✓, Test was conducted; —, Test was not conducted; ^{*}Test was conducted; [#]African multicenter study which included Cameroon, Madagascar, Morocco, Niger and Senegal; ^xAn international multicenter study which included only South Africa; *Study was conducted in both animal and human host.

and the elongation factor tu - *nuc*, *tuf* - genes) was performed in only 12.3% (7/57). The screening for antibiotic resistance and toxin/virulence genes were carried out in seven and 22 studies, respectively (**Table 1**). Furthermore, all the eligible studies analyzed MRSA using at least one genotyping technique, and 59.6% (34/57) provided adequate genotyping data on MRSA clones from 15 African countries (**Tables 1, 2**). Studies included in this systematic review did not investigate on the *mecC* gene.

Community- and Hospital- Acquired MRSA

Overall, 51% (29/57) of the eligible studies provided the potential source (hospital- or community-associated) of the MRSA strains. Only 17.5% (10/57) reported MRSA from community settings (**Table 1**). USA300 (ST8-IV [2B]) and other related sequence types were noted both in health care and community settings in nine African countries (**Tables 1, 2**). The “Brazilian/Hungarian clone” (ST239-III [3A]) was associated with hospital-acquired infections in nine countries (**Tables 1, 2**). Furthermore, the “West Australia MRSA-2” (ST88-IV [2B]) was reported in community- and hospital-acquired infections in several African countries (**Table 2**).

Detection of Panton-Valentine Leukocidin (PVL) Genes

The screening for PVL-associated genes (*lukF-PV* and *lukS-PV*) was carried out in 44 studies, and the detection of PVL genes was only reported in 32 studies (**Table 1**). In animals, PVL-positive MRSA (ST5) was described in nasal samples of pigs from Senegal (Fall et al., 2012). In humans, the proportion of PVL-positive MRSA carriage and/or infections ranged from 0.3 to 100%. Studies from Algeria and Tunisia reported higher PVL prevalence while investigations from South Africa reported the lowest prevalence (**Table 3**). Overall, PVL-positive MRSA were more frequently reported with skin and soft tissue infections, and community-associated clones (**Tables 1, 2**). There was no report on the role of PVL in necrotizing pneumonia caused by MRSA in Africa.

MRSA Clones Reported in Africa Using the Current eBURST Scheme

Figures 2, 3 summarize the MRSA clones identified in Africa based on MLST CCs. By the current eBURST scheme, six main CCs were identified: CC5, CC22, CC30, CC45, CC80, and CC88. In addition, a number of diverse *spa* types were identified among the MRSA clones in Africa (**Table 2**), but the distribution of *spa* types t042 and t044 (associated with CC80-IV [2B]) appear to be limited to three North African (Algeria, Egypt and Tunisia) countries (**Table 2**).

Clonal Complex 5

This clonal complex is considered the largest group based on the eBURST scheme (**Figure 3**). It was subdivided into three main clusters and designated as CC5-ST1, ST5, and ST8.

MRSA CC5 with Sequence Type 1

This group was reported in Nigeria (Raji et al., 2013) and Tunisia (Ben Jomaa-Jemili et al., 2013). The clonal type included the

PVL-positive ST1-V [5C] isolated from patients in a tertiary hospital in Nigeria (Raji et al., 2013), and the PVL-negative ST1 with a non-typeable *SCCmec* element (*spa* type t035 and *agr* type III) identified in Tunisia (Ben Jomaa-Jemili et al., 2013). In addition, an ST1 related sequence type (ST772-V [5C]), “the Bengal-Bay clone” has been described in Nigeria (Raji et al., 2013).

MRSA CC5 with Sequence Type 5

This clone was documented in 14 studies and consisted of diverse *SCCmec* elements (**Table 2**). The ST5-I [1B]/III [3A] were identified from clinical samples in health care institutions in South Africa (Shittu et al., 2009; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014). ST5-II [2A] has been described in Nigeria (Raji et al., 2013), and Senegal (Breurec et al., 2011b). ST5-IV [2B]-PVL-positive was the dominant clone in hospitalized patients with skin and soft tissue infections in Dakar, Senegal (Breurec et al., 2011b). In addition, ST5-IV [2B] was detected from nasal samples of pigs in the same geographical area (Fall et al., 2012). ST5-IV [2B] has also been identified in Algeria (Ramdani-bouguessa et al., 2006), Gabon (Schaumburg et al., 2011; Ateba Ngoa et al., 2012), Morocco (Breurec et al., 2011b), and South Africa (Essa et al., 2009), while the *SCCmec* IVa [2B] variant was recovered from hospitalized patients in Algeria (Ramdani-bouguessa et al., 2006; Bekkhoucha et al., 2009), Angola (Conceição et al., 2014), and São Tomé and Príncipe (Conceição et al., 2013). Moreover, ST5-IVa [2B] was reported from nasal samples of apparently healthy-hospital workers in Angola (Conceição et al., 2014). Other ST5 and related clones identified are ST5-V [5C] in Angola (Conceição et al., 2014), Cameroon (Breurec et al., 2011b), and Nigeria (Shittu et al., 2011), ST72-*SCCmec*-NT in South Africa (Jansen van Rensburg et al., 2011), ST72-V [5C] in Angola and Ghana (Egyir et al., 2013, 2014b; Conceição et al., 2014), and ST105-II [2A] from a patient in São Tomé and Príncipe (Conceição et al., 2013). Furthermore, ST650-IV [2B] was detected from clinical samples in health care institutions in South Africa (Jansen van Rensburg et al., 2011). Finally, ST2629-V [5C] was described in Angola (Conceição et al., 2014).

MRSA CC5 with Sequence Type 8

MRSA assigned to this clone are widespread and diverse across Africa as evidenced in 27 studies (**Table 2**). The first known early or ancestral clone, ST250-I [1B], was mainly associated with hospital-acquired infections in Ibadan, South-West Nigeria (Ghebremedhin et al., 2009), and recently observed in Ghana (Egyir et al., 2014b). ST8-II [2A] was only described in the KwaZulu-Natal region of South Africa (Essa et al., 2009), while a number of investigations reported ST8-IV [2B] in Angola (Conceição et al., 2014), Cameroon (Breurec et al., 2011b), Gabon (Schaumburg et al., 2011; Ateba Ngoa et al., 2012), Ghana (Egyir et al., 2014b), Madagascar (Breurec et al., 2011b), Nigeria (Adesida et al., 2005; Shittu et al., 2012), São Tomé and Príncipe (Conceição et al., 2013) and South Africa (Essa et al., 2009). The MRSA isolates from Angola possessed the *SCCmec* type IVd element (Conceição et al., 2014). ST612-IV [2B], a double locus variant (dlv) of ST8-IV [2B], and only recently reported as PVL-positive (Oosthuysen et al., 2014), is widespread

TABLE 2 | Methicillin resistant *Staphylococcus aureus* clones reported in 34 eligible studies.

Country	Clonal type ST-SCCmec	Clonal complex	spa type	PVL status	agr	References
Algeria	ST80-IV [2B]	80	ND	+	III	Ramdani-bouguessa et al., 2006
	ST37-IVa [2B]	30	ND	+	III	
	ST239-III [3A]	5	ND	-	I	
	ST239-IVa [2B]	5	ND	-	I	
	ST241-III [3A]	5	ND	-	I	
	ST637-III [3A]	5	ND	-	I	
	ST5-IV, IVa [2B]	5	ND	+, -	II	
	ST635-IV [2B]	80	ND	-	III	
	ST636-NT	22	ND	-	I	
	ST80-IV [2B]	80	t044	+	ND	Bekkhoucha et al., 2009
Angola	ST239-III [3A]	5	t037	-	ND	
	ST5-IVa [2B]	5	t311, t450	-	ND	
	ST88-NT	88	t188, t267	-	ND	
	ST80-IV [2B]	80	t044, t4143	+	III	Antri et al., 2011
	ST241-III [3A]	5	ND	-	III	
Egypt	ST8-V [5C]	5	ND	-	I	
	ST80-IVc [2B]	80	ND	+, -	ND	Djoudi et al., 2013
Cameroon	ST39-II [2A]	30	ND	-	ND	
	ST5-IVa [2B]	5	t105, t311, t11657	-	II	Conceição et al., 2014
Gabon	ST8-IVd, V [2B]	5	t104, t1774	-	I	
	ST72-V [5C]	5	t3092	-	I	
	ST88-IVa [2B]	88	t186, t325, t786, t1951, t3869	-	III	
	ST5-V [5C]	5	t6065	-	II	
	ST2629-V [5C]	5	t6065	-	II	
	ST789-V [5C]	7	t091	-	II	
	ST5-V [5C]	5	t311	+	II	Breurec et al., 2011b
Ghana	ST88-IV [2B]	88	t186	-	III	
	ST8-IV [2B]	5	t024, t121, t451	+	I	
	ST1289-V [5C]	88	t1339	+	III	
Niger	ST80-IVc [2B]	80	t042, t044, t070, t983	+	III	Enany et al., 2010
	ST30-IVa [2B]	30	t251, t318	+	III	
	ST1010-X ^a	121	t159, t312	+	IV	
Nigeria	ST8-IV [2B]	5	t121	+	I	Schaumburg et al., 2011; Ateba Ngoa et al., 2012
	ST88-IV [2B]	88	t186	-	III	
	ST5-IV [2B]	5	t653	-	II	
	ST5-IV [2B]	5	t653	-	ND	Schaumburg et al., 2014
	ST8-NT	5	t112, t121	+	ND	
Togo	ST45-V [5C]	45	t437, t8860	-	ND	
	ST88-IV [2B]	88	t4195	-	ND	
	ST72-V [5C]	5	t537	-	ND	Egyir et al., 2013
	ST8-V [5C]	5	t064	-	ND	
	ST88-IV [2B]	88	t325, t1951, t2649	-	ND	
Other	ST72-V [5C]	5	t537	-	ND	Egyir et al., 2014b
	ST8-IV [2B]	5	t121	+	ND	
	ST239-III [3A]	5	t037	-	ND	
	ST250-I [1B]	5	t928	-	ND	
	ST2021-V [5C]	5	t024	-	ND	

(Continued)

TABLE 2 | Continued

Country	Clonal type ST-SCCmec	Clonal complex	spa type	PVL status	agr	References
	ST88-IV [2B]	88	t186	—	ND	
	ST789-IV [2B]	7	t547	+	ND	
	ST508-V [5C]	45	t5132	—	ND	Egyir et al., 2014a
Kenya	ST239-III [3A]	5	t037	—	ND	Aiken et al., 2014
Madagascar	ST8-IV [2B]	5	t121	+	I	Breurec et al., 2011b
	ST30-V [5C]	30	t4686	—	III	
	ST88-IV [2B]	88	t186	—	III	
Morocco	ST239, ST241-III [3A]	5	t037, t138	—	I	
	ST5-IV [2B]	5	t311	+	II	
Niger	ST239, ST241-III [3A]	5	t138	—	I	
	ST239, ST241-V [5C]	5	t037	—	I	
	ST88-IV [2B]	88	t186	—	III	
Nigeria	ST8-IV [2B]	5	ND	—	ND	Adesida et al., 2005
	ST88-IV [2B]	88	t186	+	III	Ghebremedhin et al., 2009
	ST241-IV [2B]	5	t037	—	I	
	ST250-I [1B]	5	t194, t292	—	I	
	ST241-III [3A]	5	t037	—	ND	Shittu et al., 2011
	ST8-V [5C]	5	t064	—	ND	
	ST8-V [5C]	5	t451	—	ND	
	ST94-IV [2B]	5	t008	—	ND	
	ST5-V [5C]	5	t002	—	ND	
	ST241-III [3A]	5	t037	—	ND	Shittu et al., 2012
	ST88-IV [2B]	88	t729, t1603	—	ND	
	ST37-III [3A]	30	t074	—	ND	
	ST39-II [2A]	30	t007	—	ND	
	ST8-V [5C], IV [2B], ST8-NT	5	t064	—	ND	
	ST152-NT	152	t4690	+	ND	
	ST1-V [5C]	5	ND	+	ND	Raji et al., 2013
	ST239-III [3A] <i>mercury</i>	5	ND	—	ND	
	ST5-II [2A]	5	ND	—	ND	
	ST8-V [5C]	5	ND	—	ND	
	ST247-I [1B]	5	ND	—	ND	
	ST772-V [5C]	5	ND	+	ND	
	ST88-IV [2B]	88	ND	—	ND	
	ST241-III [3A]	5	ND	—	ND	Okon et al., 2009
	ST5-IV [2B]*	5	t311	+	ND	Fall et al., 2012
Senegal	ST88-IV [2B]*	88	t3489	—	ND	
	ST239, ST241-III [3A]	5	t037, t138	—	I	Breurec et al., 2011b
	ST5-II [2A]	5	t311	+	II	
	ST5-IV [2B]	5	t311	+	II	
South Africa	ST88-IV [2B]	88	t168	—	III	
	ST5-IV [2B]	5	ND	ND	ND	Essa et al., 2009
	ST8-IV [2B]	5	ND	ND	ND	
	ST8-II [2A]	5	ND	ND	ND	

(Continued)

TABLE 2 | Continued

Country	Clonal type ST-SCCmec	Clonal complex	spa type	PVL status	agr	References
	ST239-III [3A]	5	ND	ND	ND	
	ST45-IV [2B]	45	ND	ND	ND	
	ST612-IV [2B]	5	ND	—	ND	Goering et al., 2008
	ST36-II [2A]	30	ND	—	ND	
	ST1173-IV [2B]	5	t064	—	ND	Shittu et al., 2009
	ST1338-IV [2B]	5	t064	—	ND	
	ST239-III [3A]	5	t037	—	ND	
	ST5-III [3A]	5	t045	—	ND	
	ST239-III [3A]	5	t037	—	ND	Moodley et al., 2010
	ST612-IV [2B]	5	t064	—	ND	
	ST5-I [1B]	5	t045	—	ND	
	ST22-IV [2B]	22	t032	—	ND	
	ST22-IV [2B]	22	t891	+	ND	
	ST36-II [2A]	30	t012	—	ND	
	ST239-III [3A]	5	t037	—	ND	Jansen van Rensburg et al., 2011
	ST5-I [1B]	5	t045	ND	ND	
	ST650-IV [2B]	5	t002	ND	ND	
	ST612-IV [2B]	5	t064, t1443, t2196	ND	ND	
	ST72-NT	5	t3092	ND	ND	
	ST22-IV [2B]	22	t032	ND	ND	
	ST36-II [2A]	30	t012, t021	ND	ND	
	ST5-I [1B]	5	t002	—	II	Oosthuysen et al., 2014
	ST8-V [5C]	5	t064	—	I	
	ST612-IV [2B]	5	t064	+	I	
	ST239-III [3A]	5	t021	—	I	
	ST22-V [5C]	22	t891	+	I	
	ST22-IV [2B]	22	t891	—	I	
	ST36-II [2A]	30	t021	—	III	
São Tome and Príncipe	ST5-IVa [2B]	5	t105	—	II	Conceição et al., 2013
	ST105-II [2A]	5	t002	—	II	
	ST8-V [5C]	5	t451	—	I	
	ST8-IV [2B]	5	t451	—	I	
	ST88-IVa [2B]	88	t186, t786	—	III	
Tunisia	ST80-IV [2B]	80	t044	+	III	Ben Nejma et al., 2009
	ST80-IV [2B]	80	t044	+	III	Ben Nejma et al., 2013
	ST728-IVc [2B]	80	t042, t044	+	III	Kechrid et al., 2011
	ST8-IVc [2B]	5	t062	+	II	
	ST80-IVc [2B]	80	t203	+	III	Ben Slama et al., 2011
	ST1-NT	5	t035	—	III	Ben Jomàa-Jemili et al., 2013
	ST247-I [1B]	5	t040	—	I	
	ST239-III [3A]	5	t003	—	I	
	ST241-III [3A]	5	t125	—	I	
	ST97-NT	5	t003	—	I	
	ST1819-I [1B]	5	NS	—	I	
	ST80-IVc [2B]	80	t070	+	III	
	ST2563-IVc [2B]	80	t070	+	III	

(Continued)

TABLE 2 | Continued

Country	Clonal type ST-SCCmec	Clonal complex	spa type	PVL status	agr	References
	ST1440-IVc [2B]	80	t070	+	III	
	ST80-IVc [2B]	80	t1021	-	II	
	ST80-IVc [2B]	80	ND	-	III	
	ST22-NT	22	t998	-	II	
	ST45-NT	45	ND	-	I	
	ST153-NT	80	NST	+	III	
	ST153-NT	80	t044	ND	III	Gharsa et al., 2012

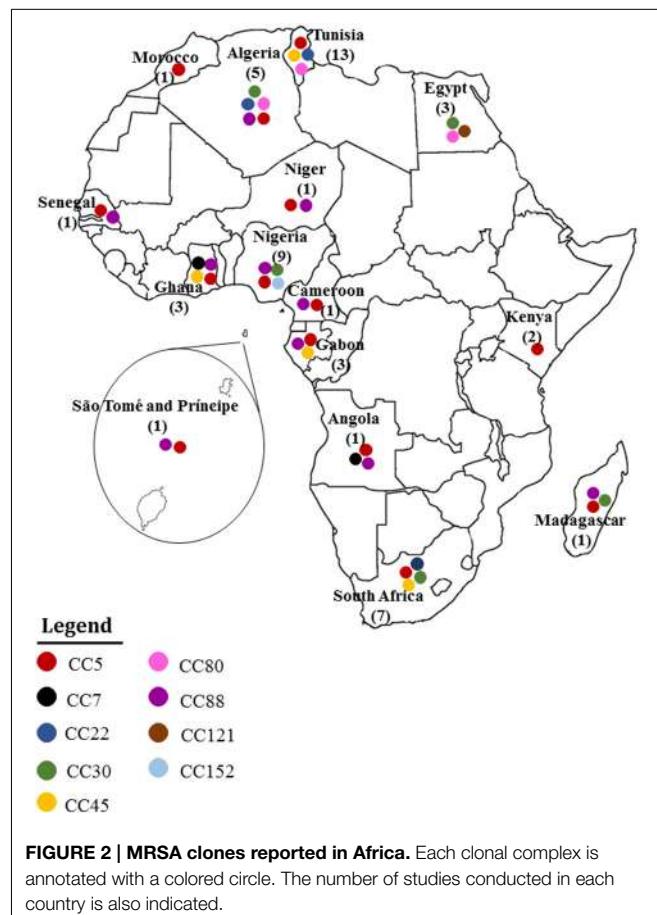
agr, Accessory gene regulator; CC, Clonal complex; NT, Non typeable; ND, Not determined; NST, New spa type; PVL, Panton-Valentine Leukocidin genes; SCCmec, Staphylococcal chromosomal cassette *mec* element; ST, Sequence type; spa, *Staphylococcus aureus* protein A gene; X^a, Unknown SCCmec type other than I, II, III, IV or V; *Clones isolated from pigs; +, PVL positive; -, PVL negative.

TABLE 3 | Panton-Valentine Leukocidin prevalence as reported by the eligible studies with sample size of 30 or above.

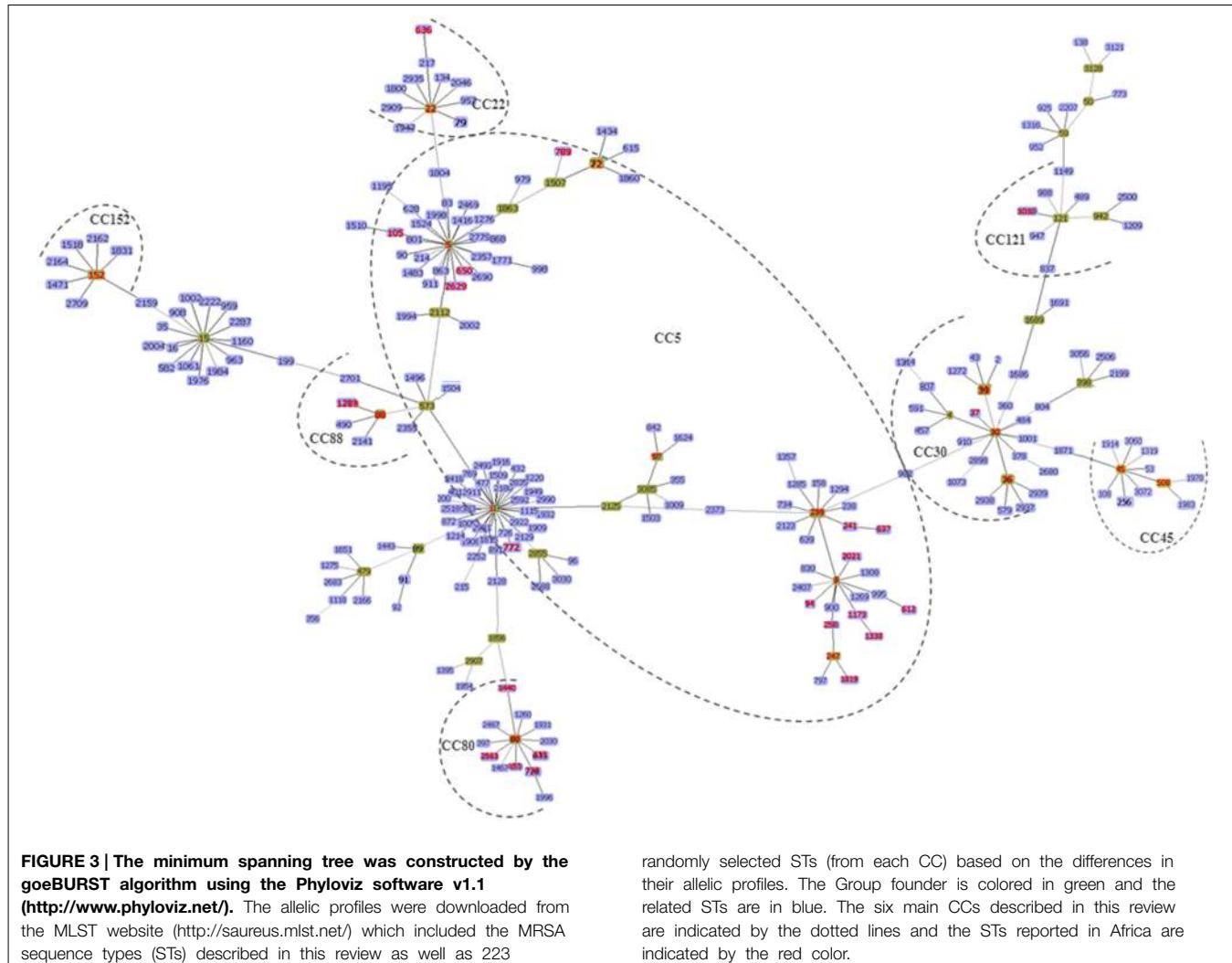
Country	PVL positive (no. positive/total tested)	Prevalence (%)	References
Algeria	46/61	75	Ramdani-bouguessa et al., 2006
	19/64	30	Ouchenane et al., 2011
	94/122	77	Antri et al., 2011
Kenya	14/69	20	Maina et al., 2013
Libya	10/35	29	Al-haddad et al., 2014
Nigeria	33/70	47	Ghebremedhin et al., 2009
South Africa	1/320	0.3	Moodley et al., 2010
	4/97	4	Makgotloho et al., 2009
	5/56	9	Oosthuysen et al., 2014
Tunisia	68/72	94	Ben Nejma et al., 2006
	64/64	100	Ben Nejma et al., 2013
	43/69	62	Ben Jomaa-Jemili et al., 2013
Uganda	30/41	73	Kateete et al., 2011
Multicenter*	20/86	23	Breurec et al., 2011b

*Multicenter study which included Cameroon, Madagascar, Morocco, Niger and Senegal.
PVL, Panton-Valentine Leukocidin; no., Number.

across South Africa (Goering et al., 2008; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014), alongside other variants such as ST1173/ST1338-IV [2B] (Shittu et al., 2009). The ST8-IV [2B] clone in South Africa was identified from a variety of clinical infections, in particular, bacteraemia, skin and soft tissue and wound infections (Shittu et al., 2009; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014). An ST8-IVc [2B] strain (PVL-positive; spa type t062) was identified from a 4 day old male child with community-acquired invasive infection in Tunisia (Kechrid et al., 2011). Furthermore, ST8-V [5C] was described in Algeria (Antri

**FIGURE 2 | MRSA clones reported in Africa.** Each clonal complex is annotated with a colored circle. The number of studies conducted in each country is also indicated.

et al., 2011), Angola (Conceição et al., 2014), Ghana (Egyir et al., 2013), Nigeria (Shittu et al., 2011, 2012; Raji et al., 2013), São Tomé and Príncipe (Conceição et al., 2013), and South Africa (Oosthuysen et al., 2014). Other STs observed within the CC5-ST8 cluster include ST8-SCCmec-NT in Gabon (Schaumburg et al., 2014) and Nigeria (Shittu et al., 2012), ST94-IV [2B] described in Nigeria (Shittu et al., 2011) and ST97-SCCmec-NT in Tunisia (Ben Jomaa-Jemili et al., 2013). In addition, ST247-I [1B] was reported only in Tunisia (Ben Jomaa-Jemili et al., 2013)



and Nigeria (Raji et al., 2013), ST637-III [3A] in Algeria (Ramdani-bouguessa et al., 2006), ST1819-I [1B] in Tunisia (Ben Jomaa-Jemili et al., 2013), and ST2021-V [5C] in Ghana (Egyir et al., 2014b).

The “Brazilian/Hungarian clone” (ST239-III [3A]) is an hybrid of CC30 and CC8 based on a single large chromosomal replacement (Robinson and Enright, 2004), and ST241-III [3A] is a single locus variant (slv) of ST239-III [3A]. These two STs were identified concurrently in Algeria (Ramdani-bouguessa et al., 2006), Morocco, Niger and Senegal (Breurec et al., 2011b), and Tunisia (Ben Jomaa-Jemili et al., 2013). ST239-III [3A] has also been described in Ghana (Egyir et al., 2014b) and Kenya (Aiken et al., 2014), and consistently since 2001 in South Africa (Essa et al., 2009; Shittu et al., 2009; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014). A recent study detected ST239 with the SCCmec type III_{mercury} [3A] in a tertiary health care facility in South-West Nigeria (Raji et al., 2013). ST241-III [3A] is the dominant clone in North-East Nigeria (Okon et al., 2009; Shittu et al., 2011, 2012). Interestingly, three

SCCmec variants, ST239-IVa [2B], ST239/ST241-V [5C], and ST241-IV [2B], and associated with hospital-acquired infections were reported in Algeria (Ramdani-bouguessa et al., 2006), Niger (Breurec et al., 2011b), and Nigeria (Ghebremedhin et al., 2009).

Clonal Complex 22

In Africa, ST22 was identified only in Algeria (Ramdani-bouguessa et al., 2006), South Africa (Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014), and Tunisia (Ben Jomaa-Jemili et al., 2013). ST22-IV [2B] was related with hospital-associated infections in the Western Cape and KwaZulu-Natal provinces of South Africa. A variant of ST22 (ST22-V [5C]-PVL-positive) was also reported in an hospital in Western Cape, South Africa (Oosthuysen et al., 2014). The ST22 identified in Tunisia possessed a non-typeable SCCmec element (Ben Jomaa-Jemili et al., 2013). Besides, an ST636-SCCmec-NT isolate has also been reported in Algeria (Ramdani-bouguessa et al., 2006).

Clonal Complex 30

ST30-IVa [2B]-PVL-positive, also known as “South-West Pacific clone,” has been reported in Egypt (Enany et al., 2010), and a multicenter African study identified ST30-V [5C] only in Antananarivo, Madagascar (Breurec et al., 2011b). The hospital associated ST36-II [2A] (UK-EMRSA-16), was described only in South Africa (Goering et al., 2008; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014), while ST39-II [2A] a dlv was identified in an hospital in Algiers, Algeria (Djoudi et al., 2013), and Ile-Ife, South-West Nigeria (Shittu et al., 2012). MRSA assigned with these groups (ST36-II [2A] and ST39-II [2A]) were PVL-negative. Furthermore, two *SCCmec* variants, ST37-IVa [2B] and ST37-III [3A], were reported in Algeria (Ramdani-bouguessa et al., 2006) and Nigeria (Shittu et al., 2012), respectively.

Clonal Complex 45

ST45-IV [2B], the “Berlin clone,” was detected in an hospital in the KwaZulu-Natal (South Africa) during a multicenter surveillance study (Essa et al., 2009) and ST45-V [5C] was reported in mother-infant pairs in Gabon (Schaumburg et al., 2014). An MRSA with a non-typeable *SCCmec* associated with community-acquired infections has been identified in Tunisia (Ben Jomàa-Jemili et al., 2013). Finally, ST508-V [5C], a slv to ST45, and also associated with community-acquired infections was described in Ghana (Egyir et al., 2014a).

Clonal Complex 80

The CC80 was limited to three North African countries: Algeria, Egypt, and Tunisia (Table 2). The European clone, ST80-IV [2B]-PVL-positive, was first described in Algeria from both hospitalized and outpatients (Ramdani-bouguessa et al., 2006), and has continued to be the leading clone in the country (Ramdani-bouguessa et al., 2006; Bekkhoucha et al., 2009; Antri et al., 2011; Djoudi et al., 2013). ST80-IVc [2B] has been identified in Egypt (Enany et al., 2010), and Tunisia (Ben Slama et al., 2011; Ben Jomàa-Jemili et al., 2013). In addition, sequence types related to ST80 have been recovered from human clinical samples (ST153-*SCCmec*-NT, ST728-IVc [2B], ST1440-IVc [2B], and ST2563-IVc [2B]) (Kechrid et al., 2011; Ben Jomàa-Jemili et al., 2013), and nasal specimen of healthy sheep (ST153-*SCCmec*-NT) (Gharsa et al., 2012) in Tunisia. The afore-mentioned sequence types, ST728, ST1440 and ST2563 belonged to accessory gene regulator (*agr*) type III and were PVL-positive. Moreover, a PVL-negative ST80-IVc [2B] with *agr* type II has also been detected in Tunisia (Ben Jomàa-Jemili et al., 2013), and a PVL-negative ST635-IV [2B] in Algeria (Ramdani-bouguessa et al., 2006).

Clonal Complex 88

The “West Australia MRSA-2 clone” (WA-MRSA-2), ST88-IV [2B], was reported in both hospital and community settings in eight African countries; Angola (Conceição et al., 2014), Cameroon (Breurec et al., 2011b), Gabon (Schaumburg et al., 2011; Ateba Ngoa et al., 2012), Ghana (Egyir et al., 2013, 2014b), Madagascar (Breurec et al., 2011b), Niger (Breurec et al., 2011b), Nigeria (Ghebremedhin et al., 2009; Shittu et al., 2012; Raji et al.,

2013) and Senegal (Breurec et al., 2011b). The MRSA isolates from Angola possessed an *SCCmec* IVa [2B] element (Conceição et al., 2014). PVL-positive ST88-IV [2B] were detected in Nigeria (Ghebremedhin et al., 2009), and an *SCCmec* subtype ST88-IVa [2B] was identified among three health care workers and a patient in São Tomé and Príncipe (Conceição et al., 2013). The ST88-IV [2B] with *spa* type t3489 was also recovered from nasal samples of swine in Senegal (Fall et al., 2012). In addition, an *SCCmec* non-typeable ST88 was described from an out-patient in Algeria (Bekkhoucha et al., 2009), and a strain related to WA-MRSA-2 (ST1289-IV [2B]) was identified in Yaoundé, Cameroon (Breurec et al., 2011b).

Other Clonal Complexes

CC7, CC121, and CC152 have been reported in Africa. The PVL-negative ST789 (assigned with CC7) was identified in Angola (with *SCCmec* IV [2B]) (Conceição et al., 2014). However, in Ghana, ST789 was PVL-positive and carried an *SCCmec* IV element [2B] (Egyir et al., 2014b). An ST1010-PVL-positive (CC121) with non-typeable *SCCmec* element has only been described in Egypt (Enany et al., 2010). Furthermore, PVL-positive MRSA assigned to CC152 (ST152-*SCCmec*-NT) was reported in Nigeria (Shittu et al., 2012).

Discussion

MRSA has been reported in Africa, at least since 1978 (Scragg et al., 1978). This systematic review showed that adequate data on the molecular epidemiology of MRSA are limited, with reports from only 15 of the 54 African countries. No *spa* type was dominant, however, t042 and t044 were the major *spa* types identified in three North African countries (Table 2). Moreover, we did not observe a clear distinction between hospital- and community-associated MRSA clones in Africa which is in agreement with other investigations worldwide (Fossum Moen et al., 2013; Pasquale et al., 2013; Sherwood et al., 2013; Tavares et al., 2013). In this systematic review, the use of the current eBURST scheme grouped several African MRSA CCs (CC1, CC5, CC8, and CC7) into a single cluster, (CC5). This raises some concern on a suitable method for discrimination and grouping of *S. aureus* isolates. To overcome the above mentioned issue, whole genome sequencing approach might be the alternative (Dabul and Camargo, 2014).

Although a combination of factors could be responsible for the dissemination of clones between continents, increased movement of human population within or between countries might be one of the potential factors (Rogers et al., 2011). International travel could play a significant role in the transmission of MRSA, particularly the replacement of existing MRSA with fitter and more transmissible clones (Zhou et al., 2014). We observed that the predominant hospital-associated epidemic clones, EMRSA-15 [ST22-IV [2B]] and (EMRSA-16 [ST36-II [2A]]), in the United Kingdom (UK) (Johnson et al., 2005) were reported only in South Africa (Goering et al., 2008; Moodley et al., 2010; Jansen van Rensburg et al., 2011; Oosthuysen et al., 2014). Moreover, ST80-IV [2B] (the European clone) has consistently been recognized as the predominant PVL-positive MRSA clone in North Africa

(Ramdani-bouguessa et al., 2006; Bekkhoucha et al., 2009; Ben Nejma et al., 2009, 2013; Enany et al., 2010; Antri et al., 2011; Ben Slama et al., 2011; Ben Jomaa-Jemili et al., 2013; Djoudi et al., 2013). A recent report based on whole genome analysis provided strong evidence that the European ST80-IV [2B] was derived from a PVL-positive MSSA ancestor in sub-Saharan Africa that acquired the SCCmec IV element, and clonal spread was enhanced by increased transnational movement (Stegger et al., 2014). However, the factors responsible for the limited spread of the ST80-IV [2B] only in North Africa observed so far are unclear.

The SCCmec IV (and its subtypes) and SCCmec V were identified in several MRSA clones, and ST5 and ST8 clearly showed more diversity in terms of SCCmec types compared with other STs in Africa. The success of these SCCmec types (IV and V) could be due to their small sizes and low fitness costs (Enright et al., 2002; Okuma et al., 2002; Monecke et al., 2011). It is also noteworthy that the SCCmec types IVa and IVc were identified in genetically unrelated clones, e.g., ST5-IVa [2B] (CC5) in Algeria (Ramdani-bouguessa et al., 2006; Bekkhoucha et al., 2009), São Tomé and Príncipe (Conceição et al., 2013), ST8-IVc [2B] in Tunisia (CC5) (Kechrid et al., 2011), and ST37-IVa [2B] (CC30) in Algeria (Ramdani-bouguessa et al., 2006). This might suggest horizontal gene transfer or independent acquisition (Mašlaňová et al., 2013). Another interesting observation was the detection of the SCCmec type IVa and V in the hospital-associated ST239/ST241-III [3A] in Algeria (Ramdani-bouguessa et al., 2006), Nigeria (Ghebremedhin et al., 2009), and Niger (Breurec et al., 2011b). Since ancient MSSA strains for this ST have not been reported (Enright et al., 2002), our observation suggests that acquisition of these SCCmec types by MSSA is less likely, and points to the possible replacement of SCCmec type III with IV and V on the ST239/241 genome (Li et al., 2013).

Data on the epidemiology of MRSA in animals are limited in Africa (EL Seedy et al., 2012; Fall et al., 2012; Gharsa et al., 2012). Moreover, the genetic relatedness between human and animal MRSA has not been investigated (Table 2). It should be noted, however, that human-associated ST5-IV [2B], ST88-IV [2B], and ST153-SCCmec-NT have been reported from animals in Tunisia (Gharsa et al., 2012) and Senegal (Fall et al., 2012). Recently, human-associated *S. aureus* lineages were described in captive Chimpanzees in Gabon, Madagascar, Uganda and Zambia (Schaumburg et al., 2012, 2013; Nagel et al., 2013). Notably, a likely case of *S. aureus* transmission from a veterinarian to a chimpanzee from the same sanctuary was demonstrated (Schaumburg et al., 2012). Zoonotic transmission may constitute a major concern in Africa, where there is often substantial exposure to domesticated animals (Fall et al., 2012; Gharsa et al., 2012). Furthermore, animal-adapted clones might undergo further host-adaptive evolutionary changes, which could result in an epidemic spread of new and more virulent strains in the human population (Spoor et al., 2013). Other risk factors for animal to human MRSA transmission, which include contaminated environment (Verkade and Kluytmans, 2013) and meat products (Hamid and Youssef, 2013), have not been investigated in Africa. Livestock-associated MRSA are widespread in Europe, but the transmission of these strains to humans is

either rare or limited to people with direct contact with MRSA infected/carrier animals (Verkade and Kluytmans, 2013). Using whole genome sequencing, evidence of zoonotic transmission of MRSA harboring *mecC* was reported in Denmark (Harrison et al., 2013). The *mecC*-positive MRSA, initially known as a livestock MRSA belonging to the CC130, is recognized in both animals and humans in Europe (Paterson et al., 2014a). In addition, this clone has been implicated in severe infections in humans (Paterson et al., 2014b), resulting in one death (García-Garrote et al., 2014). The clinical importance of *mecC*-positive MRSA is not yet clear in Africa as data is unavailable. Therefore, we suggest that surveillance for MRSA should include detection of the *mecC* gene where *mecA* is not detected in resistant isolates.

This systematic review did not seek to provide comprehensive information on the burden of PVL-positive MRSA in Africa. However, it provided some interesting observations on their epidemiology in Africa such as the identification of PVL-positive isolates assigned with CC7 (originally classified with CC152) in Ghana (Egyir et al., 2014a), CC88-IV [2B] in Cameroon (Breurec et al., 2011b) and Nigeria (Ghebremedhin et al., 2009), ST612-IV [2B] in South Africa (Oosthuysen et al., 2014), and CC152 in Nigeria (Shittu et al., 2012). Until now, CC152 was only described in the Balkan region (Francois et al., 2008). The mode of acquisition of the *mecA* gene by ST152 is still unknown, but it might be explained by either its introduction through international travel or the acquisition of the methicillin resistance gene by PVL-positive MSSA, which is prevalent in West and Central Africa (Ruijmy et al., 2008; Okon et al., 2009; Breurec et al., 2011a; Schaumburg et al., 2011; Shittu et al., 2011, 2012; Egyir et al., 2014a). These observations highlight the need for further surveillance data (including information on community-acquired necrotizing pneumonia) to understand the epidemiology of PVL-associated *S. aureus* in both hospital and community settings on the African continent.

Conclusion

A number of pandemic MRSA clones were identified in Africa. In contrast, some MRSA clones are limited to specific countries or regions. Although the eBURST snapshot provided a description of the relationship between the MRSA clones reported in Africa and other lineages submitted into the MLST database from other continents, the objective of this review was not to understand the origin of MRSA clones in Africa, as this will require in depth analysis like whole genome sequencing. However, it did show that CC5 is the largest group and predominant in Africa. Nevertheless, the limited data available on MRSA in Africa draw attention to the need for increased surveillance of MRSA and molecular epidemiological studies. We strongly recommend improved co-operation between clinicians and microbiologists in Africa. This synergy could provide an understanding on the local epidemiology of MRSA. In addition, we strongly advocate the establishment of effective diagnostic microbiology facilities that will incorporate high-throughput technologies for monitoring the clonal expansion and dissemination of MRSA. In the meantime, increased networking through collaboration with

S. aureus reference centers could provide support for genotyping services to African countries with limited resources. Finally, population-based surveillances for MRSA are needed to evaluate the situation of community associated MRSA as well as studies on MRSA from animal hosts. To understand the origin of the newly emerged clones, MSSA genotyping needs to be incorporated with MRSA surveillance studies.

Author Contributions

MK, AS, and SMA initiated the project. SMA extracted the data and reviewed the articles with MK. SMA, AS, MN, and MK wrote the manuscript. All the authors reviewed the final version of the manuscript prior to submission for publication

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An assessment on DNA microarray and sequence-based methods for the characterization of methicillin-susceptible *Staphylococcus aureus* from Nigeria

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Staphylococcus aureus is an important human pathogen causing nosocomial and community-acquired infections worldwide. In the characterization of this opportunistic pathogen, DNA microarray hybridization technique is used as an alternative to sequence based genotyping to obtain a comprehensive assessment on the virulence, resistance determinants, and population structure. The objective of this study was to characterize a defined collection of *S. aureus* isolates from Nigeria using the microarray technique, and to assess the extent that it correlates with sequence-based genotyping methods. The clonal diversity and genomic content of 52 methicillin-susceptible *Staphylococcus aureus* (MSSA) were investigated by spa typing, MLST and DNA microarray hybridization. More than half (55.8%) of these isolates were associated with clonal complexes (CCs) typically associated with methicillin-resistant *S. aureus* (MRSA) clones i.e., CC1, CC5, CC8, CC30, and CC45. Certain genes linked with virulence (*hlgA* and *clfA*) and adherence (*ebpS*, *fnaB*, *sspA*, *sspB*, and *sspP*) were detected in all isolates. A number of genes or gene clusters were associated with distinct clonal types. The enterotoxin gene cluster (egc) was linked with CC5, CC25, CC30, CC45, and CC121, enterotoxin H gene (seh) with CC1, exfoliative toxin D gene (etd) with CC25 and CC80, and the epidermal cell differentiation inhibitor B gene (edinB) with CC25, CC80, and CC152. The excellent agreement between data from DNA microarray and MLST in the delineation of Nigerian MSSA isolates indicates that the microarray technique is a useful tool to provide information on antibiotic resistance, clonal diversity and virulence factors associated with infection and disease.

Keywords: *Staphylococcus aureus*, microarray, MLST, genotyping, Nigeria

Abbreviations: Agr, accessory gene regulator; CC, Clonal complex; CLSI, Clinical Laboratory Standards Institute; MSSA, Methicillin susceptible *Staphylococcus aureus*; MLST, Multilocus sequence typing; PVL, Panton-Valentine Leukocidin; *S.aureus*, *Staphylococcus aureus*; SCCmec, Staphylococcal chromosome cassette *mec*; spa, *Staphylococcus aureus* protein A; ST, Sequence Type.

INTRODUCTION

Staphylococcus aureus is implicated in a variety of human infections with high rates of morbidity and mortality (Lowy, 1998; Corey, 2009). In infection, *S. aureus* exhibits a coordinated and regulated expression for a wide variety of cell and surface-associated virulence factors (Foster and Höök, 1998; Novick, 2006). These factors mediate adherence to host cells and damaged tissue, facilitate tissue destruction and spreading, promote iron uptake and evasion of host immune system, as well as tissue damage (Skaar and Schneewind, 2004; Grumann et al., 2014). Recent studies in Cameroon (Kihla et al., 2014), Egypt (Ahmed et al., 2014), Gabon (Alabi et al., 2013), Nigeria (Jido and Garba, 2012; Oladeinde et al., 2013), South Africa (Groome et al., 2012; Naidoo et al., 2013), and Tanzania (Kayange et al., 2010; Mhada et al., 2012) have identified *S. aureus* as the main etiological agent for various infections in Africa. Moreover, this species has been recognized as one main cause of community-acquired neonatal sepsis in Africa (Waters et al., 2011). These studies clearly establish the important role of this major human pathogen in tropical Africa.

In many health care institutions in sub-Saharan Africa, the lack of skilled laboratory manpower and resources is a major constraint in the identification of bacterial pathogens from clinical samples. If such analysis can be provided at all, identification of *S. aureus* typically relies on phenotypic methods precluding in-depth strain characterization. Molecular analysis of clonal attribution and presence of single genes contained in *S. aureus* isolates have emerged in pilot studies from select African centers, areas and populations (Ateba Ngoa et al., 2012; Shittu et al., 2012; Seni et al., 2013; Aiken et al., 2014; Egir et al., 2014; Oosthuysen et al., 2014; Conceição et al., 2015; De Boeck et al., 2015; Kraef et al., 2015; Schaumburg et al., 2015). Nevertheless, in view of the impact of *S. aureus* disease in sub-Saharan Africa, the clonal characterization in concert with a comprehensive analysis of the hitherto ill-described virulence factor armamentarium of *S. aureus* isolates from this region is urgently warranted. Such analyses should target a broad spectrum of variable staphylococcal factors such as genes or gene clusters conferring antibiotic resistance, toxins, virulence, adhesion or immune evasion factors. These analyses have not been performed on a collection of *S. aureus* isolates in Nigeria, and reports from African countries are limited and only addressed a limited and select analytical spectrum (Raji et al., 2013; Aiken et al., 2014; Rovira et al., 2015).

The DNA microarray used for this analysis is a unique and comprehensive genotyping technique based on the analysis of 334 target sequences corresponding to approximately 170 distinct genes and their allelic variants. It enables the simultaneous identification of various gene classes including species markers, genes encoding resistance and virulence properties, exotoxin and adhesion factors, accessory gene regulator (*agr*), capsule, and SCCmec types (Monecke et al., 2011). Based on the observation of a high level of genetic diversity from previous investigations on methicillin-susceptible *S. aureus* (MSSA) in Nigeria (Shittu et al., 2011, 2012; Kolawole et al., 2013), we studied MSSA isolates obtained from various clinical sources in Nigeria using

this comprehensive, array-based approach to provide an insight on the major factors associated with infection and disease.

MATERIALS AND METHODS

Identification and Antibiotic Susceptibility Testing of *S. aureus* Isolates

The isolates ($n = 52$) were obtained from samples processed as part of surveillance activities in the microbiology laboratories of six health care institutions located in Ado-Ekiti, Ille-Ife, Osogbo, Lagos, and Ibadan in South-West Nigeria, and Maiduguri in North-East Nigeria. The duration of collection of isolates was from March 2009 to April, 2010. Only the isolates were analyzed in this study. Preliminary verification as *S. aureus* was based on colony characteristics on blood agar, positive results for catalase, coagulase and DNase tests. Twelve isolates from a previous study (Shittu et al., 2011) were also included in this investigation. Identification was confirmed by Matrix-Assisted Laser Desorption/Ionization-Time Of Flight analysis (MALDI-TOF). Susceptibility testing to penicillin (10 units), cefoxitin (30 µg), doxycycline (30 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), chloramphenicol (30 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg) were determined using the disk diffusion method according to the Clinical Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute (CLSI), 2009).

DNA Extraction

S. aureus genomic DNA was extracted from an 18–24 h old culture on sheep blood agar using lysis buffer and lysis enhancer (StaphyType Kit, Alere Technologies GmbH, Jena, Germany) and processed using a DNeasy tissue kit (Qiagen, Hilden, Germany).

Molecular Typing of the Isolates

Typing of *S. aureus* was based on sequencing of the hypervariable region of the protein A gene (*spa*). The *spa* types were determined using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany, version 2.1.1) (Harmsen et al., 2003). Multilocus sequence typing (MLST) was performed for one isolate of each *spa* type (Enright et al., 2000), as a *spa* type usually belongs to one sequence type (ST) with few exceptions due to homoplasies (Basset et al., 2009, 2012). The allelic profiles and STs were assigned using the MLST *S. aureus* database (www.mlst.net), and the sequence types of the remaining isolates were inferred from the derived MLST data.

DNA Microarray Hybridization

The DNA microarray of the StaphyType™ kit (Alere Technologies GmbH, Jena, Germany) was used in this study according to previously established protocols (Monecke et al., 2008). The isolates were grouped with various clonal complexes (CCs) by the imaging software Iconoclust based on comparison of hybridization profiles to a collection of reference strains previously characterized by MLST.

Splits Graph Construction

The SplitsTree algorithm (Huson and Bryant, 2006) and software was used to analyze the similarities between hybridization patterns, and network tree construction was performed using SplitsTree 4.10 on default settings (characters transformation, uncorrected P; distance transformation, Neighbor-Net; and variance, ordinary least squares).

RESULTS

Identification of *S. aureus* Isolates

A total of 52 MSSA (3 and 49 isolates from nasal and clinical sources, respectively) were analyzed (Table 1). The clinical isolates were obtained from wounds and associated infections ($n = 29$; 59.2%), urinary tract infections ($n = 6$; 12.2%), semen/infertility diagnosis ($n = 4$; 8.2%), ocular infections ($n = 3$; 6.1%), and pneumonia ($n = 2$; 4.1%). One isolate each was from otitis media, and blood related infections, while information on three isolates was not available. The clinical isolates were obtained from health care institutions located in Ile-Ife ($n = 26$; 53.1%), Osogbo ($n = 11$; 22.4%), Maiduguri ($n = 5$; 10.2%), Lagos ($n = 4$; 8.2%), Ibadan, and Ado-Ekiti ($n = 2$ isolates each; 4.1%).

Antibiotic Susceptibility Testing

All the isolates were susceptible to cefoxitin and 98.1% ($n = 51$) were resistant to penicillin. Only two isolates each exhibited resistance to chloramphenicol and gentamicin, and four to doxycycline. Intermediate susceptibility to clindamycin and erythromycin were identified in six and 21 isolates, respectively. The predominant antibiotype was resistance only to penicillin ($n = 23$; 44.2%), and resistance to penicillin with intermediate susceptibility to erythromycin ($n = 10$; 19.2%) (Table 1).

Sequence based Typing (spa and MLST)

A total of 26 spa types were identified among the 52 MSSA isolates and the most common were t318 ($n = 7$), t311 ($n = 5$), t084, t127, and t2304 ($n = 4$ each). Based on MLST, the MSSA were classified into 13 sequence types (STs) (Table 1).

DNA Microarray Analysis

The assay confirmed the identity of the isolates (*S. aureus*) by positive results for specific markers including rRNA (domain 1 of 23S rRNA), protein A (spa), glyceraldehyde 3-phosphate dehydrogenase (gapA), catalase A (katA), thermostable nuclease (nuc), and staphylococcal accessory regulator A (sarA) (Supplementary Materials 1, 2). The hybridization profiles revealed that the 52 MSSA isolates clustered in 12 different CCs. More than half (55.8%) of the CCs were associated with the genetic background common to the major methicillin-resistant *S. aureus* (MRSA) clones i.e., CC1 ($n = 6$ isolates), CC5 ($n = 9$), CC8 ($n = 4$), CC30 ($n = 8$), and CC45 ($n = 2$). The rest were assigned with CC7 ($n = 1$), CC15 ($n = 7$), CC25 ($n = 2$), CC80 ($n = 1$), CC97 ($n = 1$), CC121 ($n = 8$), and CC152 ($n = 3$).

Antibiotic Resistance Genes

A total of 69.2% ($n = 36$) of the isolates yielded a hybridization signal for the beta-lactamase gene (blaZ) and only 10 and

three isolates were positive for the tetracycline resistance genes (tetK and tetM), respectively. The two MSSA in CC8 which exhibited phenotypic resistance to chloramphenicol and gentamicin possessed the corresponding resistance genes (cat and aacA-aphD). In addition, the single CC80 isolate was positive for the lincosamide resistance gene (lnuA).

Accessory Gene Regulator and Capsular Typing

The distribution of agr/CCs/capsule types for the MSSA is indicated in Figure 1. Overall, 13 (25%) isolates assigned to different clonal lineages (CC7, CC8, CC25, CC45, CC97, and CC152) were associated with agr group I, 16 (30.2%; CC5 and CC15) with group II, and 15 (28.8%; CC1, CC30 and CC80) with group III. CC121 was the only representative for agr group IV ($n = 8$; 15.4%) (Table 1). The capsule type 8 was the most frequent and detected in 33 (63.5%) isolates affiliated with CC1, CC7, CC15, CC30, CC45, CC80, and CC121. The remaining isolates (20; 38.5%) belonged to capsule type 5 (assigned with CC5, CC8, CC25, CC97, and CC152).

Enterotoxin Genes

PVL-positive isolates ($n = 27$) belonged to CC1, CC5, CC15, CC30, CC80, CC121, and CC152 (Supplementary Material 1). Moreover, the lukF gene (haemolysin gamma; component B) was universally detected in all the CCs and the lukE genes was a common feature except with MSSA isolates in CC30, CC45, and CC152 (Supplementary Material 3). With respect to the carriage of superantigen genes, only three MSSA (one isolate in CC1 and two in CC45) tested positive for the toxic shock syndrome toxin gene (tsst-1) (Supplementary Material 1). All the isolates in this study lacked a hybridization signal for the enterotoxin E gene (see Supplementary Material 2) and the enterotoxin genes were not detected in MSSA assigned with CC80, CC97, and CC152. In the haemolysin gene family, almost all (98.1%) the isolates in the various CCs possessed the haemolysin alpha and delta genes (hla, hld), while the haemolysin beta gene (hlb) was identified in the various CCs except in CC15, CC45, and CC152.

Microbial Surface Components Recognizing Adhesive Matrix Molecule (MSCRAMM) Genes

All the isolates were negative for the surface protein involved in biofilm production (bap), but possessed the genes for the inter-cellular adhesion protein (icaA/C/D) (CC152 isolates were icaC negative). The genes for clumping factor A (clfA), cell surface elastin binding protein (ebps), fibronectin-binding protein A (fnbA) and proteases (sspA, sspB, and sspP) were detected in all the isolates (Supplementary Materials 1, 3).

Splits Tree Analysis

The analysis identified four main clusters (CC5/CC25; CC8/CC97; CC1/CC7/CC80, and CC30/CC45) indicating the phylogenetic relationship between the isolates (Figure 2).

TABLE 1 | Characterization of the methicillin-susceptible *S. aureus* (MSSA) from Nigeria based on antibiotyping, microarray analysis, spa typing, and MLST.

Isolate Number	Location	Sample/Clinical diagnosis	Antibiogram	Score (%) (Alere)	agr/Clonal complex (Alere)	spa type	MLST
11486_24	Ile-Ife	Wound Infection	PEN	93.8	agr_III/CC1	t127 [¶]	ST1
AB5_28	Osogbo	UTI	PEN, ERY(i)	92.8	agr_III/CC1	t127	ST1
Aro_29	Osogbo	Semen	PEN	94.3	agr_III/CC1	t127	ST1
MD16_4	Not available	Not available	PEN	94.3	agr_III/CC1	t127	ST1
MD20_8*	Maiduguri	Wound infection	PEN, ERY(i), CC(i)	93.5	agr_III/CC1	t321 [¶]	ST1
6056_34	Osogbo	Urine	PEN	93.9	agr_III/CC1	t10433 [¶]	ST1
5675_6	Ile-Ife	Abscess	PEN	91.8	agr_II/CC5	t311	ST5
5221_7	Ile-Ife	Urine	PEN, ERY(i), SXT(i)	93.8	agr_II/CC5	t311	ST5
D23_15	Ile-Ife	Pneumonia	PEN	92.8	agr_II/CC5	t311	ST5
D42_17	Ile-Ife	Adenocarcinoma	PEN, ERY(i)	92.4	agr_II/CC5	t311	ST5
D46_18	Ile-Ife	Wound Infection	PEN, ERY(i)	92.2	agr_II/CC5	t311 [¶]	ST5
1423_36	Osogbo	Urine	PEN, ERY(i)	93.8	agr_II/CC5	t442 [¶]	ST5
D19_14	Ile-Ife	Not available	PEN	93.5	agr_II/CC5	t688 [¶]	ST5
Asu29_27	Osogbo	Otitis media	PEN, DO, ERY(i)	91.9	agr_II/CC5	t1277 [¶]	ST5
3211_30	Osogbo	Wound Infection	PEN	92.9	agr_II/CC5	t3235 [¶]	ST5
6773_11	Ile-Ife	Wound Infection	PEN	93.6	agr_I/CC7	t091 [¶]	ST789
N37_19	Ile-Ife	Erythematous lesion	PEN, SXT	90	agr_I/CC8	t064 [¶]	ST2427
UC45_37	Ibadan	Eye swab	PEN, GM, CHL, SXT	91.3	agr_I/CC8	t2658 [¶]	ST2427
55_40	Ado-Ekiti	Wound Infection	PEN, DO(i), GM, CHL, SXT	90.3	agr_I/CC8	t2658	ST2427
OS39_13*	Lagos	Semen/Infertility	PEN, DO(i), SXT	91.7	agr_I/CC8	t951 [¶]	ST8
11450_23	Ile-Ife	Sputum	PEN	92.9	agr_II/CC15	t084	ST15
5189_1	Ile-Ife	Advanced Cancer	PEN	94	agr_II/CC15	t084	ST15
189_2	Ile-Ife	Blood	PEN, DO(i), ERY(i)	93.9	agr_II/CC15	t084 [¶]	ST15
4013_14*	Ile-Ife	Wound infection	PEN	94.9	agr_II/CC15	t084	ST15
5828_5	Ile-Ife	Abscess	susceptible to all antibiotics tested	94.4	agr_II/CC15	t2216 [¶]	ST15
MD7_3*	Maiduguri	Semen/Infertility	PEN, ERY(i)	94.6	agr_II/CC15	t2216	ST15
MD19_11*	Maiduguri	Wound infection	PEN	94.4	agr_II/CC15	t2216	ST15
S13_6*	Lagos	Urinary Tract Infection	PEN, ERY(i), SXT	93.1	agr_I/CC25	t3772 [¶]	ST25
3925_32	Osogbo	Wound Infection	PEN, ERY(i), SXT	91.4	agr_I/CC25	t10183 [¶]	ST25
6073_3	Not available	Not available	PEN, DO	91.7	agr_III/CC30	t017 [¶]	ST30
D30_16	Ile-Ife	Cholecystitis	PEN	94.7	agr_III/CC30	t318 [¶]	ST30
6506_2	Osogbo	Wound Infection	PEN, ERY(i), CC(i)	91.4	agr_III/CC30	t318	ST30
NS7708_22	Ile-Ife	Nasal swab/screening	PEN, ERY(i)	94.7	agr_III/CC30	t318	ST30
54_39	Ado-Ekiti	Wound Infection	PEN	94.4	agr_III/CC30	t318	ST30
S12_7*	Lagos	Wound infection	PEN, ERY(i)	93.8	agr_III/CC30	t318	ST30
OS41_10*	Lagos	Wound infection	PEN	93.1	agr_III/CC30	t318	ST30
6330_4	Ile-Ife	Osteomyelitis	PEN	94.3	agr_III/CC30	t318	ST30
NS2907_21	Ile-Ife	Nasal swab/screening	PEN, ERY(i), CC(i)	91.8	agr_I/CC45	t095 [¶]	ST508
3950_33	Osogbo	Urine	PEN	91.5	agr_I/CC45	t10434 [¶]	ST508
GDC_35	Osogbo	Semen	PEN	94.9	agr_III /CC80	t934 [¶]	ST80
MD14_2*	Maiduguri	Wound infection	PEN, DO(i)	92.9	agr_I/CC97	t458 [¶]	ST97
ZU_26	Ile-Ife	Unavailable	PEN, ERY(i)	89.3	agr_IV/CC121	t159 [¶]	ST121
UC47_38	Ibadan	Eye swab	PEN, DO, ERY(i), CC(i)	92.1	agr_IV/CC121	t159	ST121
W10_5*	Ile-Ife	Wound infection	PEN, ERY(i)	91.8	agr_IV/CC121	t314 [¶]	ST121
MD_9*	Maiduguri	Wound infection	PEN, ERY(i), CC(i)	92.1	agr_IV/CC121	t314	ST121
6376_3	Ile-Ife	Abscess	PEN, DO(i)	93.1	agr_IV/CC121	t2304 [¶]	ST121
6540_10	Ile-Ife	Bone Marrow Infection	PEN	93.5	agr_IV/CC121	t2304	ST121
NS2986_20	Ile-Ife	Nasal swab/screening	PEN, DO, ERY(i), CC(i), SXT(i)	92.8	agr_IV/CC121	t2304	ST121
3920_31	Osogbo	Aspirate	PEN	92.8	agr_IV/CC121	t2304	ST121
D3_12	Ile-Ife	Cervical cancer	PEN, ERY(i)	94.6	agr_I/ST152	t355 [¶]	ST152

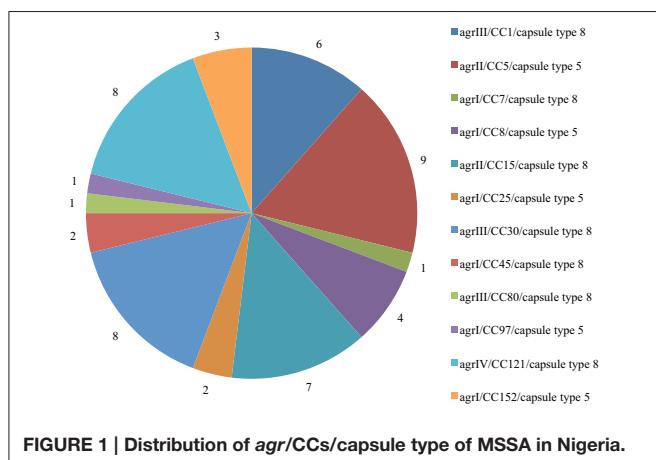
(Continued)

TABLE 1 | Continued

Isolate Number	Location	Sample/Clinical diagnosis	Antibiogram	Score (%) (Alere)	agr/Clonal complex (Alere)	spa type	MLST
D12_13	Ile-Ife	Ocular infection	PEN	94.6	agr_I/ST152	t355	ST152
W7.2_4*	Ile-Ife	Wound infection	PEN	96.3	agr_I/ST152	t355	ST152

*. *S. aureus* analyzed in a previous study; (i): intermediate susceptibility; agr, accessory gene regulator; PEN, Penicillin; DO, Doxycycline; ERY, Erythromycin; CLI, Clindamycin; GEN, Gentamicin; CHL, Chloramphenicol; SXT, trimethoprim/sulfamethoxazole; CC, Clonal Complex; ST, Sequence type.

†spa types selected for Multilocus sequence typing (MLST); Sequence types (STs) of the remaining isolates were inferred from the derived MLST data.

**FIGURE 1 |** Distribution of agr/CCs/capsule type of MSSA in Nigeria.

DISCUSSION

We observed a complete agreement between DNA microarray analysis and MLST in the delineation of the isolates (Table 1), showing that the hybridization profile could be used to predict the lineages. Furthermore, the heterogeneous and divergent nature of the isolates observed in this study provided evidence on the overall higher diversity of MSSA compared with MRSA (Deurenberg and Stobberingh, 2008; Goering et al., 2008; Ghasemzadeh-Moghaddam et al., 2011; Ruffing et al., 2012; Blomfeldt et al., 2013; Rasmussen et al., 2013, 2014). In Nigeria, many diagnostic microbiology laboratories rely on the disc diffusion technique for antibiotic susceptibility testing, but this protocol does not provide information on the nature of resistance genes. The antibiotic susceptibility results observed in this study were in accordance with the corresponding resistance gene profiles by DNA microarray. MSSA isolates that exhibited full resistance to trimethoprim-sulfamethoxazole clustered with CC8 and CC25, but were *dfrS1* negative indicating that a different mechanism could be attributed to resistance. A recent study (Nurjadi et al., 2014) has provided strong evidence that the *dfrG* gene is the predominant trimethoprim resistance determinant on *S. aureus* in Africa. Overall, resistant determinants for antibiotics, heavy metal and quaternary ammonium compounds were observed more often in CC8 than other CCs (Supplementary Materials 1, 3).

The accessory gene regulator (*agr*) and capsule typing methods are useful front-line tools for the characterization of *S. aureus* (Goerke et al., 2005). Hybridization signals for *agr* type I and IV were observed for one, three, and four isolates grouped

with CC25, CC152, and CC121, respectively (Supplementary Materials 1, 2). This could be attributed to possible cross-hybridization as the alleles for the two *agr* types are closely related (Monecke et al., 2010). Our observations on CCs and *agr* groups were similar to previous reports on MSSA in five major African towns (Breurec et al., 2010), Gabon (Ateba Ngoa et al., 2012), and Nigeria (Ghebremedhin et al., 2009; Kolawole et al., 2013). In addition, our study also support the view (Wright et al., 2005; Holtfreter et al., 2007; Rasmussen et al., 2014) that an *agr* type may be detected in isolates which are assigned to genetically diverse CCs, whereas, it is also associated with specific CCs. The dominance of capsule type 8 in MSSA is consistent with data from Gabon (Schaumburg et al., 2011), Norway (Blomfeldt et al., 2013), and Sweden (Rasmussen et al., 2013, 2014).

Staphylococcal enterotoxins are typically encoded by genes located on mobile genetic elements (Baba et al., 2002). The *egc* cluster (*seg+sei+sem+sen+seo+seu*) is located on the genomic island vSA β and reported to be associated with specific clonal types regardless of the geographical strain distribution (Lindsay and Holden, 2006). In this investigation, the *egc*-enterotoxin gene cluster was a unique feature for CC5, CC25, CC30, CC45, and CC121. Previous studies have indicated that the cluster is predominantly present in MSSA assigned with CC5, CC25, CC30, and CC45 (Van Trijp et al., 2010; Rasmussen et al., 2013). The *seh* gene is linked to the staphylococcal cassette chromosome *mec* (SCC*mec* elements) and reported to be restricted to the CC1 genomic background (Baba et al., 2002). Moreover, the *seh* gene has also been reported mainly in MSSA-CC30 (Blomfeldt et al., 2013). Nevertheless, our observation on *seh*-positive MSSA-CC1 is in agreement with previous reports (Chen et al., 2013; Rasmussen et al., 2013).

The genes associated with staphylococcal complement inhibitor (*scn*) and staphylokinase (*sak*) were also widely distributed across the CCs but CC15 isolates were *sak* gene negative. Virulence associated with the exfoliative toxins has been identified to cause epidermal cleavage in staphylococcal scalded skin syndrome (SSSS) and bullous impetigo (Ladhani et al., 1999). The exfoliative toxin D (ETD) is a 27-kDa protein which causes epidermal blisters in newborn mice (Yamasaki et al., 2006). The epidermal cell differentiation factors (EDIN) target and inhibit the small host protein RhoA, a master regulator of the host cell actin cytoskeleton (Inoue et al., 1991; Jaffe and Hall, 2005; Aktories, 2011). Furthermore, the edin-isoform (*edinB*) and *etd* genes are located in tandem in a *S. aureus* *etd* pathogenicity island in a chromosome of *etd*-positive *S. aureus* strains (Yamaguchi et al., 2002). A strong association of the *etd* gene with invasive CC25 *S. aureus* isolates has also been

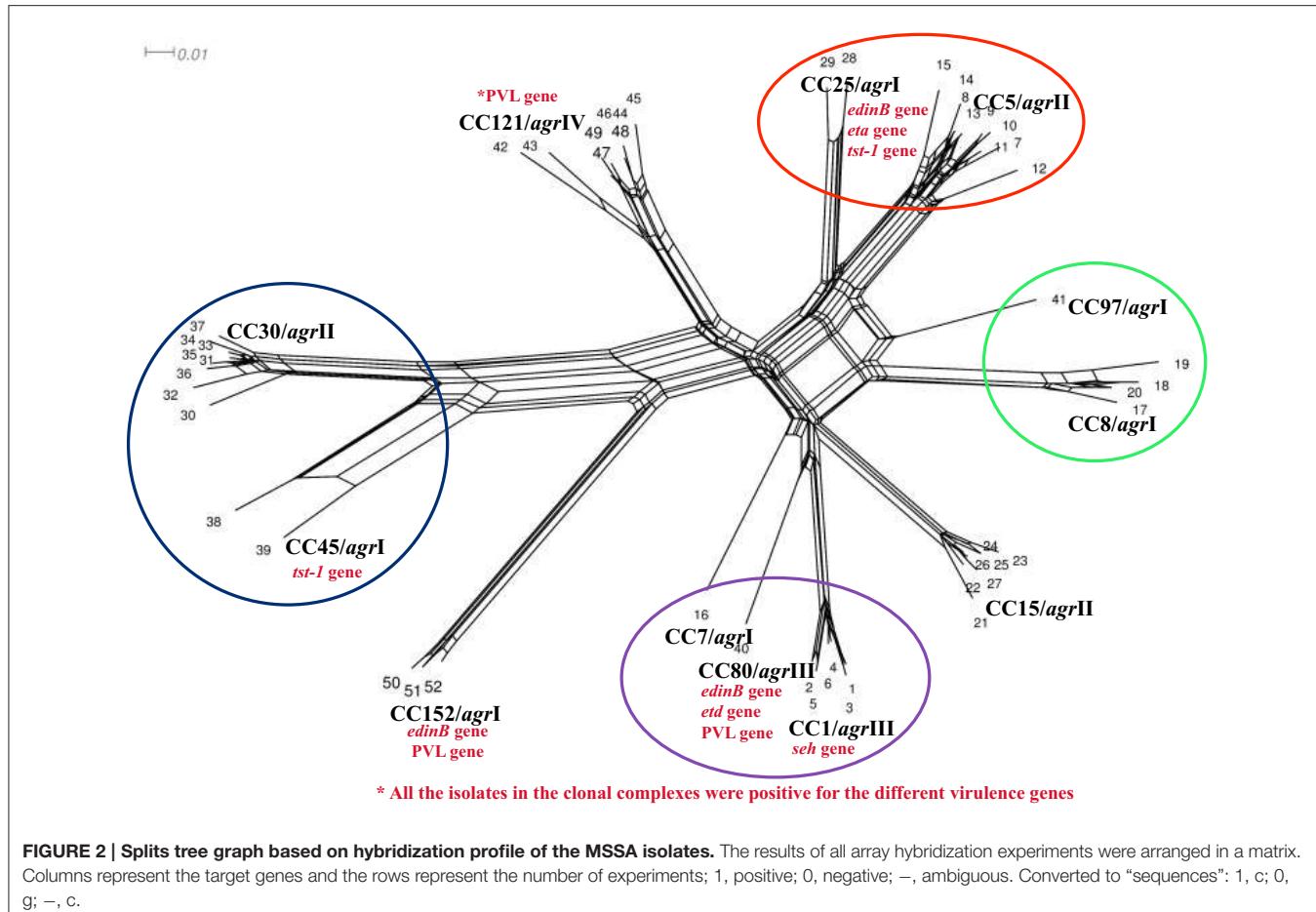


FIGURE 2 | Splits tree graph based on hybridization profile of the MSSA isolates. The results of all array hybridization experiments were arranged in a matrix. Columns represent the target genes and the rows represent the number of experiments; 1, positive; 0, negative; –, ambiguous. Converted to “sequences”: 1, c; 0, g; –, c.

reported. In this study, all the isolates assigned with CC25 and CC80 were *etd*-positive, which is in agreement with a previous study in Nigeria (Shittu et al., 2011). Moreover, MSSA grouped with CC25, CC80, and CC152 were *edinB* positive but CC152 isolates were *etd* negative. Our observations were similar to a study on the distribution of the *edin* gene in *S. aureus* from diabetic foot ulcers (Messad et al., 2013). A study in MSSA bacteremia isolates in Sweden showed that the collagen binding protein (Cna) was detected in CC1, CC30, and CC45. Our report identified the gene in isolates assigned with CC1, CC30, CC45, CC121, and CC152.

Our study has a number of limitations. Although all isolates were of human origin, and the large majority was obtained from clinical samples, a clear distinction between commensal and clinical strains could not be made based on the available information. An association of isolates within the context of endemicity i.e., nosocomial vs. community associated infections, is also not clear. Furthermore, whereas the microarray analytical database is exhaustive, well-characterized, and validated with isolates from all continents, the attribution of CCs is based on the hybridization reactions and resulting microarray profile rather than gene sequencing, and a positive signal does not necessarily imply the presence of gene product (e.g., protein). In addition, the microarray method was unable to separate ST8 from ST2427.

This might be due to the close phylogenetic relation of both STs as they are single locus variants (ST8: 3–3–1–1–4–4–3 and ST2427: 3–3–297–1–4–4–3). Finally, with a collection of 52 isolates studied, and a large number of genes and genetic profile ascertained by microarray, the potential for individual statistical comparisons is limited. Yet, with this comprehensive genetic-analytical approach performed on a clinical isolate collection obtained from patients of various medical institutions in a sub-Saharan African country, Nigeria, a number of important observations could be made which clearly characterize and demarcate the clonal distribution as well as the virulence gene equipment.

More than one half (55.8%; $n = 29$) of these MSSA isolates were associated with a genetic background which is attributable to classic methicillin-resistant *S. aureus* (MRSA) clones. PVL-positive isolates were identified in seven of the 12 CCs. Moreover, toxin genes were observed to be distributed mainly with certain clonal types, and in agreement with previous investigations (Holtfreter et al., 2007; Monecke et al., 2008). Antibiotic resistance gene profiles of the isolates by the DNA microarray demonstrated concordant results with data on antibiotic susceptibility testing. The array-based, comprehensive approach has been shown to yield such diverse CC and gene specific results on an isolate collection from sub-Saharan Africa.

Overall, microarray analysis proved to be a useful tool to provide useful information on antibiotic resistance, population structure and various virulence factor profiles associated with infection and disease. It is assumed that these findings might be useful for a better understanding of clinical staphylococcal disease presentation, patient care and for assistance in outbreak investigation in health care institutions in a country such as Nigeria. Moreover, our study also underlines the need for further trials employing well-controlled, prospectively collected clinical isolates to delineate the genetic pathogen profile in conjunction with the clinical disease presentation in sub-Saharan Africa.

AUTHOR CONTRIBUTIONS

AS, UR, GP, FS, LM, and MH conceived the study, OO, KO, AR conducted the sample collection and preliminary identification of the isolates. AS performed the microarray technique, AS and UR analyzed the microarray data, and AS wrote the manuscript (with input from all authors). All authors read and approved the final version 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/fmcb.2015.01160>

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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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MRSA Prevalence and Associated Risk Factors among Health-Care Workers in Non-outbreak Situations in the Dutch-German EUREGIO

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Preventing the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities is a major infection control target. However, only a few studies have assessed the potential role of healthcare workers (HCWs) for MRSA dissemination. To investigate the MRSA prevalence and the risk factors for MRSA colonization among HCWs, nasopharyngeal swabs were taken between June 2010 and January 2011 from 726 employees from nine acute care hospitals with different care levels within the German part of a Dutch-German border region (EUREGIO). The isolated MRSA strains were investigated using spa typing. The overall MRSA prevalence among HCWs in a non-outbreak situation was 4.6% (33 of 726), and was higher in nurses (5.6%, 29 of 514) than in physicians (1.2%, 1 of 83). Possible risk factors associated with MRSA colonization were a known history of MRSA carriage and the presence of acne. Intensive contact with patients may facilitate MRSA transmission between patients and HCWs. Furthermore, an accumulation of risk factors was accompanied by an increased MRSA prevalence in HCW.

Keywords: MRSA, decolonization, MRSA risk factors, personnel, staff, EurSafety health-net

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important hospital-associated (HA) pathogens (Voss and Doebele, 1995). Although its prevalence among patients decreased in several European countries (e.g., UK, France, and Germany) during the last years, the MRSA prevalence increased in other countries (e.g., Norway and Poland; <http://ecdc.europa.eu>). In healthcare institutions, MRSA can be transmitted between patients, or through the hands, clothes, or equipment of healthcare workers (HCWs), and the environment (Haley et al., 1982; Hardy et al., 2006; Henderson, 2006). Furthermore, it has been reported that HCWs have been the source of MRSA outbreaks in several cases (Vonberg et al., 2006). In a systematic review of Albricht et al., in which 127 studies and outbreak reports published between January 1980 and March 2006 were reviewed, transmission of MRSA from HCWs to patients was likely in 63 of 68 (93%) studies as shown by genotyping (Albricht and Harbarth, 2008). The average MRSA prevalence among HCWs in these 127 studies was 4.6%, with a broad range from 0 to 59% (95% CI 1.0–8.2%) between

countries and institutions. Notably, the MRSA prevalence rates were found to be higher in endemic situations (8.1%) compared to outbreak situations (3.9%). However, it has to be stated that different designs of the studies included limit the informative value of this review (Albrich and Harbarth, 2008).

The implementation of MRSA screening among HCWs has been shown to have a positive impact on outbreak management (Peters et al., 1999; Blok et al., 2003; Ben-David et al., 2008). Hence, infection control guidelines from many countries, including Germany, recommend a systematic screening of HCWs in the case of an acute outbreak of MRSA (Peters et al., 1999, 2014). In the Netherlands, having one of the lowest MRSA prevalence rates in Europe (<http://ecdc.europa.eu>), MRSA screening of HCWs is not conducted regularly, but in addition to outbreak cases, the national “Search-and-Destroy” strategy (www.wip.nl) recommends screening for further risk groups among personnel, such as staff returning from work in healthcare institutions abroad (Blok et al., 2003).

However, the “Search-and-Destroy” strategy remains subject of discussion (Hawkins et al., 2011; Hill, 2011) due to the lack of reliable prevalence data and an insufficient differentiation between stable and transient MRSA carriage. To the best of our knowledge, only a few studies have focused on the prevalence of MRSA colonization among HCWs in non-outbreak situations and the risk factors for stable and transient MRSA carriage in Germany. Furthermore, none of these studies involved HCWs from more than one hospital (Witte et al., 2005; Kaminski et al., 2007; Reich-Schupke et al., 2010). Therefore, the aim of the present study was to investigate the MRSA prevalence among HCWs in several hospitals in non-outbreak situations in the German part of a Dutch-German border region (“EUREGIO”) within the Dutch-German prevention network EurSafety Health-net (www.eursafety.eu) and assess data on (including non-occupational-related) risk factors for MRSA colonization among hospital staff.

MATERIALS AND METHODS

Participants

All hospitals in the German part of the EUREGIO were asked to participate in this study, and representatives of them were informed at the annual meeting of the German EUREGIO within the Dutch-German prevention network EurSafety Health-net. They discussed the study in their, respectively, hospital committees before the hospitals decided to participate in the study. Participation was voluntary for all HCWs and the directorate had to accept participation.

Between June 2010 and January 2011, nine acute care hospitals with different care levels within the German part of a Dutch-German EUREGIO participated in this study; two hospitals of basic care (No. 5 and 8), four hospitals of secondary care (No. 1, 4, 6, and 7) and three specialized clinics (No. 2, 3, and 9). The number of staff members within the hospitals ranged from 33 to 1640 and their capacities from 20 to 653 beds (Table 1). The participating hospitals had a total capacity of 2249 beds and employed 5264 staff members, including 667 physicians and 2325 nurses. In this study, both the medical staff and employees

working outside direct patient-care, such as administration, maintenance, and cleaning, were encouraged to participate, and a questionnaire was used to obtain the needed information from the participants. Overall, 726 employees participated in this study (83 physicians, 514 nurses, 109 other staff members, and 20 participants of unknown professional group), covering 12% of the hospitals physicians and 22% of the nurses, respectively. As shown in Table 1, the study’s coverage of medical staff varies between 9 and 91% between the different hospitals.

Survey of Risk Factors

The participants’ risk factors were collected using a standardized paper-based questionnaire (Supplementary Material). The following potential risk-factors for MRSA colonization were surveyed: history of MRSA carriage, profession, contact with MRSA carriers in a professional or private setting (with and without protective clothing), involvement in home-care of relatives, former professional occupation in a country known to be endemic for community-associated MRSA (CA-MRSA), dermatosis/diseases of the skin, chronic inflammatory bowel diseases (IBD), diseases of the upper respiratory tract, antibiotic therapy within the last 6 months, acute diseases (at the time of risk factor assessment), inpatient treatment within the last 6 or 12 months, contact with domestic or farm animals, and consumption of raw meat within the last 12 h. All questionnaires were processed using random pseudonyms that could only be decoded by the duty hygiene officer or the company medical officer.

Nasopharyngeal Swabs

HCWs can be MRSA positive after a working shift due to short-term contact with patients (transient MRSA carriers), while other HCWs are MRSA carriers for longer than 24 h (stable MRSA carriers) (Cookson et al., 1989; van Cleef et al., 2011). To differentiate between transient and stable MRSA carriers, swabs were taken twice on different days from all HCWs. Nasopharyngeal swabs were collected by trained personnel on Monday after an off-work weekend and subsequently on Wednesday before starting to work. To minimize the risk of considering a stable carrier as transient due to false-negative swabs, a third validation swab was collected in cases of discrepant results between the first and second sample. If the validation swab was positive, the HCW was considered to be a stable MRSA carrier, and if the validation swab was negative, the participant was regarded as a transient carrier. In addition, as skin colonization influenced decolonization-strategy, skin swabs (from the axilla and the groin) were taken from stable carriers immediately after being identified as such.

MRSA Isolation and spa Typing

Swabs were applied to chromogenic media (BioMérieux, Nürtingen, Germany) the same day as the swab was collected and incubated for 48 h at 37°C. *S. aureus* colonies were tested for antibiotic resistance using the Vitek 2 (BioMérieux, Nürtingen, Germany) and the presence of the MRSA-specific penicillin binding protein 2a was tested by

TABLE 1 | Overview of the participating hospitals and its personnel coverage shown as different professional groups.

Hospital	Beds (n)	Physicians	Nurses	Total medical staff	Others	Unknown	Total staff
1	282	Total (n)	72	347	419	346	765
		Study (n%)	15 (21)	54 (16)	69 (17)	31 (9)	2 (2)
2	20	Total (n)	11	20	31	3	34
		Study (n%)	11 (100)	14 (70)	25 (81)	2 (67)	5 (5)
3	20	Total (n)	10	33	43	4	47
		Study (n%)	6 (60)	33 (100)	39 (91)	4 (100)	1 (1)
4	582	Total (n)	231	653	884	756	1640
		Study (n%)	5 (2)	81 (12)	86 (10)	5 (1)	91 (6)
5	271	Total (n)	63	210	273	212	485
		Study (n%)	29 (46)	183 (87)	212 (78)	63 (30)	1 (1)
6	405	Total (n)	135	484	619	469	1088
		Study (n%)	2 (2)	52 (11)	54 (9)	3 (1)	0 (0)
7	361	Total (n)	95	387	482	378	860
		Study (n%)	2 (2)	58 (15)	60 (12)	0 (0)	60 (7)
8	185	Total (n)	37	120	157	37	194
		Study (n%)	6 (16)	19 (16)	25 (16)	0 (0)	6 (6)
9	123	Total (n)	13	84	97	68	165
		Study (n%)	7 (54)	20 (24)	27 (28)	1 (2)	5 (5)

a latex-agglutination-test (Oxoid, Cambridge, UK). Every first MRSA isolate was further characterized using *S. aureus* protein A gene (*spa*) typing as described by Mellmann et al. (2008). Analysis was performed using the Staph Type™ software (Ridom GmbH, Münster, Germany) (Mellmann et al., 2007).

Decolonization

If HCWs were identified as stable MRSA carriers, a decolonization protocol was started. They were asked to use mupirocin ointment intranasally and to gurgle with an octenidine-based solution, both thrice a day during a period of 6 days (Friday to Wednesday or Wednesday to Monday) and to attend a healthcare professional during the decolonization period. Moreover, all MRSA carriers were advised to daily change and wash clothes and bed-linen during the decolonization therapy. Those participants with skin colonization (additionally to nasopharyngeal colonization), as indicated by positive inguinal or axillary swabs, were also asked to daily wash themselves with octenidine-based soap.

Statistical Analysis

Data processing was performed using Microsoft Excel (Microsoft Corp., Redmont, USA) and statistical analyses were conducted with PASW Statistics (IBM Corp., Amonk, USA). Due to the small case numbers, statistical significance of risk factors was calculated in a univariate analysis with Fisher's exact test and $p < 0.05$ were regarded to be significant. To identify independent

risk-factors, a multivariate analysis was performed using multiple logistic regression for all variable with $p < 0.2$ in univariate analysis and $p < 0.05$ were considered to be significant.

Ethics

This study of screening HCW was approved by the Medical Chamber's of Westfalen-Lippe and Medical Faculty of the University of Münster's research ethic committee (2006-268-f-S). In all participating hospitals, the responsible member of the board of directors agreed to conduct the study. The employees participated in this study on a voluntary basis and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki after the study's principal investigator and local infection control manager held meetings to inform the participant about the study.

RESULTS

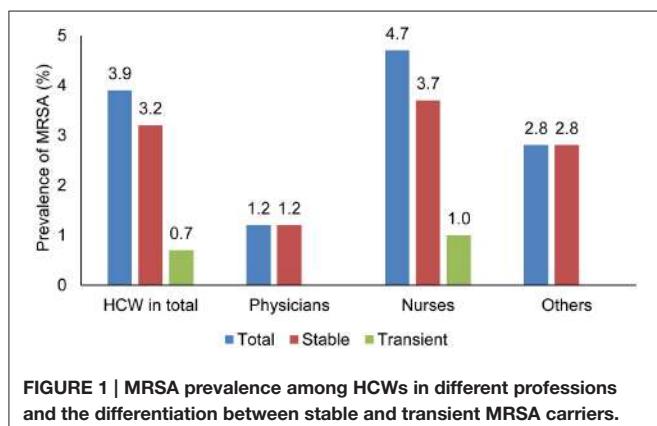
Swabs

At least two nasopharyngeal swabs from each of the 726 HCWs were collected. In 21 HCWs, both swabs were MRSA positive, and in 12 HCWs only one swab was positive for MRSA. A third "validation"-swab was required for these 12 HCWs. As five participants refused to give permission to take a third swab, and started already with decolonization, persistence of colonization could not be determined for these HCWs (Table 2).

TABLE 2 | MRSA colonization among healthcare-workers in the EUREGIO screened twice for nasopharyngeal carriage.

Result**	Number	Percentage	Validation swab*		
			Participation	Positive swab	Negative swab***
(-/-)	693	95.45	NA	NA	NA
(+/+)	21	2.89	NA	NA	NA
(-/+)	4	0.55	2 of 4	0	2
(+/-)	8	1.11	5 of 8	2	3
Total	726	-	-	-	-

*Validation swab to confirm or exclude stable MRSA colonization among HCWs with discordant results of initial two nasopharyngeal swabs; **MRSA test result (-/-), negative in two nasopharyngeal swabs; (+/+) MRSA detected in two nasopharyngeal swabs; (-/+ and (+/-), MRSA detected in one of two nasopharyngeal swabs, a validation swab was taken in these cases; ***NA, not applicable.

**FIGURE 1 |** MRSA prevalence among HCWs in different professions and the differentiation between stable and transient MRSA carriers.

MRSA Prevalence

In 33 of the 726 HCWs at least one swab was MRSA positive. Consequently, the MRSA prevalence was 4.6%. Two of the seven participants with discrepant swab results had a positive validation swab. Thus, 23 HCWs appeared to be stable carriers, whereas 5 HCWs were transient carriers resulting in a prevalence of 3.2% for stable carriers and 0.7% for transient carriers. In total, 17.9% of the detected cases of MRSA colonization were transient according to the applied definition.

The prevalence of MRSA differed for the type of healthcare profession. Among physicians, the MRSA prevalence was lower (1.2%, 1 of 83) compared to nurses (5.6%, 29 of 514). In nurses, stable MRSA carriage was found in 3.7% (19 of 514) of the cases, while transient MRSA carriage was observed in 1.0% (5 of 514) (**Figure 1**). Interestingly, stable MRSA positivity was observed in 2.8% (3 of 109) of other staff members who had no direct contact with patients.

spa Typing

Nine different *spa* types were observed among the 34 MRSA isolates [t003 ($n = 13$), t032 ($n = 11$), t151 ($n = 3$), t004 ($n = 1$), t020 ($n = 1$), t022 ($n = 2$), t179 ($n = 1$), t2261 ($n = 1$), and t6015 ($n = 1$)]. One HCW was colonized with two genetically non-related MRSA strains (*spa* type t004 and t032). Most frequently,

typical HA-MRSA strains with *spa* type t003 (38%) and t032 (32%) were observed. The observation of these *spa* types is in accordance with the most prevalent *spa* types found among patients in the study region (Köck et al., 2009).

Risk Factors for MRSA Colonization

For analysis of the risk factors for MRSA colonization in HCWs, all participants with at least one positive swab were included. The statistical analysis of the risk factors is shown in **Table 3**. Previous MRSA colonization ($p = 0.01$, CI = 2.2–24.8), acne ($p = 0.049$, CI = 1.008–19.07) and chronic IBD ($p = 0.005$, CI = 3.66–1457.33) were significantly associated with MRSA carriage among HCWs in the multivariate analysis.

For the evaluation of risk factor groups, all risk factors with an odds ratio >1 in the univariate analysis were taken into account. In order to analyze a possible impact of the accumulation of risk factors, all participants were grouped in three categories: 0, 1, to 3, or >3 risk factors. In total, 12.4% of the staff members were free of all risk factors analyzed, for the majority of participants (78.4%) 1–3 risk factors were applicable, and 9.2% of the HCWs were associated with more than three risk factors. The differences in MRSA prevalence according to the risk factor group are shown in **Figure 2**.

Decolonization

Only stable MRSA carriers were decolonized. Five participants who did not participate in the validation screening were excluded from this analysis, and two HCWs wished to be treated outside the study. So, 21 HCWs participated in the controlled decolonization. Two were colonized with MRSA on the skin and underwent the entire decolonization measures including skin washes. Nineteen HCWs (90%) underwent nasopharyngeal decolonization only. All control swabs after the decolonization attempt were negative. Six of the 21 HCWs (29%), among those the HCWs with skin-colonization, agreed on re-swabbing after 1 month, while nine HCWs (39%) were re-swabbed after 6 months. All of these control swabs were negative and confirmed the initial test result.

DISCUSSION

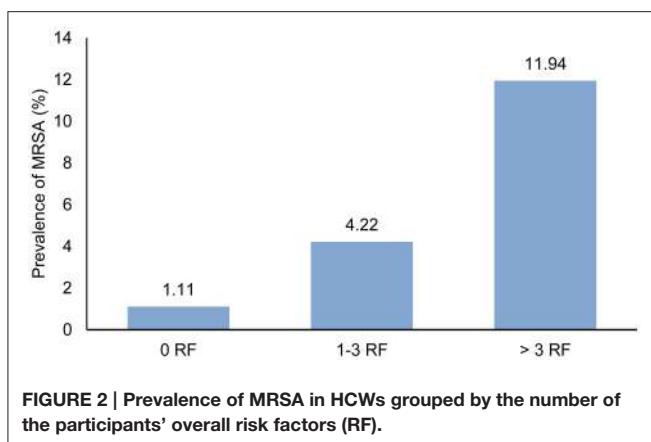
A 4.6% MRSA prevalence was observed among HCWs in the present study, which was lower than described by Albrich et al. (8.1% outside outbreak situations in several different countries), but similar to the results of Kaminski et al. (4.6% inside outbreak situations) in Germany in 2001 and 2002 (Kaminski et al., 2007; Albrich and Harbarth, 2008). Intensive training of healthcare personnel and an increased public awareness toward MRSA in the framework of the EUREGIO MRSA-net (Friedrich et al., 2008) may have contributed to the successful prevention of MRSA transmission in- and out-side outbreak situations.

The MRSA prevalence among HCWs in the EUREGIO is high compared to the MRSA prevalence observed among patients upon hospital admission. As Köck et al. have shown, MRSA admission prevalence rates in patients are approximately 2.5 times lower than in HCWs (Köck et al., 2010). In consequence, HCWs are at higher risk for MRSA colonization than the general

TABLE 3 | Risk-factor analysis for MRSA colonization in HCWs.

Risk factor	MRSA negative (<i>n</i> = 693)		MRSA positive (<i>n</i> = 33)		Fisher's exact test		Logistic regression*	
	Odds ratio	<i>p</i> (FE)*	Odds ratio	<i>p</i> (FE)*	95%CI (FE)	Odds ratio	<i>p</i> (FE)	95%CI (FE)
Physicians	82	1	0.23	0.162	0.31–1.72	0.7	0.7	0.63–7.79
Nurses	485	29	3.11	0.030	1.08–8.96	2.81	0.15	0.69–11.43
Staff without direct contact with patients	109	3	0.55	0.460	0.17–1.85	—	—	—
Unknown profession	20	0	0.97	1.000	0.96–0.98	—	—	—
Previous MRSA carrier	16	5	7.56	0.002	2.58–22.09	7.44	0.01	2.2–24.8
Occupation on ICU	102	9	2.17	0.077	0.98–4.81	2.05	0.12	0.84–4.96
Occupation with new-born	16	1	1.32	0.551	0.17–10.28	—	—	—
Occupation in foreign countries	3	0	1.00	1.000	1–1	—	—	—
Contact with MRSA carriers without protective clothing	68	4	1.31	0.549	0.45–3.84	—	—	—
Contact with MRSA carriers with protective clothing	227	16	1.92	0.088	0.95–8.87	1.04	0.94	0.39–2.7
Home-care of relatives	14	2	3.13	0.162	0.68–14.37	4.28	0.097	0.77–23.76
Contact with MRSA carriers at home	7	2	6.32	0.059	1.26–31.7	6.63	0.063	0.9–48.91
Atopic dermatitis	33	3	2.00	0.221	0.58–6.90	—	—	—
Paronchymia	1	0	1.00	1.000	1–1	—	—	—
Acne	13	3	5.23	0.032	1.42–19.34	4.38	0.049	1.008–19.07
Otitis	4	1	5.38	0.208	0.59–49.55	—	—	—
Open wounds	5	0	1.00	1.000	1–1	—	—	—
Chronic inflammatory bowel disease	1	1	21.63	0.089	1.32–353.62	73.12	0.005	3.66–1457.33
Sinusitis	23	0	0.97	0.619	0.96–0.98	—	—	—
Diabetes mellitus	8	1	2.68	0.344	0.325–22.05	—	—	—
Rhinitis	25	2	1.72	0.350	0.39–7.61	—	—	—
Other chronic diseases	64	3	0.98	1.000	0.29–3.3	—	—	—
Antibiotics	81	4	1.04	1.000	0.36–3.04	—	—	—
Hospitalization for >24 h in the last 6 months	23	1	0.91	1.000	0.12–6.95	—	—	—
Hospitalization for >24 h in the last 6–12 months	24	0	0.97	0.620	0.95–0.98	—	—	—
Hospitalization for >24 h in a foreign country	0	1	0.45	0.715	0.6–3.37	—	—	—
Contact with farm animals	27	2	1.59	0.384	0.36–7	—	—	—
Thereof contact with pigs	17	1	1.24	0.570	0.16–9.63	—	—	—
Contact with domestic animals	265	16	1.52	0.270	0.76–3.06	—	—	—
Contact with raw meat within last 12 h	62	5	1.82	0.218	0.678–4.88	—	—	—
Acute illness	32	3	2.07	0.209	0.6–7.13	—	—	—

*Risk factors with *p* < 0.2 in univariate analysis (Fisher's exact test) were included in logistic regression.



population due to the constant contact exposure to known or unknown MRSA-positive patients. This is also underpinned by the typing results of MRSA isolated from HCWs. In this study, mainly *spa* types t003 and t032, associated with the classical German healthcare-associated MRSA lineages CC5 and CC22, respectively, were observed. Transmission between patients and HCWs is likely, as these *spa* types also predominated among regional patients at the same time (Köck et al., 2013). By contrast, livestock-associated MRSA (LA-MRSA), e.g., *spa* type t011 and t034, which are the third and fourth most prevalent *spa* types among patients in the German part of the EUREGIO (Köck et al., 2011, 2013), could not be detected at all in HCWs. Since all MRSA isolates from patients in the EUREGIO are characterized, *spa* types observed in HCWs and patients in the corresponding hospital could be compared. The most prevalent *spa* types observed among HCWs were: t003 (38.2%), t032 (32.4%), t151 (8.8%), t022 (5.9%), t004, t020, t179, t2261, and t6015 (each 2.9%), while *spa* types t003 (24.7%), t032 (19.1%), t011 (13.1%), t034 (9.2%), and t020 (2.5%) were most prevalent in patients in the EUREGIO. This discrepancy of the distribution of *spa* types in patients and HCWs can be explained by the observations that the risk for LA-MRSA-colonization is 168 times higher for people who work daily with pigs than for family members living on a farm and that transmission of LA-MRSA between humans outside farms is limited (Cuny et al., 2009). The reason therefore is still unknown. Among the HCWs screened in this study, only 4% had contact with livestock.

In the different professional groups, our observations confirm the results found in previous studies, which showed a higher MRSA prevalence in nurses compared to medical doctors (Kaminski et al., 2007; Albrich and Harbarth, 2008). HCWs are likely to act more frequently as vectors, rather than being the main source of MRSA transmission. Furthermore, MRSA can be transmitted both from nurses to patients and from patients to nurses. Although colonized HCWs appeared to be most often transiently carriers, they may become stable carriers if they have sinusitis, or chronic dermatitis, leading to MRSA transmission over a longer time period (Dulon et al., 2014). The discussion on who is transmitting MRSA to whom has also been discussed in relation with MRSA observed in (pet) animals and possible transmission to humans. A Dutch study

showed that a dog living in close contact with a nurse became colonized with MRSA resulting in recurrent MRSA colonization of the nurse (van Duijkeren et al., 2004). In addition, a narrative review concluded that both dogs and cats can serve as vectors for MRSA transmission (Bramble et al., 2011). Consequently, it can be hypothesized that close physical contact with patients, e.g., washing and dressing, contributes to a higher MRSA transmission rate between patients and HCWs. The high MRSA prevalence among employees working in non-patient related activities (2.8%), also higher than in medical doctors (1.2%), seems to contradict this assumption. A possible explanation for this could be that non-patient related activities, e.g., cleaning personnel, is exposed to patients and especially patient fluids and materials in toilets and bathrooms, but may be less informed about the protocols to prevent indirect MRSA transmission. Furthermore, two of the three MRSA-positive samples from people with non-patient related activities in this study were either in contact with MRSA-positive relatives, or cared for relatives at home. Both these risk factors had a high odds ratio in the univariate analysis, but were not statistically significant risk factors for MRSA colonization.

It would be interesting to know the MRSA prevalence among surgeons and the staff from surgical wards. Unfortunately, the present study was underpowered to detect such possible differences. This was due to the fact that collection of data on the profession of the participants was not mandatory. From 85 participants, it is known that they were medical doctors. Of these 85 medical doctors, it was possible in only 39 cases to distinguish between surgeons and non-surgeons, and analyses resulted in 15 surgeons and 24 non-surgeons. Only one of these 15 surgeons was MRSA positive.

S. aureus colonization may be dependent on both host and bacterial factors. Furthermore, it has been reported that after decolonization, stable carriers often become re-colonized with the same *S. aureus* strain, whereas non-carriers resist experimental colonization. Long-term carriers of *S. aureus* were reported to carry *S. aureus* between 70 days and 8 years (Brown et al., 2014). Two MRSA swabs were taken from HCWs in order to differentiate between transient and stable carriage. Previously, it has been shown that transient carriage is especially found when HCWs are swabbed during or after their shifts (Cookson et al., 1989). Even though HCWs were sampled before starting their shifts, we observed heterogeneous swab results, indicating transient carriage among 5 of the 28 MRSA-positive HCWs. Besides transient carriage, heterogeneous results may also be the consequence of false-negative results in one swab of an actual stable carrier due to a sampling error, the less than 100% sensitivity of the nasopharyngeal swabs (Kunori et al., 2002), or the lack of the use of a semi-selective broth before application of the swabs on chromogenic media (Bocher et al., 2010) as used in the present study. To exclude false-negative results, a third validation swab was taken. This validation swab confirmed negative swab results, and thereby a stable carriage rate of 69.7%. So, 17.9% of all MRSA carriers proved to be transient carriers, and we conclude that differentiation between transient and stable carriers seems appropriate and reduces the number of HCWs who needed to be included in decolonization therapies.

By contrast, abdication of differentiation might overestimate actual MRSA prevalence in HCWs and lead to unnecessary decolonization.

According to the multivariate analysis, a positive anamnesis of MRSA carriage, acne and chronic IBD were possible risk factors for MRSA colonization in HCWs. As the latter only refers to one MRSA positive HCW and one MRSA negative HCW, its risk impact is not clear and should be confirmed using a larger cohort of individuals suffering from IBD. The other two possible risk factors confirmed observations in previous studies (Albrich and Harbarth, 2008). The fact that a positive anamnesis is a highly significant ($p = 0.01$) risk factor, encourages attempts for long-term control of MRSA-positive HCWs. Although we have shown that all HCWs could be decolonized initially, a positive MRSA anamnesis still increases the risk for re-colonization or resurgence of suppressed colonization. The reason for re-colonization/resurge is unclear, but a possible vulnerability for MRSA colonization and frequent long-term failure of topical decolonization therapies might contribute to it (Ammerlaan et al., 2009). As shown before, chronic skin diseases, such as acne in the present study, are known risk factors for MRSA colonization (Berthelot et al., 2003). As the number of individuals included in our study is rather small, the factors identified as possible risk factors should be confirmed in a larger group.

The drawback of this study was the great variation in the participation rate of HCWs among the hospitals, e.g., in hospital no. 6 only 9% of the HCWs participated, whereas in hospital no. 3 the participation rate was 91%. This variation in the participation rate of HCWs was due to different factors. Our aim was to screen approximately 100 persons per hospital, or less if the hospital was smaller. We argued with hospital directorates that not less than 100 persons per hospital should participate in order to enlarge the acceptability of the study. The directorate was concerned that the hospital would be understaffed because of the expected high rates of MRSA-positive employees and the fact that MRSA-positive employees had to be decolonized and could not work for a few days. In the future, well-organized campaigns focusing on the benefit for the hospital to participate in studies, such as ours, may take away their concern. Furthermore, the number of participants

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was also dependent on the commitment and the networking of the representatives of the hospitals. If these representatives were committed and networked well, which is much easier in smaller hospitals, more employees could be screened. To the best of our knowledge, there were no studies in Germany in 2012 in which more physicians were included for MRSA screening.

In this study, the MRSA prevalence among HCWs in a non-outbreak situation is not as high as observed in other studies, but still higher compared to the MRSA prevalence in patients at hospital admission. We showed that 70% of HCWs were stable MRSA carriers, whereas 30% “lost” MRSA in control swabs taken without decolonization attempts and indicating transient colonization/contamination. Since a higher MRSA prevalence in nurses compared to medical doctors, it can be suggested that close physical contact with patients contributes to a higher MRSA transmission rate between patients and HCWs.

AUTHOR CONTRIBUTIONS

RS, RD, RK, RH, AJ, JR, and AF planned the study, collected and analyzed the data and wrote the manuscript.

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The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmib.2016.01273>

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Dissemination of Novel Antimicrobial Resistance Mechanisms through the Insertion Sequence Mediated Spread of Metabolic Genes

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The widely used biocide triclosan selectively targets FabI, the NADH-dependent trans-2-enoyl-acyl carrier protein (ACP) reductase, which is also an important target for the development of narrow spectrum antibiotics. The analysis of triclosan resistant *Staphylococcus aureus* isolates had previously shown that in about half of the strains, the mechanism of triclosan resistance consists on the heterologous duplication of the triclosan target gene due to the acquisition of an additional *fabI* allele derived from *Staphylococcus haemolyticus* (*sh-fabI*). In the current work, the genomic sequencing of 10 of these strains allowed the characterization of two novel composite transposons TnSha1 and TnSha2 involved in the spread of *sh-fabI*. TnSha1 harbors one copy of IS1272, whereas TnSha2 is a 11.7 kb plasmid carrying TnSha1 present either as plasmid or in an integrated form generally flanked by two IS1272 elements. The target and mechanism of integration for IS1272 and TnSha1 are novel and include targeting of DNA secondary structures, generation of blunt-end deletions of the stem-loop and absence of target duplication. Database analyses showed widespread occurrence of these two elements in chromosomes and plasmids, with TnSha1 mainly in *S. aureus* and with TnSha2 mainly in *S. haemolyticus* and *S. epidermidis*. The acquisition of resistance by means of an insertion sequence-based mobilization and consequent duplication of drug-target metabolic genes, as observed here for *sh-fabI*, is highly reminiscent of the situation with the *ileS2* gene conferring mupirocin resistance, and the *dfrA* and *dfrG* genes conferring trimethoprim resistance both of which are mobilized by IS257. These three examples, which show similar mechanisms and levels of spread of metabolic genes linked to IS elements, highlight the importance of this genetic strategy for recruitment and rapid distribution of novel resistance mechanisms in staphylococci.

Keywords: insertion sequence, transposon, antimicrobial drug resistance, metabolism, resistance risk, *fabI*, IS5 family, IS1182 family

INTRODUCTION

The NADH-dependent *trans*-2-enoyl-acyl carrier protein (ACP) reductase FabI is one of the highly conserved enzymes of the bacterial fatty-acids biosynthesis. The FabI enzyme has been recognized as a novel and promising candidate drug target (Payne et al., 2001; Lu and Tonge, 2008) given the absence of a eukaryotic orthologue and its essential role in the growth of bacterial cells (Heath et al., 2001; Ji et al., 2004); a concept recently challenged by the observation that some bacteria do not require biosynthesis of fatty acids during infection of the host (Brinster et al., 2009). Due to the interest in using FabI as a drug target concerns have been raised about the large scale use of the biocide triclosan which targets the active site of FabI (Schweizer, 2001; Hijazi et al., 2016); these concerns also encompass the wider risk that biocide use *per se* may have on antimicrobial drug resistance (Oggioni et al., 2012, 2013, 2015; Coelho et al., 2013; Maillard et al., 2013; Morrissey et al., 2014). In the specific case considered here the concern is due to the fact that resistance to triclosan is in most bacterial species mediated by mutation of the promoter region or coding sequence of *fabI* (Heath et al., 1999; Slater-Radost et al., 2001; Ciusa et al., 2012; Oggioni et al., 2012; Grandgirard et al., 2015). In *Staphylococcus aureus* about half of resistant isolates have a novel type of triclosan resistance mechanism which is based on the presence of an alternative copy of *fabI* derived from *Staphylococcus haemolyticus* (*sh-fabI*) (Ciusa et al., 2012). In previous work, we reported that the *sh-fabI* gene appears to be part of a 3022 bp transposable element most probably mobilized by a single copy of the insertion sequence (IS) 1272 (Ciusa et al., 2012). IS1272 was originally identified in *S. haemolyticus* during investigation of homology matches to a truncated IS which is part of the *mec* cassette (Archer et al., 1994, 1996; Tonouchi et al., 1994). IS1272 is part of the IS1182 family of insertion sequences (Siguier et al., 2015) and apart from that truncated version found in the *mec* element is absent from *S. aureus*.

It has long been known that IS elements are able to transpose and/or change the expression of nearby or neighboring genes, that they are significantly involved in plasmid and chromosomal recombination, and that they provide the basic structure of many transposons and mobile resistance elements (Siguier et al., 2014, 2015). Soon after the discovery of ISs, it was observed that IS elements were able to transpose resistance genes between replicons (Hedges and Jacob, 1974; Barth et al., 1976). Most of the attention in the more recent sequence based work on antimicrobial resistance has still focussed on acquired “resistance”-genes which confer resistance either by target modification, inactivation of the antimicrobial compound or efflux; this is in part due to the difficulty of defining housekeeping genes identified in high throughput or metagenomic datasets as true “resistance” genes (Martinez et al., 2015). All of this “resistance”-gene oriented work has therefore overshadowed other mechanisms of resistance including those based on heterodiploidy for a metabolic gene (either resistant or susceptible alleles) which, especially in staphylococci, appears to be a mechanism with low fitness cost (Andersson, 2006; Oggioni et al., 2012). The classic example of such heterodiploidy is the presence of an additional copy of a dihydrofolate reductase

(*dhfA*) gene on conjugative elements, which thereby confers resistance to trimethoprim; these include the plasmid located *dhfA* gene which is transposed in *E. coli* by Tn7 (Barth et al., 1976), the Tn4003 transposon of *S. aureus* where the *dfrA* gene is mobilized by IS257 (Needham et al., 1995), and the more recently discovered transposable unit that comprises *dfrG* and IS256 which is located on the Tn5801 of different species (León-Sampedro et al., 2016). A more recent example in *S. aureus* involves resistance to mupirocin (pseudomonic acid), a potent inhibitor of the isoleucyl tRNA synthetase; this is conferred by an additional plasmid-encoded *mupA/ileS2* gene, which is again mobilized by IS257 (Gilbart et al., 1993; Woodford et al., 1998). With mupirocin, a disinfectant utilized for skin decontamination of MRSA staphylococci, this increased occurrence of *mupA/ileS* genes in clinical settings where mupirocin was used for decolonization has led to changes in disinfection policies (Hetem and Bonten, 2013). Even if the presence of additional *mupA/ileS* genes were found to confer a fitness defect to *S. aureus*, recent modeling experiments have predicted long-term increases in the prevalence of mupirocin-resistant phenotypes (MupR) given the “universal” use of mupirocin (Deeny et al., 2015).

In order to investigate the nature, sequence conservation, epidemiological distribution, and target site specificity of the 3022 bp transposable element carrying the *sh-fabI* gene we have sequenced the genomes of a series of triclosan resistant *S. aureus* isolates (Ciusa et al., 2012) and compared these data to the vast database of published genomes. The aims of this study were to conduct an in depth characterization of the *sh-fabI* carrying element, and to put this work into context with the other resistance mechanisms in *S. aureus* also based on IS mobilization of metabolic genes, which, in this species, appears to be a highly flexible means for the recruitment and rapid spread of novel resistance traits.

MATERIALS AND METHODS

Bacterial Strains

Sixty-five *S. aureus* strains with reduced susceptibility to triclosan were previously identified by performing standard MIC and MBC assays upon a collection of 1602 clinical isolates (Ciusa et al., 2012; Furi et al., 2013; Grandgirard et al., 2015; Oggioni et al., 2015). Ten out of the 28 isolates carrying the *sh-fabI* gene were selected from this collection for this work (Table 1; Ciusa et al., 2012).

Whole Genome Sequencing and Bioinformatic Analysis

The entire genome of 10 *S. aureus* clinical isolates (Table 1) with reduced susceptibility to triclosan and positive for *sh-fabI* detection by PCR were sequenced as previously described (Ciusa et al., 2012; Table 1). Short reads were assembled using ABySS (British Columbia Cancer Agency, Vancouver, Canada; ver. 1.3.5), improved using the multi-reference based scaffolder MeDuSa (Bosi et al., 2015) and subsequent TnShal sequence identification was performed using NCBI’s BLAST. 2.3.0+ (Altschul et al., 1997). The sequence of the prototype TnShal element corresponds to position 3908 to 887 of GenBank

TABLE 1 | Relevant information of the ten sequenced *S. aureus* isolates (Ciusa et al., 2012).

Strain	TnSha1 insertion site	MLST	Country	Year	MIC (mg/L)	MBC (mg/L)	MRSA
QBR-102278-1091	D	12	Japan	2002	4	32	MSSA
QBR-102278-1107	G	1	Australia	2002	4	32	MSSA
QBR-102278-1203	E	1	France	2002	2	16	MSSA
QBR-102278-1619	A	8	Spain	2002	4	32	MSSA
QBR-102278-2092	A2	8	Canada	2003	4	32	MSSA
QBR-102278-2210	F	83	Mexico	2003	1	32	MSSA
QBR-102278-2351	A2, and B	3	Brazil	2003	8	32	MSSA
QBR-102278-2365	C	8	Brazil	2003	2	32	MSSA
QBR-102278-2376	C	8	Argentina	2003	4	32	MSSA
QBR-102278-2605	A2	8	Japan	2003	32	64	MRSA

accession JQ712986 relative to *S. aureus* strain QBR-102278-1619. No complete sequence of TnSha2 is present in existing complete genomes in GenBank. One of the plasmid versions of TnSha2, which shows a contig break within *fabI*, corresponds to GenBank accession JCAZ01000023 of *S. aureus* strain M0227 (adzpz-supercont1.20.C23). Genbank BLAST searches for TnSha1 and TnSha2 elements were last accessed in December 2015. DNA secondary structures have been predicted by means of the RNAstructure Web Server (Reuter and Mathews, 2010). TnSha1 targets A through G have been confirmed as gene terminator loops by direct visualization of RNA-seq alignment data retrieved from the NCBI Sequence Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/Traces/sra/>). More precisely Illumina HiSeq data which were previously generated by sequencing of whole RNA (rRNA depleted) extracted from *S. aureus* ATCC51811 (SRA Experiment ID: ERP005246; SRA Sample ID: ERS421566) (Fagerlund et al., 2014) were aligned to genomes of *S. aureus* COL, GenBank accession no. CP000046, and *S. aureus* MW2, GenBank accession no. BA000033, using BWA-MEM (Li, 2014). The bootstrap maximum likelihood tree was obtained using MEGA6 with default parameters. Sequence Types (ST) were defined using the Multilocus Sequence Typing (MLST) web-based service of the Center for Genomic Epidemiology (Lyngby, Denmark; MLST allele sequence and profile data were obtained from: <http://pubmlst.org/>; Larsen et al., 2012). Contigs containing the TnSha2 element were screened to identify plasmid sequences by means of the PlasmidFinder web-based service of the Center for Genomic Epidemiology (Ver. 1.3; Lyngby, Denmark).

Molecular Analysis of Potential Transfer Intermediates

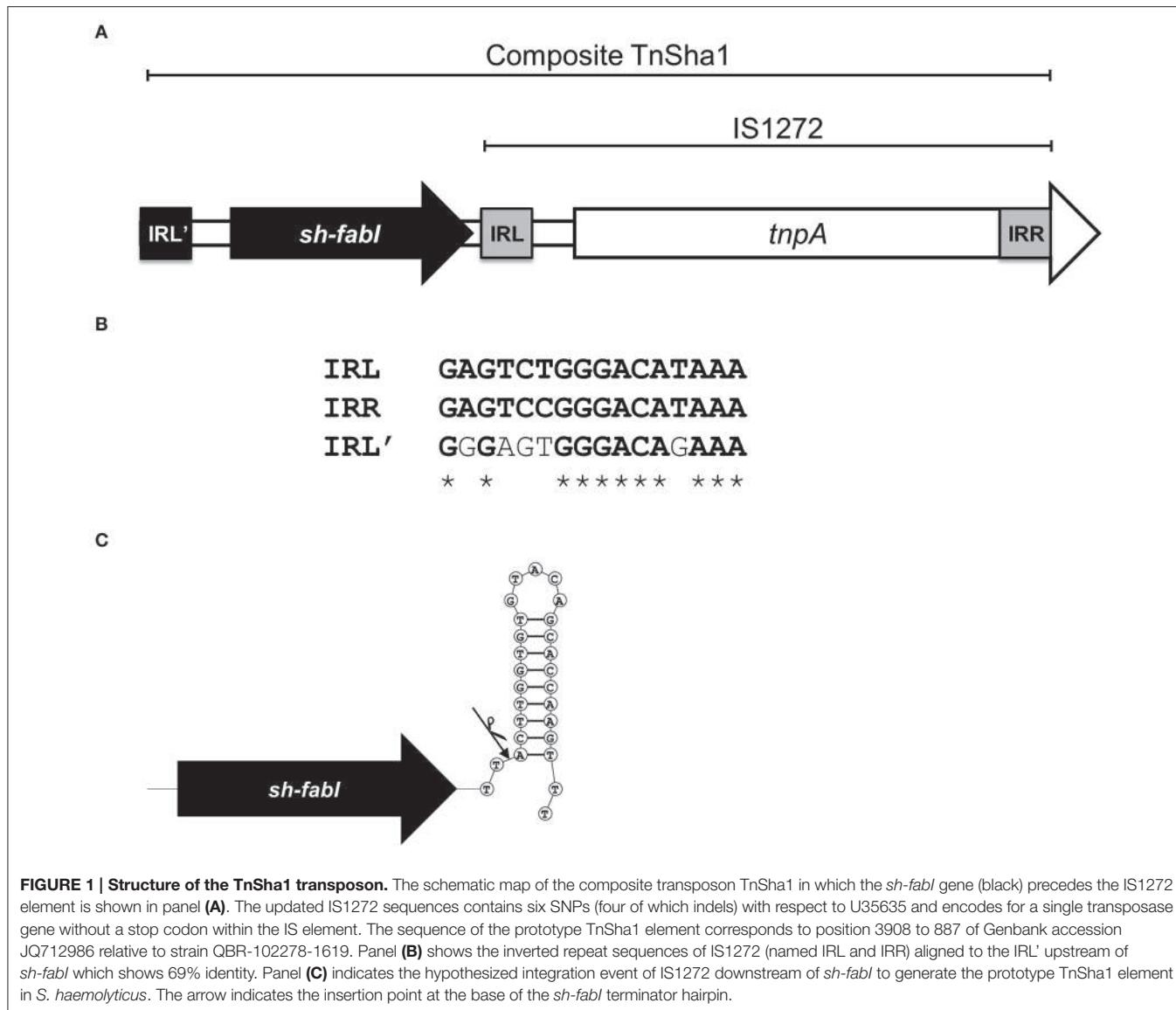
Genomic DNA was extracted from *S. aureus* strains in the exponential phase of growth using the High Pure PCR template preparation kit (Roche Diagnostics, Germany). Prior to DNA purification the bacterial strains were grown in MHB (Muller Hinton Broth; Beckton Dickinson) in the presence of sub-MIC concentrations of triclosan (2 mg/L for strain QBR-102278-1619 and 4 mg/L for QBR-102278-2351) or following Mitomycin C induction (1 µg/L) (triclosan PHR1338; Sigma-Aldrich) (Ciusa et al., 2012; Oggioni et al., 2012). Negative controls grown in

MHB medium only were also used. A FAM labeled TaqMan probe was designed to detect the circular form of the IS1272 and the composite transposon TnSha1 when both were excised from the chromosome (Table 2). Detection of strains carrying TnSha1 in either the A or B integration sites via real-time PCR amplification was performed in a LightCycler 480 system (Roche Diagnostics, Germany) using primers annealing between the integration site and the transposon (Table 2; Oggioni et al., 2002; Isola et al., 2005; Yesilkaya et al., 2006). Primers LF_30 and LF_31 were used to detect the presence of bacteria with a transposon free integration site A (Table 2).

RESULTS

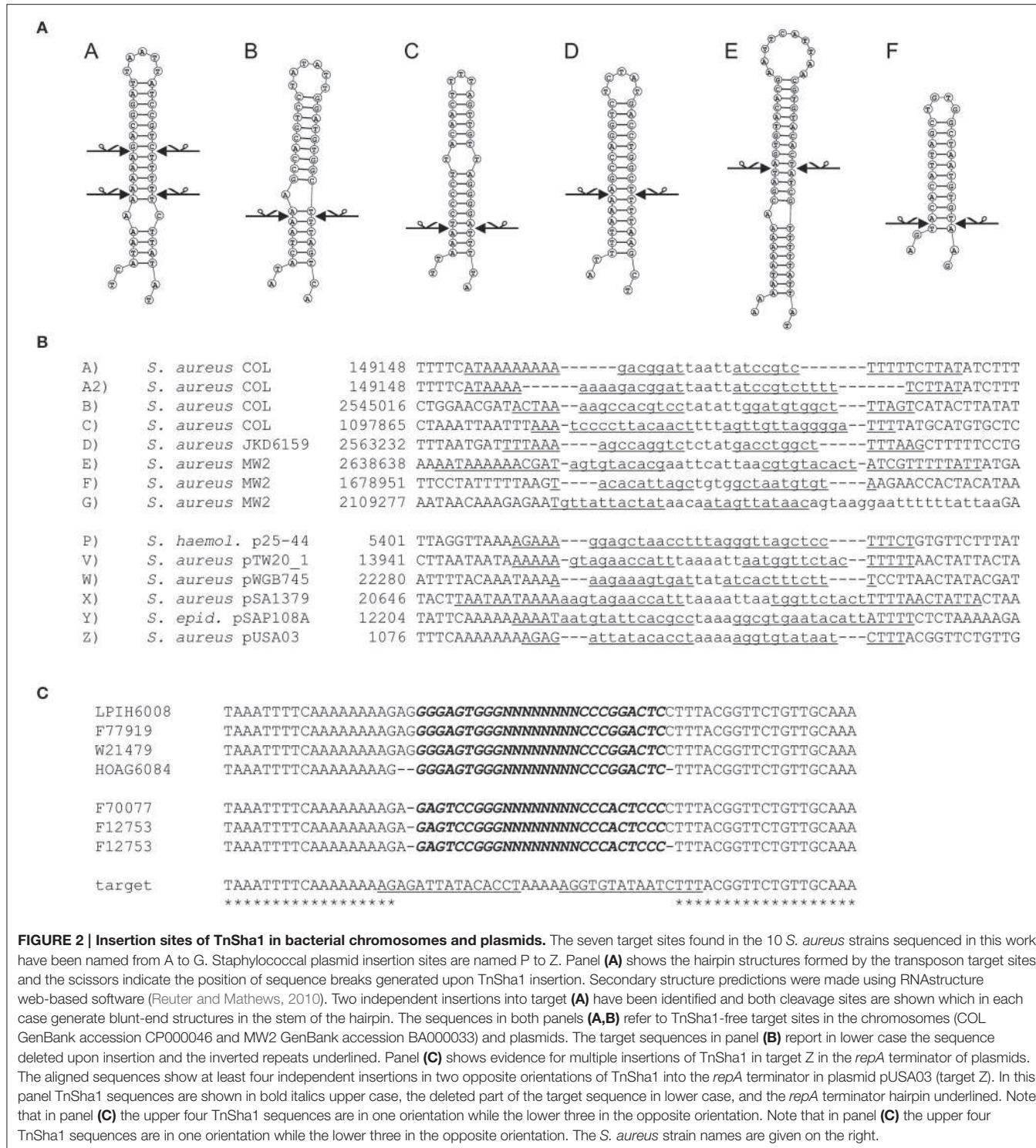
We have recently reported that a 3022 bp chromosomal element composed of the insertion sequence IS1272 and a *fabI* gene of *S. haemolyticus* (*sh-fabI*) confers resistance to triclosan in *S. aureus* (Figure 1A; GenBank accession no. JQ712986) (Ciusa et al., 2012). The potential for transposition of this unit is conferred by the presence of an alternative inverted repeat (IRL) in the *S. haemolyticus* chromosome upstream of *sh-fabI* with a high degree of similarity to the inverted repeats of IS1272 (Figure 1B). This functional unit, which is composed of the insertion sequence IS1272 and the *sh-fabI* gene, has now being renamed TnSha1 (Figure 1A). It should be noted that none of our genomes, nor any deposited sequence, match the originally deposited sequence of IS1272 (Genbank accession U35635), instead all of the hundreds of IS1272 copies show at least six SNPs (four of which are indels) with respect to U35635 (Archer et al., 1996). These differences mean that the updated IS1272 element encodes for a single transposase gene without a stop codon (Figure 1A).

In this work the sequence conservation, epidemiological distribution, and target site specificity of TnSha1 were analyzed across a panel of ten sequenced *S. aureus* strains previously reported to carry the *sh-fabI* gene (Ciusa et al., 2012). Sequencing has identified seven different integration sites within the *S. aureus* genomes which have been named A through G (Figure 2, Table 1). The discovery of seven different TnSha1 integration sites in just 10 strains suggests that there is little or no insertion site preference. Analysis of the intact target sites in the reference strains COL or MW2 showed that TnSha1 integration always

**TABLE 2 | List of primers.**

Name	Position (GenBank ID)	Sequence
LF_26	Taqman probe for TnSha1 (JQ712986)	6FAM-TTCACTTATCCAAGA ACTTTATGTCGGGGA-BHQ1
LF_27	IRL' of TnSha1 (JQ712986)	ATCCTTGCGGGGTAA ACAAAC
LF_28	IRL of TnSha1 (JQ712986)	AAAGCGAGCCAACAATACG GAGTA
LF_29	IRR of TnSha1 (JQ712986)	TAGTAGCTCAACGAGCTGA AAATAATC
LF_30	Upstream region flanking the TnSha1 integration site A/A2 (NC_002951)	TTGATTATTTCCAGCCTATT CTTTTCA
LF_31	Downstream region flanking the TnSha1 integration site A/A2 (NC_002951)	AGGATGTCGATTGATTAT ATTTTTGTACAT
LF_32	Downstream region flanking the TnSha1 integration site B (NC_002951)	ATCATTCGTTTATATAGCA GACATGATAGA

occurs into hairpin structures and, importantly, that the mode of insertion of TnSha1 always produces a partial deletion of the target sequence (**Figure 2B**). This mode of integration into the stem of secondary structures is novel to IS elements (Siguiér et al., 2015). For one of the targets sequenced in this work there were two different transposition events which could be detected; these lead to deletions of different sizes in the same target site (A and A2; **Figure 2B**). All seven of the target sites identified by sequencing of our clinical isolates, in addition to other sites subsequently identified in published sequences, were composed of inverted repeat sequences and thus have the potential to form secondary structures with the repeats representing the stems of hairpins (**Figure 2A**). In each case insertion of TnSha1 produced a partial deletion of the hairpin. This type of cleavage of a secondary structure forming sequences appears to indicate that TnSha1 makes blunt and not staggered cuts, which would have resulted in target duplications. Hairpins A through E have been



confirmed as transcriptional terminators by mapping of RNaseq data (Fagerlund et al., 2014; **Figure 3**). When analysing the target sites of a further 63 complete TnSha1 elements from published *S. aureus* genomes, we identified an additional 12 target sites confirming a lack of any primary sequence specificity within the target. All of the aforementioned features of TnSha1 are

consistent with the known behavior of the IS1272 element alone (Archer et al., 1996); i.e., such as the insertion of IS1272 which occurred into the terminator of *sh-fabI* to generate the prototype TnSha1 (**Figure 1C**), or as in the case of two target sites selected from IS1272 insertions in the genomes of *S. haemolyticus* or *S. warneri* (**Figure 4**). While most sequenced strains contained

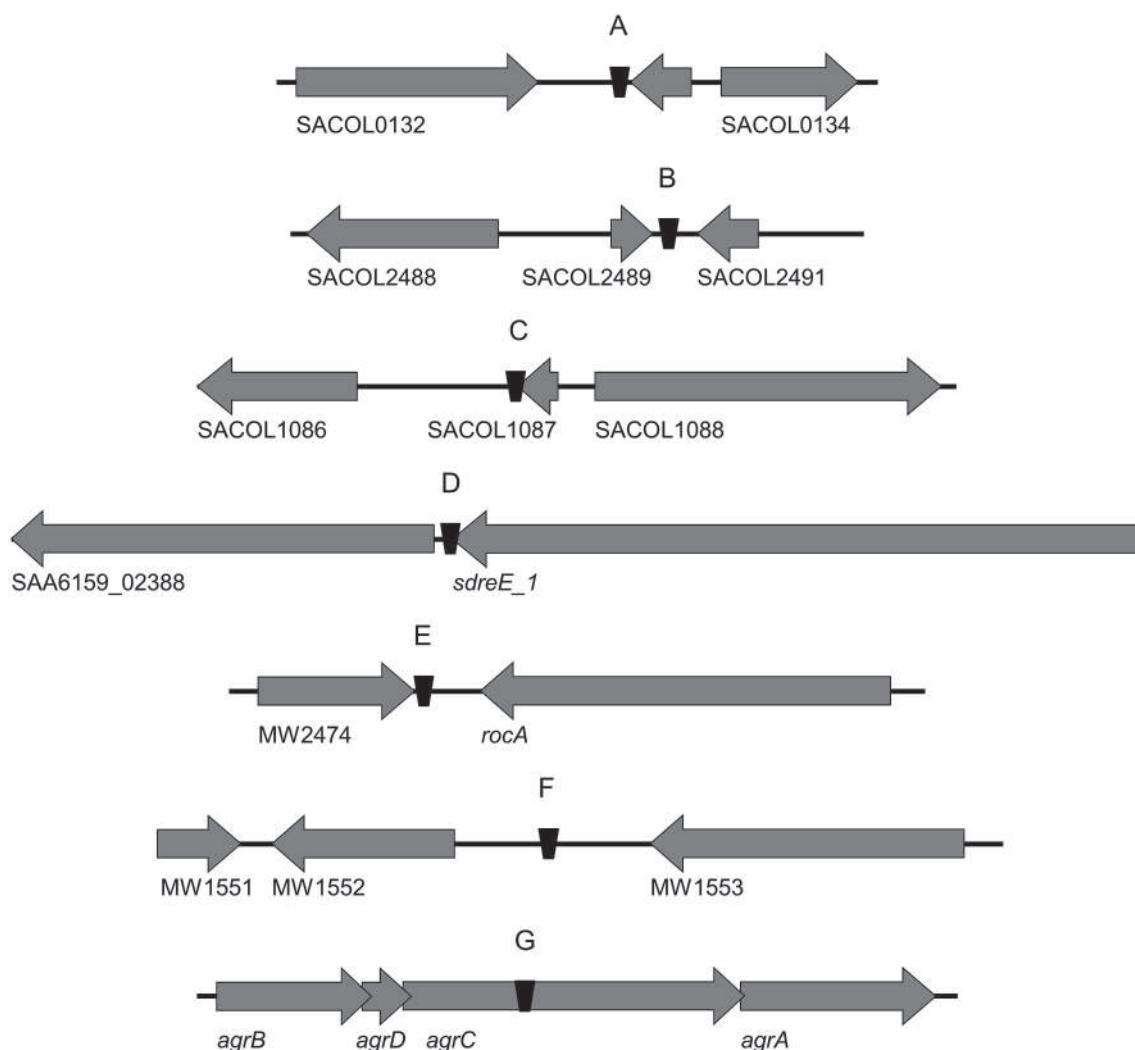


FIGURE 3 | The seven TnSha1 integration sites found in the sequenced *S. aureus* clinical isolates mapped onto annotated genomes. The *S. aureus* reference genomes included COL (GenBank accession no. CP000046; **A–C** insertion sites), JKD6159 (GenBank accession no. CP002114; **D** insertion site), and MW2 (GenBank accession no. BA000033; **E,F** insertion sites). 3/7 TnSha1 elements target predicted terminator hairpin structures (**C–E**), 1/7 targets the *agrC* coding region (**G**), and 3/7 target intergenic regions (**A,B,F**). Hairpins (**A**) through (**E**) have been confirmed as transcriptional terminators by mapping RNAseq data.

just a single copy of TnSha1 in the chromosome, strain QBR-102278-2351 was an exception and carried two TnSha1 elements each in two distinct integration sites (Figure 2, Table 1).

The dynamics and epidemiology of transposition between strains was addressed by searching for other TnSha1 elements present in Genbank. Database searches identified 133 complete or partial TnSha1 elements in the genomes of the species *S. aureus*, *S. argenteus*, *S. haemolyticus*, *S. epidermidis*, *S. saprophyticus*, and *S. warneri*, and in a series of staphylococcal plasmids (Tables 3–5). Of this latter group the integration between *repA* and IS257 in pUSA03 (target Z in Table 4) where TnSha1 was inserted in two different orientations warrants particular attention (Figure 2C). Multi locus sequence typing (MLST) of the strains showed that TnSha1 was present in *S. aureus* in sequence type 8 ($n = 23$), ST5 ($n = 18$), ST239 ($n = 6$), and

ST290 ($n = 6$) strains and in *S. epidermidis* in ST59 ($n = 10$) and ST2 ($n = 6$) strains (Table 4, Figure 5). Upon determination of the targets of these newly identified TnSha1 elements it was found that only seven out of the 66 complete TnSha1 elements were integrated into the same seven target sites, A to G, that we had previously identified (Figure 2, Tables 1, 4). Alignment of our previous 10 TnSha1 elements with the seven new TnSha1 elements that were integrated at the same target sites showed clustering of the TnSha1-SNPs (some elements differed by up to 11 SNPs from the consensus) together with their target sites (Figure 6). This pattern is consistent with the insertion of the element into a particular target followed by clonal spread. This scenario appears to be the rule in most cases except for target A2 and Z where the identical TnSha1 element is present in the same target but in strains of different ST (Table 4, Figure 5). To test for

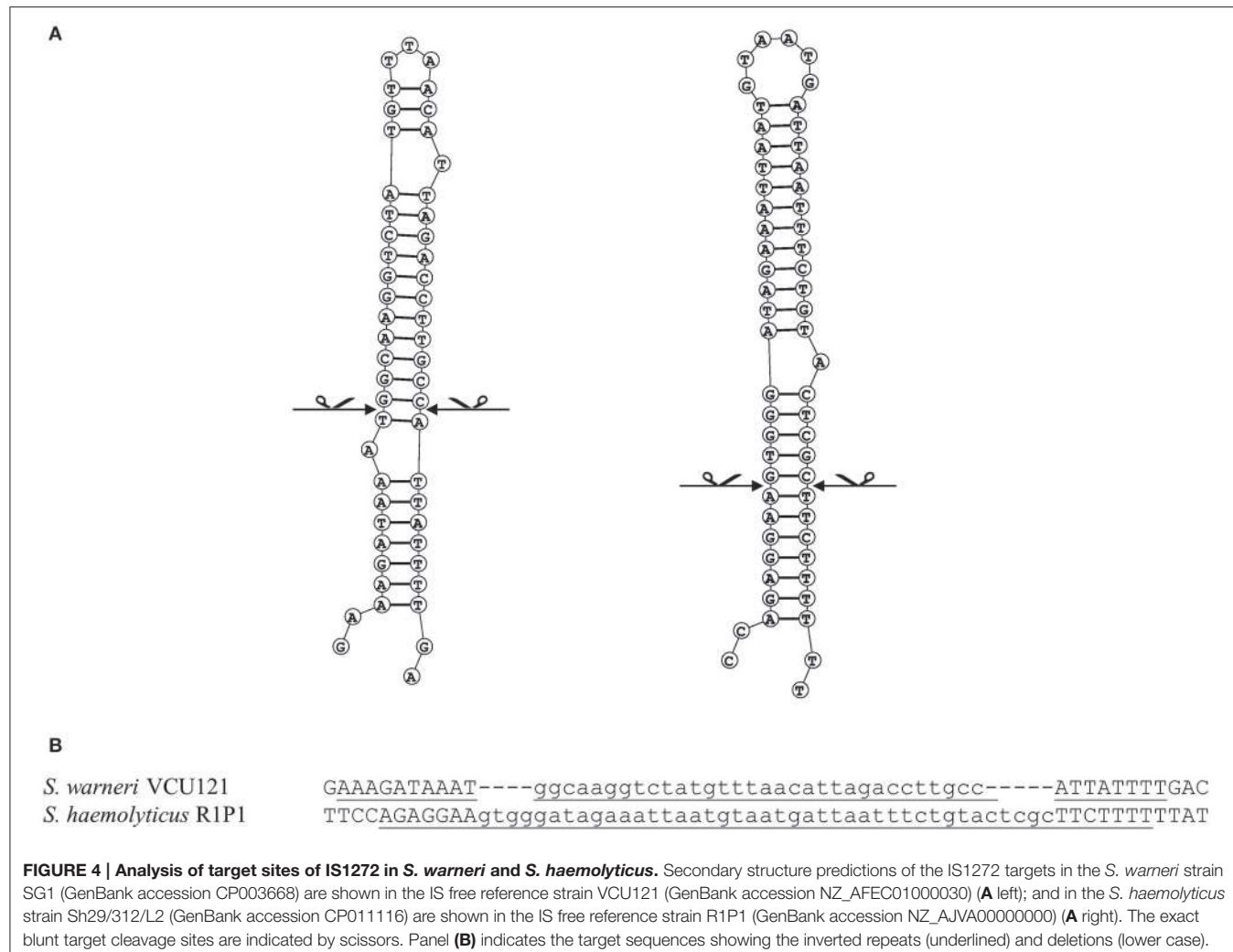


TABLE 3 | BLAST search of staphylococcal genomes for TnSha1 and TnSha2.

Species	Element	Chromosomal*	Plasmid**	Total	Total number of genomes	%
<i>S. argenteus</i>	TnSha1	1	1	2	6	33
	TnSha2	–	–	–		–
<i>S. aureus</i>	TnSha1	61	3	64	4127	1.5
	TnSha2	1	–	1		0.02
<i>S. epidermidis</i>	TnSha1	30	1	31	286	10.8
	TnSha2	19	–	19		6.6
<i>S. haemolyticus</i>	TnSha1	3	–	3	141	2.1
	TnSha2	10	–	10		7
<i>S. warneri</i>	TnSha1	2	–	2	7	28.6
	TnSha2	–	–	–		–
<i>S. saprophyticus</i>	TnSha1	1	–	1	5	20
	TnSha2	–	–	–		–
Total		128	5	133	4567	–

*The localization designated as chromosomal may also include plasmids present in genome contigs. **Only includes plasmids deposited as complete plasmids.

TABLE 4 | List of strains carrying the TnSha1 element on the chromosome.

Species	GenBank ID	Strain name	Insertion site	MLST
<i>S. argenteus</i>	CCEM01000001	Sa_LBSA043	M	-
<i>S. aureus</i>	AIDT01000009	DR10	H	398
<i>S. aureus</i>	AUPV01000018	S100	H	398
<i>S. aureus</i>	CAVU01000033	S1805	E	80
<i>S. aureus</i>	CAWA01000053	S2396	E	1
<i>S. aureus</i>	CFPN01000018	USFL079	L	8
<i>S. aureus</i>	CGGX01000004	USFL145	A	8
<i>S. aureus</i>	CIAK01000002	USFL234	J	8
<i>S. aureus</i>	CIGG01000001	USFL129	C	8
<i>S. aureus</i>	CP003045	71193	H	398
<i>S. aureus</i>	CSDA01000011	USFL101	L	8
<i>S. aureus</i>	CSDP01000002	USFL046	J	8
<i>S. aureus</i>	CSEJ01000001	USFL123	C	8
<i>S. aureus</i>	CSEV01000003	USFL189	A2	828
<i>S. aureus</i>	CSFL01000012	USFL078	L	8
<i>S. aureus</i>	CSHX01000003	USFL190	A2	828
<i>S. aureus</i>	CSJZ01000023	USFL050	nd	8 slv*
<i>S. aureus</i>	CTWU01000023	M705	K	239
<i>S. aureus</i>	CTWW01000088	H211	K	239
<i>S. aureus</i>	CTX00100001	M1229	K	239
<i>S. aureus</i>	CTXZ01000061	H216	K	239
<i>S. aureus</i>	CTYD01000081	H202	K	239
<i>S. aureus</i>	CVOP01000018	SH3	M	630
<i>S. aureus</i>	CVOU01000018	SH1	M	630
<i>S. aureus</i>	CVRW01000028	M170	K	239
<i>S. aureus</i>	JBFG01000011	KINW6058	I	5
<i>S. aureus</i>	JBGB01000006	FVRH6130	nd	8
<i>S. aureus</i>	JBGS01000014	GGMC6026	I	5
<i>S. aureus</i>	JBLE01000006	SCOA6048	H	8
<i>S. aureus</i>	JBMX01000012	SCOA6012	H	8
<i>S. aureus</i>	JDLS01000019	T78544	I	5
<i>S. aureus</i>	JDOK01000060	F35307	nd	5
<i>S. aureus</i>	JDOU01000017	H27862	I	5
<i>S. aureus</i>	JDOV01000017	H27872	I	5
<i>S. aureus</i>	JDPI01000020	H67656	I	5
<i>S. aureus</i>	JECS01000014	T34011	I	5
<i>S. aureus</i>	JEDV01000017	H64967	I	5
<i>S. aureus</i>	JETJ01000039	T22051	nd	5
<i>S. aureus</i>	JGNE01000030	W41757	nd	5
<i>S. aureus</i>	JHTT01000046	CO-86	L	8
<i>S. aureus</i>	JIXI01000011	PA57	H	398
<i>S. aureus</i>	JIYX01000004	C5086	H	398 slv
<i>S. aureus</i>	JJDE01000001	122	H	398
<i>S. aureus</i>	JURB01000090	84_SAUR	F	5
<i>S. aureus</i>	JUTG01000033	78_SAUR	F	5
<i>S. aureus</i>	JVUC01000150	1315_SAUR	nd	15
<i>S. aureus</i>	JZAL01000002	LHSKBClinical	C	8
<i>S. aureus</i>	LAMP01000014	99-06	L	8
<i>S. aureus</i>	LAMS01000038	99-48	L	8
<i>S. epidermidis</i>	ACHE01000003	BCM-HMP0060	-	59

(Continued)

TABLE 4 | Continued

Species	GenBank ID	Strain name	Insertion site	MLST
<i>S. epidermidis</i>	AGUC01000094	14.1.R1.SE	-	-
<i>S. epidermidis</i>	AHLC01000060	VCU120	-	22
<i>S. epidermidis</i>	AHLF01000017	VCU125	-	384
<i>S. epidermidis</i>	AKGM01000041	NIHLM067	-	333
<i>S. epidermidis</i>	AKGN01000056	NIHLM061	-	332
<i>S. epidermidis</i>	AKGW01000049	NIHLM020	-	7
<i>S. epidermidis</i>	APHT01000038	528m	-	2
<i>S. epidermidis</i>	APHU01000037	41tr	-	2
<i>S. epidermidis</i>	ARWU01000031	UC7032	-	595
<i>S. epidermidis</i>	JUMV01000141	938_SEPI	-	59
<i>S. epidermidis</i>	JUNI01000550	926_SEPI	-	59
<i>S. epidermidis</i>	JUVK01000069	73_SEPI	-	16
<i>S. epidermidis</i>	JUYJ01000034	655_SEPI	-	59
<i>S. epidermidis</i>	JUYK01000190	654_SEPI	-	59
<i>S. epidermidis</i>	JVQK01000079	196_SEPI	-	59
<i>S. epidermidis</i>	JVSC01000077	154_SEPI	-	59
<i>S. epidermidis</i>	JVSC01000077	154_SEPI	-	59
<i>S. epidermidis</i>	JVSZ01000046	134_SEPI	-	59
<i>S. epidermidis</i>	JVT01000122	1321_SEPI	-	59
<i>S. epidermidis</i>	JWBR01000039	114_SEPI	-	-
<i>S. epidermidis</i>	JWCR01000091	1115_SEPI	-	88
<i>S. epidermidis</i>	JWEH01000024	1063_SEPI	-	2
<i>S. epidermidis</i>	JWFU01000070	1024_SEPI	-	2
<i>S. epidermidis</i>	JWFV01000104	1023_SEPI	-	2
<i>S. epidermidis</i>	JZUK01000044	NGS-ED-1107	-	2
<i>S. epidermidis</i>	JZUL01000024	NGS-ED-1109	-	439
<i>S. haemolyticus</i>	CP011116	Sh29/312/L2	-	-
<i>S. haemolyticus</i>	CUEZ01000014	CN1197	-	-
<i>S. saprophyticus</i>	JXBG01000015	SU8	-	-
<i>S. warneri</i>	CANQ01000015	A487	-	-
<i>S. warneri</i>	JPOW01000002	NGS-ED-1001	-	-

*slv = single locus variant; targets A–G from our genome sequences; targets H–Z from Genbank, nd not determined.

TnSha1 mobilization we used PCR with a set of divergent primers on TnSha1, another set on IS1272, and a third set of primers targeting the “A” insertion site. Unfortunately, no excision events producing a circular intermediate could be observed when two *S. aureus* strains were tested with or without the presence of triclosan in the culture medium, and after induction with mitomycin C.

Within the multiple entries retrieved from Genbank a subgroup showed identical sequences upstream of TnSha1. Investigation of this genomic region showed that in 30 of the strains the TnSha1 element forms part of a larger element (named TnSha2), which in almost all cases is a plasmid, but which can also be found integrated into the genome where it is generally flanked by a second copy of IS1272 (Tables 4, 5). The prototype sequence of TnSha2 (*S. aureus* JCAZ01000023) is an 11.7 kb plasmid in which TnSha1 is inserted into the terminator of *traQ* gene of an 8.7 kb replicon (without *fabI* and IS1272)

TABLE 5 | List of strains carrying the TnSha1 element on a plasmid.

Species	GenBank ID	Strain name	Plasmid	Insertion site	MLST
<i>S. argenteus</i>	FR821778	MSHR1132	pST75	X2	-
<i>S. aureus</i>	AHVE01000013	CIG1165	Plasmid	X	5
<i>S. aureus</i>	CP012121	USA300_2014.C02	pUSA300_2014.C02	X	8
<i>S. aureus</i>	CP012594	HOU1444-VR	pVR-MSSA_01	W	5
<i>S. aureus</i>	CSBT01000019	USFL338	Plasmid	V	8
<i>S. aureus</i>	JBGG01000015	LAMC6115	Plasmid	Zrc	8
<i>S. aureus</i>	JBJX01000010	SJUD6114	Plasmid	X	8
<i>S. aureus</i>	JBRD01000004	AMMC6015	Plasmid	Z	8
<i>S. aureus</i>	JBSH01000015	HOAG6084	Plasmid	Z	8
<i>S. aureus</i>	JCAZ01000023	M0227	TnSha2	P	5
<i>S. aureus</i>	JDIX01000007	LPIH6008	Plasmid	Z	8
<i>S. aureus</i>	JEEN01000028	F77919	Plasmid	Z	5
<i>S. aureus</i>	JEOL01000012	W21479	Plasmid	Z	5
<i>S. aureus</i>	JEQM01000030	F70077	Plasmid	Zrc	5
<i>S. aureus</i>	JGFR01000050	F12753	Plasmid	Zrc	5
<i>S. aureus</i>	JGIT01000028	T28653	Plasmid	X	8
<i>S. aureus</i>	JICL01000103	880	phMPREF1625_3	W	-
<i>S. aureus</i>	JVUX01000076	1299_SAUR	Plasmid	Z	2250
<i>S. epidermidis</i>	GQ900465	SK6536	pSAP110A	Y	-
<i>S. epidermidis</i>	JUPD01000185	890_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JUUP01000151	749_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JUYI01000150	656_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JUZG01000070	634_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JUZR01000081	623_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVBT01000060	568_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVHA01000086	439_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVHR01000056	422.rep2_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVHS01000079	422.rep1_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVKK01000090	354_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JVXB01000116	1249_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWBN01000071	1143_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWDK01000004	1088_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWFS01000043	1026_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWFW01000102	1022_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWFX01000149	1021.rep2_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWFY01000144	1021.rep1_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWGH01000004	1013_SEPI	TnSha2	P	2
<i>S. epidermidis</i>	JWGL01000037	101_SEPI	TnSha2	P	2
<i>S. haemolyticus</i>	CUCK01000025	25-38	TnSha2 in chromosome*	P	29
<i>S. haemolyticus</i>	CUCN01000051	25-60	TnSha2	P	-
<i>S. haemolyticus</i>	CUCZ01000051	51-13	TnSha2	P	3
<i>S. haemolyticus</i>	CUDB01000007	51-11	TnSha2 in chromosome	P	3
<i>S. haemolyticus</i>	CUDQ01000054	51-33	TnSha2	P	3
<i>S. haemolyticus</i>	CUDW01000051	51-41	TnSha2	P	2
<i>S. haemolyticus</i>	CUDY01000029	51-43	TnSha2 in chromosome	P	2
<i>S. haemolyticus</i>	CUFB01000040	51-15	TnSha2	P	3
<i>S. haemolyticus</i>	CUGD01000039	127925	TnSha2 in chromosome	P	3
<i>S. haemolyticus</i>	CUGG01000054	113101	TnSha2	P	3

*Chromosomally integrated TnSha2 elements are reported for clarity in this plasmid table.

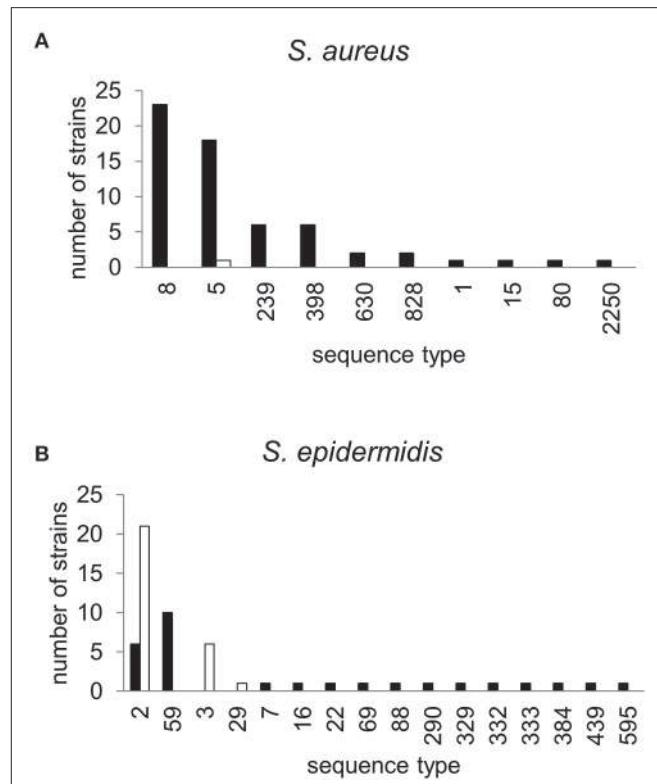
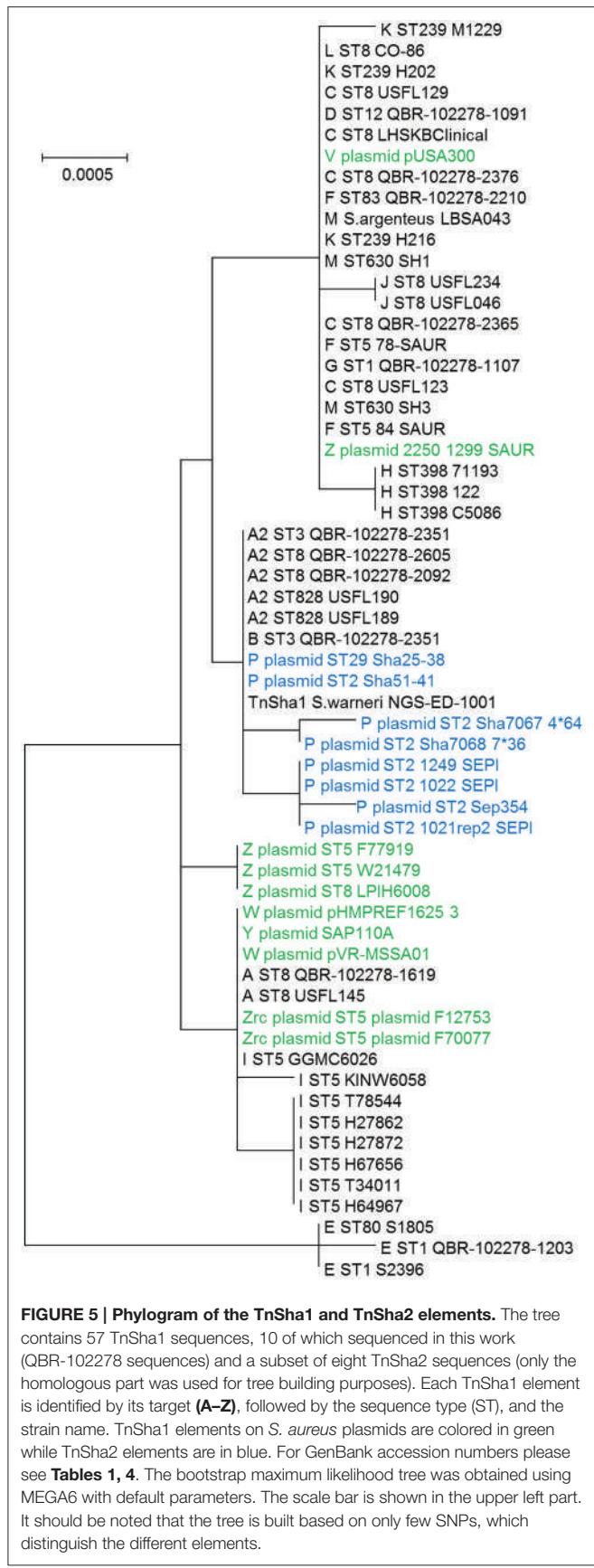


FIGURE 6 | MLST profiles of staphylococcal strains carrying TnSha1 and TnSha2. The sequence type (ST) of 62 *S. aureus* (**A**) and 47 *S. epidermidis* strains (**B**) which carry the *sh-fabI* elements TnSha1 (filled bars) and TnSha2 (open bars) are shown. Data are from staphylococcal genomes accessed in GenBank in December 2015 and sequence types were defined using the MLST web-service of the Center for Genomic Epidemiology (Larsen et al., 2012).

present in *S. haemolyticus* (CUCL01000044), but also in *S. aureus* (JJAQ01000025; **Figure 7**). The *rep* of the plasmid belongs to the Rep_2 family and the DNA polymerase, recombinase and mobilization protein show high sequence identity to those of plasmid p-12228-03 of the *S. epidermidis* reference strain ATCC 12228 (**Figure 7**; Zhang et al., 2003; Lanza et al., 2015). ThSha2 was detected in the genomes of clonally diverse *S. haemolyticus*, and *S. epidermidis* (ST3 $n = 6$, and ST2 $n = 21$, respectively), but only in a single *S. aureus* (ST5) genome, thereby reflecting different dissemination routes for different species (**Table 5**). Upon investigating the origin of deposited genome-strains it was observed that the *S. epidermidis* ST2 strains are of hospital origin (Schoenfelder et al., 2010; Roach et al., 2015) and that all of the *S. haemolyticus* strains with TnSha2 and ST3 that we found in this study are clinical isolates (**Table 5**). Interestingly the database search for IS1272 alone detected no intact elements in *S. aureus*, but did show that about 1/10 of *S. epidermidis* isolates and the majority of *S. haemolyticus* isolates carried IS1272 thereby ensuring that the contigs with the 11.7 kb plasmid in these species are always interrupted in the IS element.

In a phylogenetic tree based on the TnSha1-part of TnSha2, the two elements group into separate branches due to a series of SNPs both in *fabI* and the IS1272 element (**Figure 5**). In

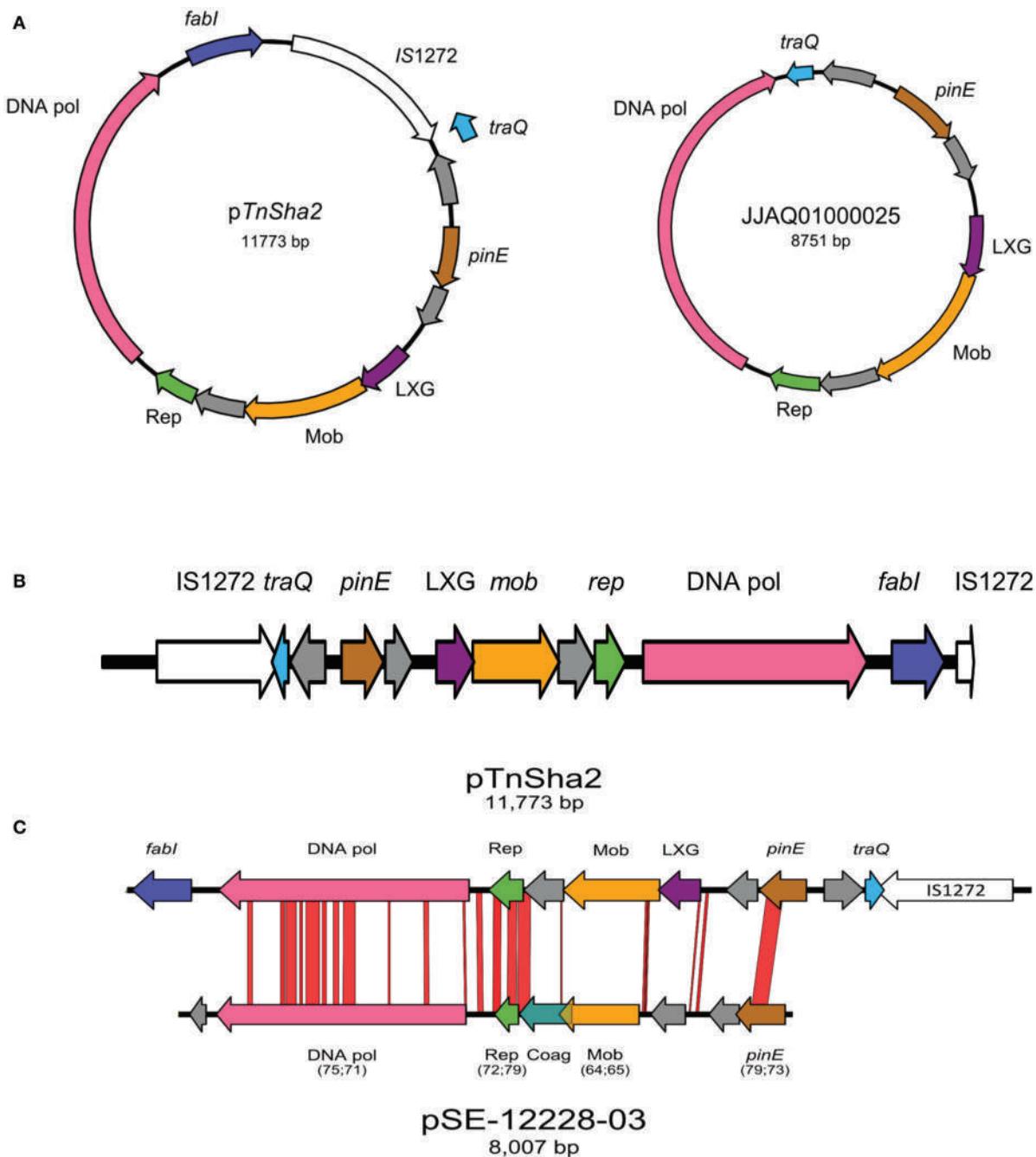


FIGURE 7 | Schematic map of TnSha2 elements. The circular maps of the prototype 11.7 kb plasmid sequence of TnSha2 (*S. aureus* strain M0227, GenBank accession JCAZ01000023) and the target 8.7 kb TnSha1-free plasmid (*S. haemolyticus* CUCLO1000044 or *S. aureus* JJAQ01000025) are shown in panel **(A)**. The map of TnSha2 integrated into the *S. haemolyticus* chromosome at a position occupied by a single IS1272 in the reference strain is shown in panel **(B)**. In three occasions the resulting chromosomal island is bordered by two IS1272 elements (CUCK01000025 at position 590129 of strain JCSC1435, CUDY01000029 at 1828271, and CUGD01000039 at 2443315) while in one case only by the “left” IS1272 element (CUDB01000007 at 1322312). Comparison between pTnSha2 element (*S. aureus* JCAZ01000023) and pSE-12228-03 (*S. epidermidis* NC_005006) is shown in panel **(C)**. Numbers between brackets indicate coverage percentage and identity percentage respectively. The genes encode for: a putative DNA-directed DNA polymerase (DNA pol), a replication protein involved in a theta-type replication mechanism (Rep), a putative mobilization protein (Mob), a domain of a group of polymorphic toxin proteins (LXG), a Site-specific DNA recombinase related to the DNA invertase Pin (PinE), and a conjugal transfer pilin chaperone (TraQ).

four cases the whole 11.7 kb TnSha2 plasmid appears to be integrated into the chromosome of *S. haemolyticus* in the same position that IS1272 elements are found in reference strain JCSC1435 resulting in a chromosomal island flanked in three cases by two IS1272 copies (CUCK01000025 at position 590.150 of JCSC1435, CUDB01000007 at 1.322.311, CUDY01000029 at 1.828.279, and CUGD01000039 at 2.443.339; **Figure 7**; Cavanagh et al., 2014).

In the original publication, which described *sh-fabI* in *S. aureus* QBR-102278-1619 (Ciusa et al., 2012), the TnSha1 element was reported to carry a *sh-fabI* gene with an A577T SNP with respect to the sequence of the core genome *fabI* of *S. haemolyticus* (**Table 6**). Since, this SNP had not previously been associated with triclosan resistance of *sa-fabI* in *S. aureus* the gene was reported to represent a susceptible allele (Ciusa et al., 2012). Whilst analysing the panel of *sh-fabI* genes carried by TnSha1 these were all found to show at least one SNP compared to the core-genome triclosan-susceptible *sh-fabI* of *S. haemolyticus* (**Table 6**). All of these SNPs, except A577T and T578A (see above), have previously been associated with triclosan resistance in the *S. aureus* *sa-fabI* (**Table 6**; Ciusa et al., 2012; Grandgirard et al., 2015). The only exception is *S. aureus* strain T22051 which carried a wild type *sh-fabI* gene in TnSha1 thus indicating that the second (or third) IS-element mobilized genomic *fabI* copy generally appears to be a resistant allele.

DISCUSSION

A novel mobile chromosomal element based upon IS1272 and containing the *sh-fabI* gene of *S. haemolyticus* has been identified in a large-scale screen for biocide resistance across more than 1600 *S. aureus* isolates (Ciusa et al., 2012). This element, now named TnSha1, had been found to be present in one third of triclosan resistant strains of *S. aureus* while the other triclosan resistant strains of that study had mutations either within or upstream of the enoyl-acyl carrier protein reductase *fabI* gene of the core genome (Ciusa et al., 2012; Grandgirard et al., 2015). A more detailed analysis of these *S. aureus* strains, and of other available staphylococcal genomes, has shown that the situation is actually more complex. The main finding of this was that *sh-fabI* appears to be transferred by two types of elements; TnSha1, which has a single IS1272 element, and the composite TnSha1-carrying plasmid TnSha2 which can also integrate into chromosomal IS1272 copies generating in most cases an IS1272 bordered island (**Figures 1, 7**). Similar congruent formations where reported for other similar elements as for example IS257 mobilizing the trimethoprim resistance determinant *dfrA* (Leelaporn et al., 1996). As the two elements do not show any obvious species specific characteristics it was somewhat unexpected to observe such a strong divergence in distribution for these elements, with *S. aureus* almost exclusively harboring TnSha1, *S. haemolyticus* prevalently harboring TnSha2, and only *S. epidermidis* which was found to commonly carry both elements. Neither the GC content (TnSha1 is 31.4%, TnSha2 is 31.8%, *S. aureus*, *S. haemolyticus* 33%, and *S. epidermidis* 32%), nor the presence of restriction modification systems nor the origin of the isolates

can explain this observation; however we admit that this is as yet based on 4127 *S. aureus* genomes, but upon just 286 *S. epidermidis* and 141 *S. haemolyticus* genomes. Similar dynamics are seen with trimethoprim resistance were the housekeeping *dfrA* gene was acquired from other species and successfully transferred by IS257 across different staphylococcal species (Dale et al., 1995; Leelaporn et al., 1996). Whilst not explaining this difference as such, the observation that the intact IS1272 elements are present in most *S. haemolyticus* isolates, only in a few *S. epidermidis* strains and not at all in *S. aureus* already indicates a species specific “behavior” of the IS element. Future experiments on the transposition of the elements in the three species, will hopefully be more successful than the attempted detection of circular intermediates in *S. aureus* and therefore shed light on the observed differences in the epidemiology of the elements.

The mechanism of TnSha1 and IS1272 target recognition and integration appears to be novel. Both TnSha1 and IS1272 are seen to target hairpin secondary structures with little primary sequence conservation. In addition this insertion into the target appears to produce a blunt-end cut leading to insertion of the element and deletion of part of the target (**Figures 1–4**). This is in line with the original description of IS1272 which reported that the IS did not produce target duplications (Archer et al., 1996). Interestingly the recent review on IS elements by Siguier and colleagues reports that some members of the IS1182 family, of which IS1272 is part, also target palindromic sequences (Siguier et al., 2015).

Albeit that no primary sequence consensus was detected, the hairpin structures targeted by TnSha1 must have some important features in common as we found three targets with evidence for multiple independent insertions i.e., the targets “A” and “X” or the target “Z” where we also observed multiple insertions in both orientations (**Figure 2C**). The absence of insertion into rolling circle replicating plasmids, coupled to the observation of insertions in opposite directions appear to indicate the absence of an orientation bias observed for IS elements inserting into the replication fork (Siguier et al., 2014). In the case of TnSha2 the situation appears to be more varied. The few available data show that TnSha2 can integrate like IS1272. In addition the few and incomplete genomic data we have could indicate that TnSha2 could be mobilized by the hypothetical ORFs present leading to duplications and co-integrate formation (Needham et al., 1995). The capacity to utilize different integration mechanisms has been described also for other transposons like Tn7 (Siguier et al., 2014).

The large number of both TnSha1 and TnSha2 element sequences available in the database has allowed us to investigate the genesis of this recently generated and spreading element. This was hampered in part by the fact that both elements included IS1272 and that these ISs, like most other repeat sequences, tend to generate contig breaks during the assembly of bacterial genomes. Given the absolute conservation of the position of IS1272 in all of the TnSha1 and TnSha2 elements, we favor the hypothesis that the initial steps leading to the formation of these mobile elements were very few or possibly even only a single event. The initial events in the assembly of the element

TABLE 6 | *Sh-fabI* nucleotide sequence of 89 TnSha1 and TnSha2 elements.

Species	Strain	Polymorphic sites in <i>sh-fabI</i> *						Phenotype**	Comment
		1	5	5	5	6			
		8	0	7	7	9	1		
		1	6	7	8	3	1		
<i>S. haemolyticus</i>	JCSC1435	T	G	A	T	C	T	S	Reference strain***
<i>S. haemolyticus</i>	0281	S	Triclosan susceptible clinical isolate***
<i>S. aureus</i>	T22051	S	wt sequence
<i>S. aureus</i>	KINW6058	C	.	T	.	.	.	R	GTA81GCA V27A; ATT577TTT I193F
<i>S. epidermidis</i>	354_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	422.rep1_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	422.rep2_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	493_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	568_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	623_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	634_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	656_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	749_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	890_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	1013_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	1021.rep2_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	1022_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	1088_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. epidermidis</i>	1143_SEPI	.	A	.	.	.	C	R	GTT106ATT V36I; TC611TCC F204S
<i>S. aureus</i>	F12753	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	F70077	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	GGMC6026	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	H27862	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	H27872	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	H64967	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	H67656	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	pHMPREF1625_3	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	pVR_MSSA_01	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	QBR-102278-1619	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	T34011	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	T78544	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	USFL050	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	USFL145	.	.	T	.	.	.		ATT577TTT I193F
<i>S. epidermidis</i>	134_SEPI	.	.	T	.	.	.		ATT577TTT I193F
<i>S. epidermidis</i>	154_SEPI	.	.	T	.	.	.		ATT577TTT I193F
<i>S. epidermidis</i>	196_SEPI	.	.	T	.	.	.		ATT577TTT I193F
<i>S. epidermidis</i>	SAP110A	.	.	T	.	.	.		ATT577TTT I193F
<i>S. aureus</i>	F77919	.	.	.	A	.	.		ATT578AAT I193N
<i>S. aureus</i>	LPIH6008	.	.	.	A	.	.		ATT578AAT I193N
<i>S. aureus</i>	W21479	.	.	.	A	.	.		ATT578AAT I193N
<i>S. aureus</i>	QBR-102278-1203	.	.	.	G	.	.	R	GCT593GGT A198G
<i>S. aureus</i>	S1805	.	.	.	G	.	.	R	GCT593GGT A198G
<i>S. aureus</i>	S2396	.	.	.	G	.	.	R	GCT593GGT A198G
<i>S. argenteus</i>	pST75	G	.	R	TTC611TGC F204C
<i>S. argenteus</i>	Sa_LBSA043	G	.	R	TTC611TGC F204C
<i>S. aureus</i>	122	G	.	R	TTC611TGC F204C
<i>S. aureus</i>	1299_SAUR	G	.	R	TTC611TGC F204C

(Continued)

TABLE 6 | Continued

Species	Strain	Polymorphic sites in <i>sh-fabl</i> *					Phenotype**	Comment
		1	5	5	5	6		
		8	0	7	7	9		
<i>S. aureus</i>	1315_SAUR	G	R
<i>S. aureus</i>	71193	G	R
<i>S. aureus</i>	78_SAUR	G	R
<i>S. aureus</i>	84_SAUR	G	R
<i>S. aureus</i>	C5086	G	R
<i>S. aureus</i>	CO-86	G	R
<i>S. aureus</i>	H202	G	R
<i>S. aureus</i>	H216	G	R
<i>S. aureus</i>	LHSKBClinical	G	R
<i>S. aureus</i>	M1229	G	R
<i>S. aureus</i>	QBR-102278-1091	G	R
<i>S. aureus</i>	QBR-102278-1107	G	R
<i>S. aureus</i>	QBR-102278-2210	G	R
<i>S. aureus</i>	QBR-102278-2365	G	R
<i>S. aureus</i>	QBR-102278-2376	G	R
<i>S. aureus</i>	SH1	G	R
<i>S. aureus</i>	SH3	G	R
<i>S. aureus</i>	USA300_2014.C02	G	R
<i>S. aureus</i>	USFL046	G	R
<i>S. aureus</i>	USFL123	G	R
<i>S. aureus</i>	USFL129	G	R
<i>S. aureus</i>	USFL234	G	R
<i>S. epidermidis</i>	1249_SEPI	G	R
<i>S. epidermidis</i>	14.1.R1.SE	G	R
<i>S. epidermidis</i>	NIHLM020	G	R
<i>S. haemolyticus</i>	Sh29/312/L2	G	R
<i>S. aureus</i>	QBR-102278-2092	C	R
<i>S. aureus</i>	QBR-102278-2351	C	R
<i>S. aureus</i>	QBR-102278-2605	C	R
<i>S. aureus</i>	M0277	C	R
<i>S. aureus</i>	USFL189	C	R
<i>S. aureus</i>	USFL190	C	R
<i>S. aureus</i>	W41757	C	R
<i>S. epidermidis</i>	1026_SEPI	C	R
<i>S. haemolyticus</i>	25-38	C	R
<i>S. haemolyticus</i>	25-60	C	R
<i>S. haemolyticus</i>	51-11	C	R
<i>S. haemolyticus</i>	51-13	C	R
<i>S. haemolyticus</i>	51-43	C	R
<i>S. haemolyticus</i>	51-41	C	R
<i>S. haemolyticus</i>	113101	C	R
<i>S. haemolyticus</i>	127925	C	R
<i>S. warneri</i>	NGS-ED-1001	C	R

*Polymorphic sites are indicated with respect to the *sh-fabl* sequence of *S. haemolyticus* strain JCSC1435. **Phenotype as defined in references: (Clusa et al., 2012; Grandgirard et al., 2015). ***These wt *S. haemolyticus* strains do not carry the *TnSha1* element, therefore the *fabl* sequence reported is the one of the core genome.

must presumably have happened in *S. haemolyticus* since the *sh-fabI* derives from the core genome of this species and both elements are also present in this species. It is again not easy to hypothesize any rationale for the almost exclusive presence of TnSha1 in *S. aureus*, nor for the preferential presence of TnSha2 in *S. haemolyticus*. The lack of primary target sequence specificity and the peculiarity of targeting hairpin secondary structures make the elements very versatile and would allow for transposition to many locations within the chromosome and on plasmids. While intact IS1272 itself is not present in *S. aureus* the apparent selection for TnSha1 has very efficiently allowed spread of the element in this species. In the case of TnSha2 the presence of mobilization genes could indicate that this plasmid may be horizontally transferable. Unfortunately, as in the case of the *dfr* genes (Dale et al., 1995; Leelaporn et al., 1996), none of these data provide a solid molecular basis to explain the difference in distribution of the two elements among staphylococcal species. The explanation may however come from a closer look at the structure of the elements themselves. Indeed the very limited range of the diversity in the elements, and the SNPs present in them, indicates they have a recent origin. Another important aspect of the dynamics of spread of this element is related to the different triclosan-resistance conferring SNPs which have accumulated in *sh-fabI*. The presence of triclosan susceptible and resistant alleles in the studied population suggests the first event was acquisition of a susceptible *sh-fabI* allele followed by the selection of resistant alleles after the transfer event by a *fabI* targeting agent, most probably triclosan (Ciusa et al., 2012; Oggioni et al., 2012, 2013; Morrissey et al., 2014; Grandgirard et al., 2015). This sequence of events in which the acquisition of a resistance element is followed by the selection of more efficient alleles is also the basis of the diversification of other resistance elements as plasmids-encoded beta-lactamases (Bush, 2013).

Type II fatty acid metabolism, and in particular *FabI*, are among the most highly investigated targets for development of antimicrobial compounds (Banerjee et al., 1994; Park et al., 2007; Escaich et al., 2011; Kaplan et al., 2012). This remains true even though there is now debate on the essentiality of bacterial fatty acid metabolism during invasive infection because, at least for group B streptococci, it has been reported that it is possible to forage host derived fatty acids (Brinster et al., 2009; Balemans et al., 2010). In this respect it is therefore of great interest to monitor the spread of genetic elements which could produce reduced susceptibility to new antibiotics whose action is based on targeting and inhibition of *FabI*. In this regard it is worth noting that our experimental screening of a collection of 1602 world-wide clinical *S. aureus* isolates detected *sh-fabI* in 24/1602 of strains (1.5%; Ciusa et al., 2012), which matched perfectly with the *in silico* screen of a database of microbial genomes that detected *sh-fabI* carrying elements in 65/4127 *S. aureus* strains (1.57%). This exact overlap in prevalence indicates that genome databases of such size can now serve as suitable datasets for epidemiological investigation, even though databases generally lack detailed background information on strains. While 1.5% may not appear to be a high background resistance level in a population, it could still be a worrying presence when introducing a new *FabI*-targeting agent. Even worse is the

detection of *sh-fabI* elements in 14% of *S. epidermidis* isolates; this appears to reach levels at which clinical use, including the use of triclosan as disinfectant for decontamination, could be jeopardized. It remains possible however that the lower sample size, of <300 genomes screened, and the absence of clinical information on these strains may limit the relevance of this observation.

In order to correlate our data with two well-known examples of IS-mobilizable metabolic genes, we have checked the relative occurrence of the *dfrA-thyE* genes (Rouch et al., 1989), conferring trimethoprim resistance, and *ileS2* genes (Needham et al., 1994), conferring mupirocin resistance, in the same 4800 staphylococcal genomes. BLAST searches for *dfrA-thyE* yielded 279 hits in *S. aureus* and 134 in *S. epidermidis*, while *ileS2* yielded 207 hits in *S. aureus* and 18 in *S. epidermidis*. This compares well with the detection rate in our work of 65 TnSha1/2 elements in *S. aureus* and 50 in *S. epidermidis*. These numbers indicate that IS mediated transfer of metabolic resistance genes, in our case *sh-fabI* genes and triclosan resistance, is a highly relevant mechanism for the acquisition and spread of antibiotic resistance. These data show that it is not only plasmids which serve as vectors of IS mediated resistance gene transfers, but that the spread of composite transposons can also be a highly efficient mechanism for such a goal. A similar well-described mechanisms also exist in staphylococci for the antibiotic resistance genes for example in Tn4001, Tn4002, and Tn4003, (Lanza et al., 2015).

In conclusion our data show that IS mediated transposition of metabolic genes represents a vast and growing antimicrobial resistance phenomenon. In addition to the well-described Tn4003 element, which mobilizes *dfrA* by way of three IS257 thereby conferring trimethoprim resistance (Rouch et al., 1989), or the IS257 mediated mobilization of *ileS2* conferring mupirocin resistance (Needham et al., 1995), this arsenal now includes TnSha1 and TnSha2; these elements utilize IS transposition, IS-targeted integration and plasmid mobilization to allow transfer of the *fabI* gene of *S. haemolyticus* to different staphylococci and thereby contribute to triclosan resistance and, potentially, to resistance for other *FabI*-targeting drugs. These data show that IS mobilization of metabolic genes is a powerful and highly flexible mechanism that can very rapidly provide resistance phenotypes to vast numbers of strains and species. In this era where thousands of genomes are readily available in public databases the analysis of such IS mediated mobilization of core genome metabolic genes may warrant a more detailed and larger scale investigation.

AUTHOR CONTRIBUTIONS

LF performed the genome sequencing and bioinformatic analysis, wrote the manuscript, and approved the final version. RH participated in bioinformatic analysis, wrote the manuscript, and approved the final version. ZA performed wet lab experiments, participated in bioinformatic analysis, participated in the revision of the manuscript, and approved the final version. HO preformed bioinformatic analysis, discussed results and implication and helped in revision of the manuscript

and approved the final version. IM had input in the initial study design, participated in the generation and analysis of data, the revision of the manuscript, and approved the final version. RL preformed bioinformatic analysis, discussed results and implication and helped in revision of the manuscript and approved the final version. JM had input in the initial study design, participated in the analysis of data, participated in the revision of the manuscript, and approved the final version. TC had input in the initial study design, participated in the analysis of data, participated in the revision of the manuscript, and approved the final version. MO designed the study, participated in bioinformatic analysis, wrote the manuscript, and is accountable for all aspects of the work.

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Co-diversification of *Enterococcus faecium* Core Genomes and PBP5: Evidences of *pbp5* Horizontal Transfer

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Ampicillin resistance has greatly contributed to the recent dramatic increase of a cluster of human adapted *Enterococcus faecium* lineages (ST17, ST18, and ST78) in hospital-based infections. Changes in the chromosomal *pbp5* gene have been associated with different levels of ampicillin susceptibility, leading to protein variants (designated as PBP5 C-types to keep the nomenclature used in previous works) with diverse degrees of reduction in penicillin affinity. Our goal was to use a comparative genomics approach to evaluate the relationship between the diversity of PBP5 among *E. faecium* isolates of different phylogenomic groups as well as to assess the *pbp5* transferability among isolates of disparate clonal lineages. The analyses of 78 selected *E. faecium* strains as well as published *E. faecium* genomes, suggested that the diversity of *pbp5* mirrors the phylogenomic diversification of *E. faecium*. The presence of identical PBP5 C-types as well as similar *pbp5* genetic environments in different *E. faecium* lineages and clones from quite different geographical and environmental origin was also documented and would indicate their horizontal gene transfer among *E. faecium* populations. This was supported by experimental assays showing transfer of large (\approx 180–280 kb) chromosomal genetic platforms containing *pbp5* alleles, *ponA* (transglycosilase) and other metabolic and adaptive features, from *E. faecium* donor isolates to suitable *E. faecium* recipient strains. Mutation profile analysis of PBP5 from available genomes and strains from this study suggests that the spread of PBP5 C-types might have occurred even in the absence of a significant ampicillin resistance phenotype. In summary, genetic platforms containing *pbp5* sequences were stably maintained in particular *E. faecium* lineages, but were also able to be transferred among *E. faecium* clones of different origins, emphasizing the growing risk of further spread of ampicillin resistance in this nosocomial pathogen.

Keywords: ampicillin resistance, PBP5 mutations, PonA, chromosomal transfer, phylogenomic diversification

INTRODUCTION

Infections caused by *Enterococcus faecium* have increasingly been reported since the early 1980s, as have the number of antibiotic resistant isolates of this species (Top et al., 2007). Currently, most clinical strains of *E. faecium* are ampicillin resistant (AmpR), which often also acquire transferable mobile genetic elements, some encoding vancomycin resistance (Arias and Murray, 2012; Wagenvoort et al., 2015). Multidrug resistant *E. faecium* isolates are responsible for infections associated with treatment failures and high mortality rates (Arias et al., 2010; Centers for Disease Control and Prevention, 2013). However, the influence of ampicillin resistance in the population structure of *E. faecium* remains largely unexplored.

Enterococcus faecium is intrinsically resistant to cephalosporins and exhibit natural reduced susceptibility to penicillins. This species has six penicillin binding proteins (PBPs) belonging either to class B (monofunctional D,D-transpeptidases), or class A (bifunctional enzymes with glycosyltransferase and D,D-transpeptidase activity), some of them being associated with resistance to β -lactams (Zorzi et al., 1996; Rice et al., 2009). In *E. faecium* resistance to β -lactam antibiotics is conferred by the low-affinity class B PBP5, that requires the participation of class A PBPs (PonA, PbpF) to synthesize the cell wall in the presence of cephalosporins (Williamson et al., 1983; Rice et al., 2009). Resistance to high ampicillin concentrations in *E. faecium* was initially explained by either the enhanced production of PBP5, and/or by polymorphisms in the beta subunit of this protein (Fontana et al., 1996). Soon after, it was demonstrated that such changes were frequently strain specific and did not necessarily correlate with differences in the ampicillin MIC values (Fontana et al., 1996; Zorzi et al., 1996; Rybkine et al., 1998; Rice et al., 2001, 2004; Sifaoui et al., 2001; Belhaj et al., 2016). Further analysis of *E. faecium* strains with different levels of ampicillin susceptibility ($0,5\text{--}128\text{ mg/L}$) revealed that the variability of PBP5 sequences is mostly due to changes in 21 specific positions of the protein, suggesting that a sequential acquisition of mutations could have contributed to the progressive resistance to ampicillin from the early 1980s (Galloway-Peña et al., 2011; Pietta et al., 2014). Mutations in genes encoding other species-specific proteins that participate in the cell wall synthesis, such as D,D-carboxypeptidases (Ddcp and DdcY), L,D-transpeptidases (Ldt_{fm}), glycosyltransferases (PgtA) and acetyl muramoyl-L-alanine amidase (LytG) may also slightly increase the MIC values (Rice et al., 2007; Zhang et al., 2012; Kristich et al., 2014), even in the absence of PBP5 (Sacco et al., 2014). Although the occurrence of β -lactamases has been documented in *E. faecium*, β -lactamases producing *E. faecium* remain rare (Coudron et al., 1992; Klare et al., 1992; Zhang et al., 2012; Hendrickx et al., 2013).

Enterococcus faecium population biology is dominated by two main phylogenomic groups, clade A and clade B. Most AmpR isolates belong to clade A or “hospital-associated clade” mainly comprising *E. faecium* from hospitalized patients (Galloway-Peña et al., 2012; Palmer et al., 2012; Lebreton et al., 2013). A subgroup within the clade A, clade A1, is enriched in mobile genetic elements and have enhanced ability to colonize and

persist in human hosts due to the presence of adhesins and specific metabolic traits. In contrast, the clade B, or “community-associated *E. faecium*,” mostly comprises ampicillin susceptible (AmpS) isolates from healthy, non-hospitalized individuals (Willems et al., 2005; Lebreton et al., 2013; Tedim et al., 2015; Freitas et al., 2016; Guzman Prieto et al., 2016). The increasing detection of AmpR among isolates from hosts not associated with the hospital setting (Gonçalves et al., 2011; de Regt et al., 2012; Novais et al., 2013; Santos et al., 2013; Tremblay et al., 2013) is of concern, as the acquisition of transferable genes encoding AmpR might facilitate its further spread into AmpS *E. faecium* populations or to other less frequently recovered enterococcal species for which AmpR has been rarely documented (Raze et al., 1998). A relevant observation was the identification of a transferable chromosomal region of 60 kb comprising *pbp5* and a transposon that confers resistance to glycopeptides (*vanB2-CTn5386*) (Carias et al., 1998; Rice et al., 2005b). Homologous recombination between similar chromosomal regions carrying *pbp5* of different strains potentially associated with a conjugation mechanism has been recently suggested (García-Solache et al., 2016).

The known diversity of genotypes of AmpR *E. faecium* is mainly focused on the PBP5 polymorphisms analyzed in a limited number of strains (Poeta et al., 2007; Klibi et al., 2008; Galloway-Peña et al., 2011). Similarly, knowledge related to the *pbp5* transferability is limited to the characterization of conjugative Tn916-like elements (CTn5382 or the interaction of CTn916 and CTn5386) in a few *E. faecium* strains (Carias et al., 1998; Rice et al., 2005a,b; García-Solache et al., 2016). Here, we extend previous knowledge on these issues by using a comparative genomics approach of the chromosomal regions containing *pbp5*, that demonstrate a relationship between the diversity of PBP5 and *E. faecium* phylogenomic groups. We also assess the *pbp5* transferability among isolates from different clonal lineages.

MATERIALS AND METHODS

Bacterial Strains

Seventy-eight isolates from a collection of 205 AmpR *E. faecium* ($\text{MIC} \geq 16\text{ mg/L}$) recovered in Portugal from the last decades were analyzed (Novais et al., 2005a,b,c, 2006, 2013). The 78 *E. faecium* were selected in order to include isolates with different antibiotic resistant phenotypes, from diverse origins (39 from different patients admitted in five hospitals from different cities, 18 from swine feces and piggeries, 4 from retail poultry carcasses, 2 from healthy human feces and 15 from hospital wastewater), isolation date and, whenever possible, clonal relatedness. Susceptibility to ampicillin and 10 other antibiotics of different classes was evaluated by disk diffusion and/or agar dilution method (Clinical and Laboratory Standards Institute, 2012). β -lactamase production was tested in AmpR *E. faecium* isolates by the nitrocefin test (5 μl were directly placed in bacteria growing around the ampicillin disk) and PCR amplification of *blaZ* using primers based on the GenBank sequence no. M25257.1 (*blaZF-3'-TTGCCTATGCTTCGACTTCA-5'*, *blaZR-3'-AGTGAACCGCCAAGAGTGT-5'*). Clonal relatedness was

established by Pulsed-Field Gel Electrophoresis (PFGE) and analysis of Multilocus Sequence Typing (MLST) data (Tenover et al., 1995; Homan et al., 2002; Freitas et al., 2013)¹ using the Bayesian Analysis of Population Structure (BAPS) algorithm (Willems et al., 2012; Tedim et al., 2015).

Transferability of Ampicillin Resistance

Filter-mating assays were performed in plain Brain Heart Infusion (BHI) not supplemented with antibiotics at 37°C overnight, using a donor/recipient ratio of 1:1 (1-3 experiments per isolate) and *E. faecium* GE1 as the recipient strain. Further filter mating assays under the same experimental conditions but using *E. faecium* BM4105RF and *E. faecium* 64/3 strains as recipients, were performed for those field isolates able to transfer *pbp5* to *E. faecium* GE1. The three laboratory recipient strains used differed in the susceptibility against ampicillin and the presence of *pbp5*: GE1 [$\Delta p b p 5$; MIC_{Amp} < 0.016 mg/L; tetracycline (Tet), rifampicin (Rif) and fusidic acid (Fus) resistant (^R); ST515/BAPS 2.3b], BM4105RF (*pbp5*; Rif^R, Fus^R; MIC_{Amp} = 0.5 mg/L; ST172/BAPS 1.3) and 64/3 (*pbp5*; Rif^R, Fus^R; MIC_{Amp} = 1 mg/L; ST21/BAPS 2.3a). Recipients acquiring *pbp5* are from this point named as transconjugants, based on the most likely transfer mechanism related to *pbp5* (conjugation) published during the revision of this article (García-Solache et al., 2016). Transconjugants were selected on BHI agar supplemented with antibiotics (ampicillin-10 mg/L, fusidic acid-25 mg/L; rifampicin-30 mg/L) and incubated for 24 up to 96 h (37°C) to recover potential transconjugants growing at different times. Transconjugants were confirmed by comparison of their antibiotic resistance phenotype and PFGE profile with those of the donors and the recipients. Stability of acquired *pbp5* chromosomal regions (from now on designated *pbp5* genetic platforms) after serial daily passages (x30) on antibiotic free BHI agar was evaluated in both donor strains and transconjugants. Colonies from each passage were inoculated on plates containing the same agar medium and tested for ampicillin susceptibility by disk diffusion (Clinical and Laboratory Standards Institute, 2012).

Characterization of the Region Conferring AmpR

Total DNA from a donor strain (AmpR *E. faecium* clinical isolate HPH2), a recipient strain (AmpS *E. faecium* GE1) and the resulting GE1 transconjugant (AmpR *E. faecium* TCGEHPH2.1) was extracted with PureEluteTM Bacterial Genomic Kit (EdgeBio, Gaithersburg, MD, USA). DNA concentration was measured with Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) and Qubit 2.0 fluorometer (Life Technologies). Genomic DNA (250–350 ng) was sequenced on the Illumina MiSeq system using the MiSeq reagent kit v3 and a read length configuration of 2 × 300 bp (Illumina, San Diego, CA, USA). Sequencing was carried out using a standard 2 × 71 base protocol (300–400 bp insert size) in a Genome Analyzer IIx (Illumina, San Diego, CA, USA) at the sequencing facility of the University of Newcastle (United Kingdom). The main statistics for the three sequence

datasets (number of reads and coverage) analyzed are shown in Supplementary Table S1. Assembly of sequence data was done using Newbler software (454 life sciences, Roche, Branford, CT, USA). Supplementary Table S2 shows the final assembly results.

Gene prediction was performed using GeneMark.hmm 3.05 (Besemer et al., 2001). Similarity searches for potential protein-coding regions were carried out against a UniRef100 database² using Best Blast Hit approach (Supplementary Table S3). The sequence of the transferred region containing the *pbp5* gene was predicted by a gene-by-gene comparison strategy using *blastn* (genes present in transconjugant strain TCGEHPH2.1 and donor strain HPH2 but not in recipient strain GE1) (Supplementary Table S4). Sequence of the putative “transferred region” was mapped against the genome of the *E. faecium* strain DO (RefSeq Accession: NC_017960) using Easyfig 1.2 software (Sullivan et al., 2011). In addition to *E. faecium* DO, the “transferred region” was also aligned against fully sequenced and closed genomes of *E. faecium* Aus0004, *E. faecium* NRRL B-2354 and *E. faecium* Aus0085 strains (RefSeq accessions numbers: NC_017022, NC_020207 and NC_021994, respectively) with Mauve (Darling et al., 2004). Functional annotation of the query sequences was performed using Blast2GO and Uniprot and KEGG databases³.

Analysis of the *pbp5* Genetic Environment

An 8–10 kb DNA fragment comprising the *pbp5* gene and its boundaries was characterized by PCR assays in 15 transconjugants and 21 field isolates (Table 1). PCR conditions were adapted according to the expected amplicon size (<3 kb/>3 kb): 0.5/1 mM of each primer, 2/2.5 mM MgCl₂, 1x of reaction buffer, 0.2/0.4 mM of each deoxynucleoside triphosphate, 1.25U GoTaq[®] Flexi DNA Polymerase [Promega Corporation, Madison, WI, USA]/2.5U Takara LA Taq polymerase (TakaraTM Bio Inc., Shiga, Japan). The amplification program was 25 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C; 1 cycle 10 min at 72°C (for fragments <3 kb) or 35 cycles of 30 s at 96°C, 1 min at 55°C, 7 min at 72°C; 1 cycle 10 min at 72°C (for fragments >3 kb). Amplicons were further discriminated by comparing the corresponding RFLP patterns obtained after digestion with *Dde*I or *Apa*I and sequences. Genomic location of *pbp5* was identified by hybridization of I-CeuI and *Sma*I-digested genomic DNA using 23S rDNA and/or *pbp5* probes (Liu et al., 1993; Freitas et al., 2013). Labeling and detection were carried out with Gene Images Alkphos direct labeling system kit following the manufacturer's instructions (Amersham GB/GE Healthcare Life Sciences UK limited). The presence of CTn916, CTn5386 and CTn5382, previously associated with transferable AmpR, was determined by analyzing the presence of specific sequences (integrases, excisionases, non-integrase left region) (Carias et al., 1998; León-Sampedro et al., 2016). The transferred 8–10 kb genetic regions carrying *pbp5* characterized in this study were compared with those of all *E. faecium* available genomes at the National Center for

²<http://www.uniprot.org/uniref>

³<http://www.genome.jp/kegg/genome.html>

¹<http://pubmlst.org/>

TABLE 1 | Epidemiological background, *pbp5* gene characterization and antibiotic resistance among *E. faecium* strains and transconjugants from this study.

Isolate type (isolate name)	<i>pbp5</i> genetic environment type (<i>pbp5</i> -fstW)		Epidemiological background						Resistance to other antibiotics	
	(RFIP_DdeI)	Sequencing	PBP5 alleles	PFGE-SmaI/ <i>pbp5</i> hybridization (kb)	ST	BAPS	PFGE ^a	Date	Source/ sample ^d	
<i>E. faecium</i> recipient strains										
Rc (<i>Efc</i> GE1)	-	-	-	-	515	2.3b	A			≤ 0.016
Rc (<i>Efm</i> BM4105RF)	A	-	-	290	172	1.3	B			0.5
Rc (<i>Efc</i> 64/3)	B	-		250	21	2.3a	C			RIF, FA
<i>E. faecium</i> donor strains and their corresponding transconjugants										
WT (H323)	C	^b	210	280	3.1	100		2002	H(C)/urine	128
TCEf _m GE1 (GEH323.3)	C	-	C12	210					VAN, TEC, ERY, CIP, GEN, STR	32
WT (HPH2)	C	^b	210	125	3.1	126		2007	H(A)/urine	128
TCEf _m GE1 (GEHPH2.1)	C	-	C20	210					VAN, TEC, ERY, CIP, NIT	64
TCEf _m 64/3 (64HPH2.1)	ND		170 + 250						VAN, TEC, TET, ERY, RIF, FA	8
WT(70411)	^b		210	670	3.3a	90		1997	H(B)/urine	32
TCEf _m GE1 (GE70411.2)	D		C4	210					VAN, TEC, TET, ERY, RIF, FA	32
TCEf _m BM4105RF (BM70411.5)	D		C4	170 + 200					VAN, TEC, TET, ERY, RIF, FA	8
WT (E4)	D	^b	210	132	3.3a	Ζ		2001	HS(E)/water	128
TCEf _m GE1 (GEE4.1)	D		C19	210					TET, ERY, CIP, GEN, STR	16
WT (28798)	D	^b	210	132	3.3a	88		1999	H(B)/blood	64
TCEf _m GE1 (GE28798.1)	D		C8	210					ERY, QD, CIP, GEN, STR	32
WT (E233)	D	^b		210	132	3.3a	88.1	2002	H(S(E))/water	64
TCEf _m GE1 (GEE233.1)	D			210					VAN, TEC, TET, ERY, STR, RIF, FA	32
WT (E169)	D	^b		210	132	3.3a	88.2	2001	HS(C)/water	64

(Continued)

TABLE 1 | Continued

Isolate type (isolate name)	<i>pbp5</i> genetic environment		Epidemiological background						Resistance to other antibiotics			
	(RFLP_Ddel)	type (<i>pbp5</i> -rstW)	Sequencing	PBP5 alleles	PFGE-SmaI <i>pbp5</i> hybridization (kb)	ST	BAPS	PFGE ^a	Date	Source/ sample ^d	MIC to Amp (mg/L)	
TCEfmGE1 (GEE169.3)	D			210							32	TET, RIF, FA
TCEfmBM4 105RF (BME169.3)	WT (E49)	ND		200 + 240							8	RIF, FA
TCEfmGE1 (GEE49.1)	D	^b		210	132	3.3a	C3	2001	HSE/water		128	VAN, TEC, ERY, CIP, STR
WT (H207)	ND		C8	210							32	VAN, TEC, TET, RIF, FA
TCEfmGE1 (GEH207.1)	ND	ND	C4	210	280	3.1	150	2002	H(C)/exudate		32	TET, ERY, CIP, GEN, STR
TCEfmBM4 105RF (BMH207.3)	ND	ND	Unknown ^c	180							32	TET, RIF, FA
WT (SN71, SN133)	C	^b		210							8	RIF, FA
				200	393	2.1a	SN208	2006	PG/n = 4- waste lagoon, animal faeces		64	TET, ERY, STR, NIT
TCEfmGE1 (GESN71.1; GESN133.1)	C	-	C9	200							64	TET, RIF, FA
TCEfmBM4 105RF (BMSN71.1)	C	-		200							8	RIF, FA
TCEfm64/3 (64SN71.1)	C	-		210							64	RIF, FA
WT (VD79C1)	D	^b		200	18	3.3a	59	2001	HH/faeces		128	VAN, TEC, TET, ERY, CIP, GEN
TCEfmGE1 (GEVD79C1.5)	D		C7	210							128	VAN, TEC, TET, ERY, GEN, RIF, FA
TCEfmBM4 105RF (BM/D79C1.6)	D		C7	200							128	VAN, TEC, RIF, FA
Representative <i>E. faecium</i> without <i>pbp5</i> transfer												
WT (529940)	ND		C6	ND	16	3.3a	74	2000	H(B)/urine		> 64	VAN, TEC, TET, ERY, STR
WT (H352)	ND		C4	ND	280	3.1	71	2000	H(D)/sputum		> 32	VAN, TEC, ERY, CIP, GEN
WT (HPH6)	ND		C5	ND	18	3.3a	128	2007	H(A)/pus		> 32	VAN, TEC, ERY, CIP, NIT

(Continued)

TABLE 1 | Continued

Isolate type (isolate name)	<i>pbp5</i> genetic environment		Epidemiological background								
	(RFLP_Ddel)	Sequencing	PBP5 alleles	PFGE-Smal <i>pbp5</i> hybridization (kb)	ST	BAPS	PFGE ^a	Date	Source/ sample ^d	MIC to Amp (mg/L)	Resistance to other antibiotics
WT (E197)	ND		C8	ND	368	3.3a	H4	2001	HS(C)/water	>32	VAN, ERY, CIP, GEN, STR
WT (SN446)	ND		C8	ND	132	3.3a	H119.5	2007	PG/ water	>32	VAN, TEC, ERY, GEN
WT (H196)	ND	∨	C1	ND	390	3.1	129	2002	H(D)/unknown	>32	TET, ERY, STR
WT (C373)	ND	—	C11	ND	148	2.1b	36.2	2001	HH/faeces	>32	TET, ERY, CIP, GEN, STR, NIT
WT (SE97F1)	ND	—	C10	ND	<i>purK6</i>	ND	ND	2001	P/skin	>32	TET, ERY, CIP, STR
WT (164306)	ND	∨	C2	ND	190	2.3a	98	1998	H(B)/urine	32	VAN, TEC, TET, ERY, CIP, STR
WT (SN194)	ND	∨	C3	ND	264	2.3a	ND	2006	PG/residual water	32	TET, CIP, CHL, STR

^aEach different PFGE number or letter represent a different clone. In clones 88.1 and 88.2, the number after a dot represents the number of bands differing from the more ancient clone 88. ^bThe *pbp5* genetic platform type of these wild strains was determined by RFLP, which pattern was the same as the respective transconjugants with *pbp5* platform characterized by sequencing. ^cIn this strain we could not amplify the region between *pbp5* and *fstW* or *pbp5* and *N-acylglucosamine-6-phosphate 2-epimerase*. Abbreviations: RC, recipient strain; WT, wild type; TC, transconjugant; Efm, *E. faecium*; H, hospital; HS, hospital sewage; HH, healthy human; PG, piggery; P, poultry; VAN, vancomycin; CIP, ciprofloxacin; TET, tetracycline; TEC, teicoplanin; FA, fusidic acid; NIT, nitrofurantoin; RIF, rifampicin; FA, Fusidic Acid; ND, not determined. ^dLetters appearing in parenthesis of H (hospitals) and HS (hospital sewage) represent the five hospitals included (A-E).

Biotechnology Information (NCBI) database (Supplementary Table S5).

Phylogenetic Analysis of PBP5

Comparative analysis of all PBP5 protein sequences available in the GenBank database with those identified in this study was performed using the basic local alignment sequence tool (BLAST) (Altschul et al., 1997) and the ClustalW2 program for multiple sequence alignment (Larkin et al., 2007; Goujon et al., 2010), hosted by the National Center for Biotechnology Information (NCBI) and the European Bioinformatic Centre (EBI), respectively. The PBP5 amino acid sequences were designated by a “C” followed by a number in agreement with the nomenclature used in previous works (Supplementary Table S6) (Galloway-Peña et al., 2011). Multiple sequence alignments of all PBP5 sequences were performed through MEGA 7 software⁴ and the topology of the phylogenetic tree was inferred by maximum-likelihood algorithm using PhyML (Guindon et al., 2010) with bootstrap analysis based in 1000 permutations and a cut-off of ≥70%.

Phylogenetic congruence of core genomes and *pbp5* sequences was established by comparative analysis of their gene tree topologies. Core genomes for the 233 *E. faecium* strains whose draft genome sequences were available at NCBI Trace Database (last updated on February 2014), were reconstructed independently using an all-against-all reciprocal BLAST approach. Briefly, each CDS was used as a BLAST query against a local database of CDS from all genomes in the sample analyzed. Afterward, we re-annotated the chromosome sequences with GeneMarkS v2.8 (Besemer et al., 2001). The core genome was defined as the non-redundant genes present in all strains using CD-HIT (Li and Godzik, 2006) with the 80% coverage, 80% identity cut-offs, and parsed by homemade Perl script. All core genome genes were concatenated and aligned using MAFFT (Katoh and Toh, 2010). The phylogenetic tree was built by FastTree2 (Price et al., 2010). Tree comparison was carried out by *cophyloplot* command of APE (Analyses of Phylogenetics and Evolution) (Paradis et al., 2004) using R software.

RESULTS

Transferability of AmpR (*pbp5*)

Twelve isolates ($n = 12/78$; 15%; Table 1) were able to transfer under our experimental conditions a chromosomal genetic platform containing *pbp5* (associated with AmpR) to *E. faecium* strain GE1. Five of them were also able to transfer *pbp5* to *E. faecium* BM4105RF and two, to *E. faecium* 64/3. All donor strains belonged to major *E. faecium* human lineages mostly associated with clinical isolates causing hospital infections, namely BAPS subgroups 3.3a (ST670, ST132, ST280), 3.1 (ST280, ST125), and 2.1a (ST393). They were obtained from samples of hospitalized humans but also from samples of hospital sewage, healthy humans and piggeries. β -lactamase production was not detected in any of the isolates.

⁴www.megasoftware.net

All transconjugants had similar PFGE profiles to recipient strains (**Figure 1**), were resistant to rifampicin and fusidic acid and exhibited a variable susceptibility to ampicillin (MICs = 8–128 mg/L), which was often lower than that of their corresponding donor isolates (8 mg/L or 16 vs. 32 mg/L to >256 mg/L). Besides the AmpR phenotype, some transconjugants also exhibited resistance to vancomycin ($n = 8$), teicoplanin ($n = 8$), erythromycin ($n = 6$), tetracycline ($n = 1$; in non-GE1 transconjugants; as GE1 strain is tetracycline-resistant), streptomycin ($n = 1$) and/or gentamicin ($n = 1$). Transconjugants carrying the *pbp5* genetic platform either alone or with plasmids harboring genes encoding resistance to different antibiotics were recovered from selection plates supplemented with ampicillin and not with other antibiotics (data not shown).

The *pbp5* Gene of AmpR Isolates Is Located on Large and Transferable Chromosomal Genetic Platforms Containing Metabolic Traits

Hybridization of *pbp5* and 23S rDNA probes with a digested I-CeuI DNA band of high MW revealed a chromosomal location of *pbp5*. Further hybridization of *Sma*I-digested genomic DNA with

the *pbp5* probe showed hybridization to fragments of ~210 kb in all but one GE1 transconjugants, for which the *pbp5* probe hybridized to a band of ~180 kb (**Figure 1**). Hybridization of the same probe with fragments of *Sma*I-digested genomic DNA of *E. faecium* BM4105RF and *E. faecium* 64/3 transconjugants of different sizes reflects independent transfer events. Similarity in the PFGE patterns of the transconjugants obtained using different donors indicates that DNA acquisition occurs within a particular region of the genome (**Figure 1**). The donor strains lack integrases/excisionases of Tn916-like conjugative transposons (Tn916, Tn5386, Tn5382).

For whole genome sequence a representative donor:recipient pair and its transconjugant were analyzed. The donor was the clinical HPH2 strain; the recipient, *E. faecium* GE1 which did not originally carry *pbp5*, and thus limiting the possibilities of recombination events between the acquired and natural *pbp5* genetic platform in the recipient strain; and the size of the *Sma*I digested genomic DNA fragment, the one that most commonly hybridizing with the *pbp5* probe (~210 kb). Sequencing of the recipient (*E. faecium* GE1), the donor (*E. faecium* HPH2) and the transconjugant (*E. faecium* TCCEHPH2.1) allowed us to identify 7 contigs with genes present in the donor and its transconjugant but absent in the

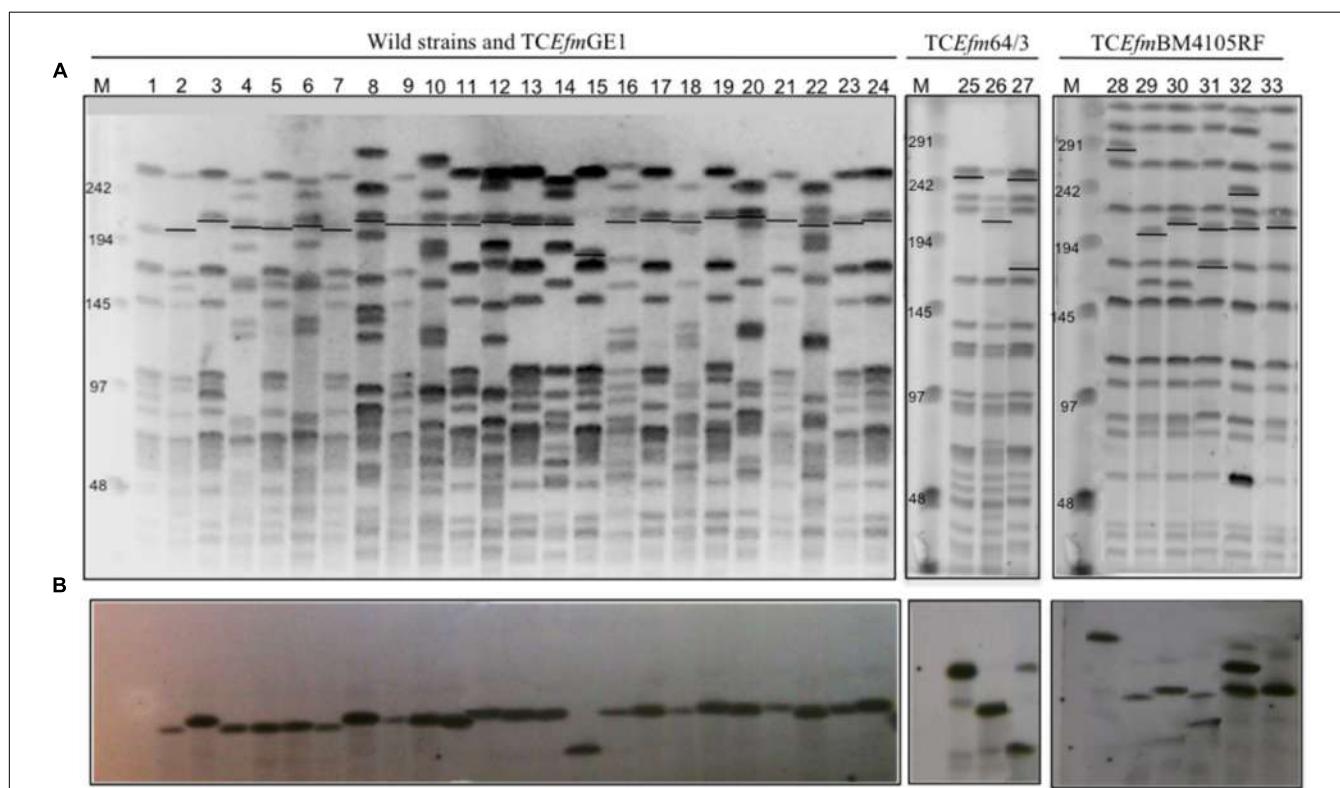
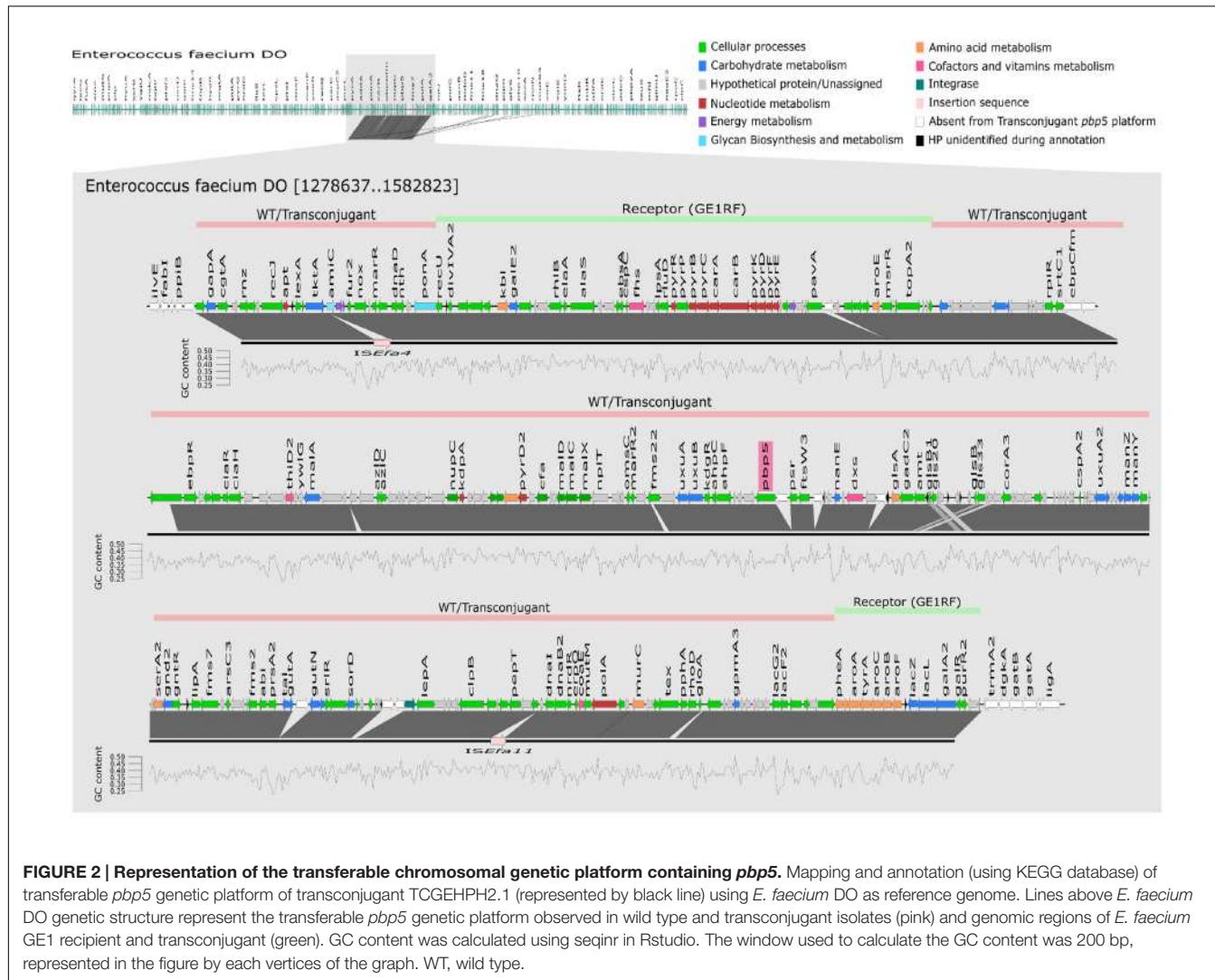


FIGURE 1 | Clonality and hybridization assays with *pbp5* probes of wild type, recipients and transconjugants strains. (A) PFGE *Sma*I digested DNA of recipient strains (1-*E. faecium* GE1, 25-*E. faecium* 64/3, 28-*E. faecium* BM4105RF), wild type (2-VD79C1, 4-SN71, 6-SN133, 8-HPH2, 10-H323, 12-70411, 14-H207, 16-E49, 18-E233, 20-E169, 22-E4) and transconjugants (3-GEVD79C1.5, 5-GESN71.1, 7-GESN133.1, 9-GEHPH2.1, 11-GEH323.3, 13-GE70411.2, 15-GE207.1, 17-GEE49.1, 19-GEE233.1, 21-GEE169.3, 23-GE28798.1, 24-GEE4.1, 26-64SN71.1, 27-64HPH2.1, 29-BMSN71.1, 30-BMH207.3, 31-BM70411.5, 32-BME169.3, 33-BMVD79C1.6). **(B)** Hybridization assays with a *pbp5* probe (primers P1 and P2-Figure 4). M- Low Range PFGE Marker, kb (New England, BioLabs). Abbreviations: TCCEfm- Transconjugant *E. faecium*.

recipient strain. The fragments which comprised the *pbp5* gene, represent a genetic platform of approximately 280 kb that is slightly larger than that inferred from the *Sma*I-PFGE gels. A more detailed analysis of the contigs revealed that two of them (contig00158 and to a lesser extent contig00068) also contained genes present in the recipient and transconjugants but not in the donor strain, which could be explained either by the partial transfer of the *pbp5* genetic platform and/or post-transfer recombination events (Figure 2). The comparative analysis of the genomes sequenced in this work with the four closed *E. faecium* genomes available at the NCBI database at the time of writing this manuscript [DO (NC_017960.1), NRRL B-2354 (CP004063.1), Aus0004 (NC_017022.1), Aus0085 (NC_021994.1) strains], revealed a common region of 153 kb for our donor/transconjugant strains and the four genomes analyzed, which was absent in the recipient *E. faecium* GE1 genome (Figure 3). The remaining sequence of the 280 kb “transferred chromosomal region” was variably present and located in different genomes (Figure 3).

Figure 2 shows a detailed characterization of the 280 kb transferred genetic platform, with ORFs of a G + C content ranging from 24 to 41%. Besides the *pbp5*-related resistance to β -lactams, this chromosomal transferable genetic platform contains genes involved in different cellular functions including amino acids and carbohydrate transport, redox processes, survival under stressful conditions in the intestinal environment (e.g., acid and bile tolerance) and also other genes related to β -lactam resistance. Among the last category is a gene (named *ponA*) encoding a bifunctional class A PBP with transpeptidase and transglycosylase activity, which is involved in the synthesis of peptidoglycan, allowing the survival in the presence of cell wall inhibitors (Rice et al., 2009). A copy of the *CiaRH* operon, coding for a two-component signal-transduction system responsive to cell envelope lysis-stress response and restricted to *Streptococcus* spp. to date, was also detected. The 280 kb genetic platform containing *pbp5* also had genes with presumed influence on microbe-host interactions. These include three phosphotransferase systems (PTS), namely



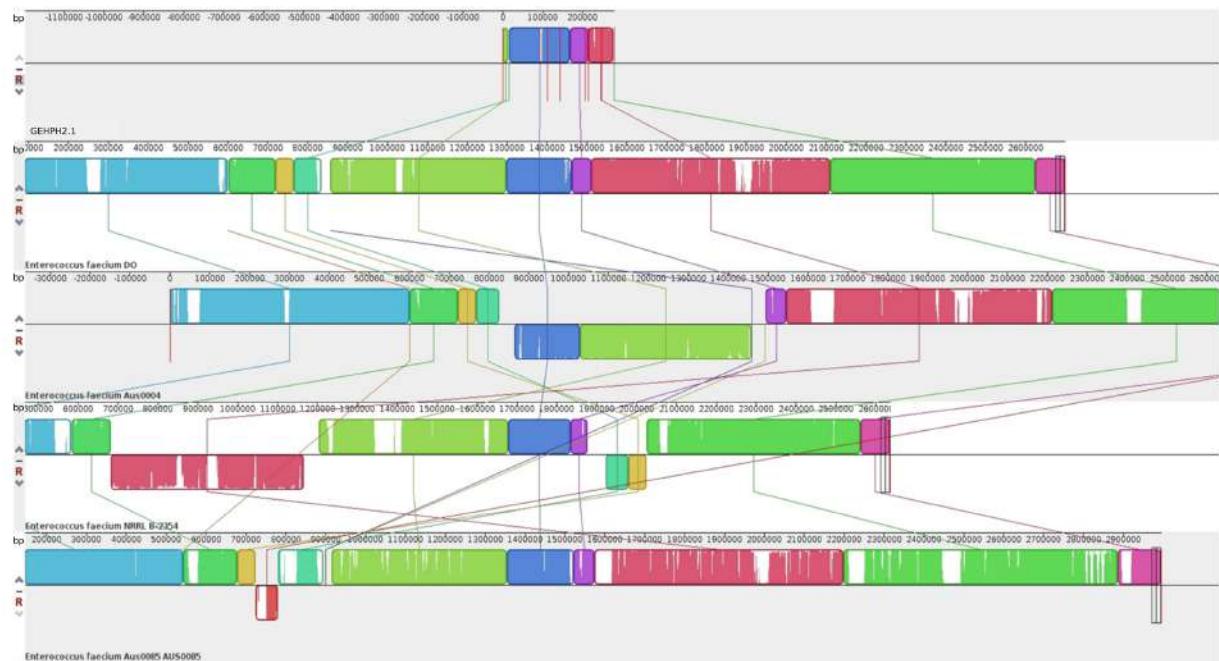


FIGURE 3 | Comparison by MAUVE of the transferable genetic platform containing *pbp5* (TCGEHPh2.1) with the four close *E. faecium* genomes (DO, Aus 0004, Aus 0085 and NRRL B-2354), present in GenBank database. Each color block represents a genome region present in at least two of the sequences analyzed. These similar blocks can be identified by their color. Blank regions within the block represent point mutations or even regions that are absent in the same color blocks of the other sequences.

glucitol/sorbitol, L-ascorbate and mannose/fructose/sorbose), the ABC transport and transformation system of maltodextrines (*malACDX*) and the *N*-acetylmannosamine-6-phosphate epimerase (*NanE*; part of a pathway that allows the usage of sialic acids, major components of glycoproteins, gangliosides, and other sialoglyco-conjugates). Other metabolic genes in the *pbp5* genetic platform could be involved in tolerance to the intestinal acidic environment, including glyceraldehyde-3-phosphate dehydrogenase, ATP synthase subunit α , NADH dehydrogenase, glutaminase and genes encoding enzymes involved in the production of ammonia from glutamine and deamination or transport of branched/nitrogenated amino acids. Finally, five stress response proteins including two belonging to the Csp (cold shock proteins) system (*CspA* and *CspC*) and the small chaperone Hsp20, and Gls33 (also present in *E. faecium* genomes available in the GenBank databases) were also identified. They are involved in stress responses to salts, pH and ethanol exposure in *Clostridium*. Hsp20 is a small chaperone protein involved in survival of different abiotic stress conditions including heat (55°C) and salt (5 mM) in *Bifidobacterium longum* (Khaskheli et al., 2015).

To date, only two *E. faecium* strains lack *pbp5*. These are strains GE1 and D344SRF, which are AmpS isolates. The D344SRF strain is susceptible to cephalosporin and ampicillin due to the spontaneous deletion of a 170 kb genome fragment that includes *pbp5* and other *pbp* genes. This deletion occurred by the interaction of CTn5386 (a 60 kb element that comprises *pbp5* and *vanB2*) with Tn916 (Rice et al., 2007). The GE1 laboratory strain

does only harbor the integrase of Tn5801. The causes of the loss of the *pbp5* genetic platform are still unknown.

The Chromosomal Region Harboring *pbp5* Exhibit Hotspots for Insertions

The occurrence of different insertion sequences (ISs), (Figure 4) in the boundaries of the *pbp5* gene and the presence of a >3027 bp *psr-pbp5* fragment in *E. hirae* (99.9% identical to that found on *E. faecium*) suggest the presence of hot spots that could facilitate recombination of regions containing the *pbp5*. To test this possibility, we further analyzed the 8–10 Kb genetic environment of *pbp5* from 21 wild type and 15 transconjugant isolates included in this study as well as available enterococcal genomes. Identical RFLP patterns of these 8–10 kb fragment were observed for each pair of donor and its corresponding transconjugants, but were different from those naturally occurring in the *pbp5* carrying recipient strains *E. faecium* BM4105RF and 64/3 (Figure 4). Sequencing of fragments representing distinct RFLP patterns and comparative analysis with similar *pbp5* genetic environment in GenBank available genomes, revealed 21 variants of such 8–10 kb chromosomal fragments (designated by roman numerals), which differed in the number and type of ISs (Figure 4). Three variants (types I, II, III) corresponded to *pbp5* transferable platforms described in this study (Figure 4, Table 1).

The predominant 8–10 kb fragment identified in both AmpR and AmpS *E. faecium* analyzed in this study did not contain indels and is considered here as the *pbp5* genetic environment

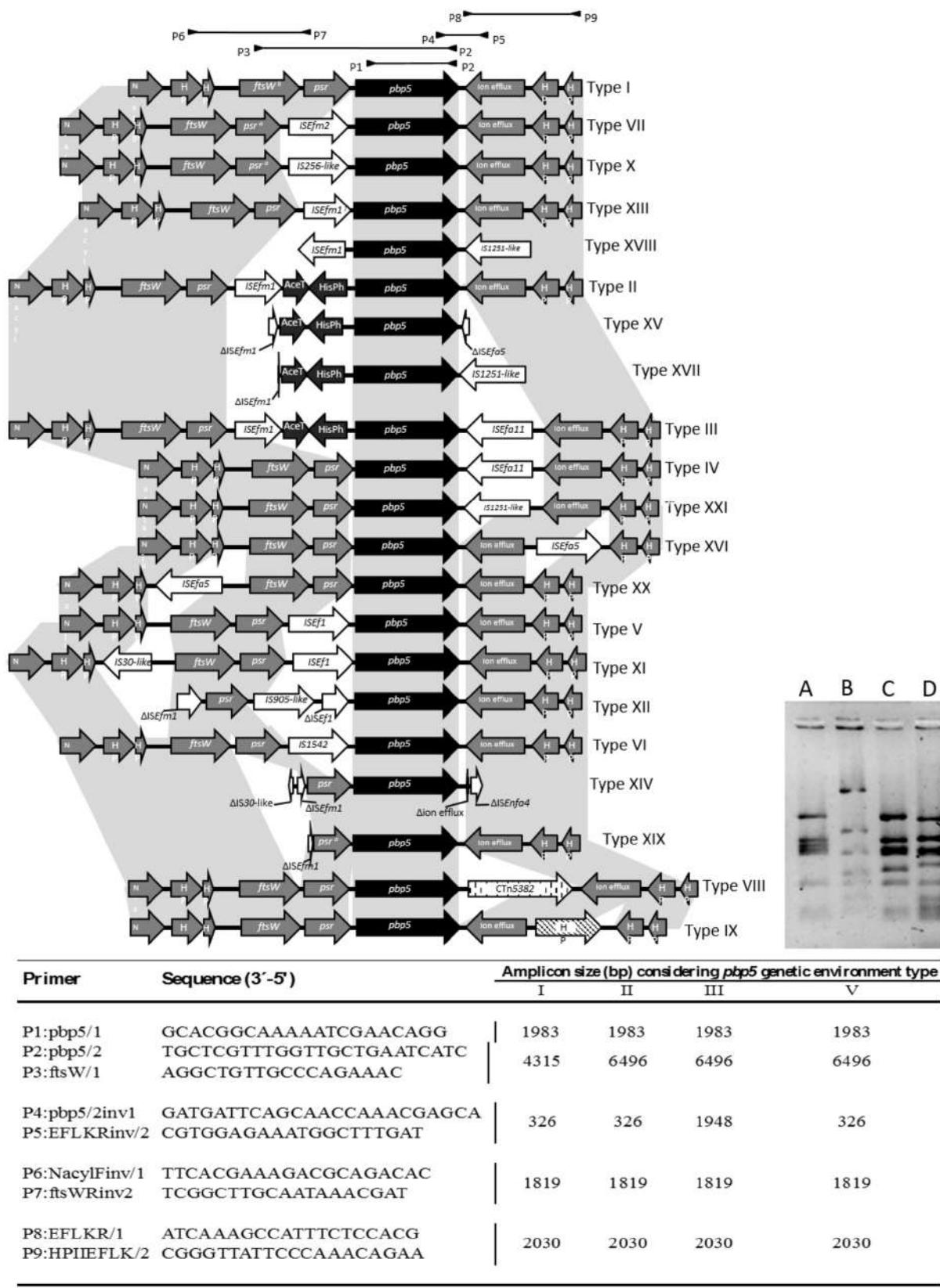


FIGURE 4 | Continued

FIGURE 4 | Characterization of *E. faecium* *pbp5* genetic environment by PCR and sequencing. The Roman numbers I, II, III, and V represent the different *pbp5* genetic platforms detected in *E. faecium* from this study. The numbers IV, VI to XXI were detected in available genomes from GenBank database. The different types were named according to diversity of insertions sequences, genomic fragments or conjugative transposons within genes or intergenic regions. Mutations or recombinations within genes or intergenic regions were not considered for type classification. The Table indicates the primers used (designed for this study; P1/P2 described by Dahl et al., 2000) and the size of PCR amplicons from genetic environment of types I–III and V. The A, B, C and D lines of the bottom right side figure represent RFLP patterns of amplicon P3-P2 of mobile platforms I (pattern C) and II/III (pattern D) of isolates included in this study, when digested with Ddel restriction enzyme. The patterns A and B correspond to the amplicons of the recipient strains *E. faecium* BM4105RF and 64/3, respectively.^a These gene has an extra stop codon within its sequence. Abbreviations: N-acyl, (N-acyl-glucosamine-6-phosphate-2-epimerase); HP (hypothetical protein); *ftsW* (cell cycle protein); *psr*, (*pbp5* synthesis repressor); *pbp5* (gene encoding penicillin binding protein 5); AceT (acetiltransferase); HisPh (Histidinol Phosphate Phosphatase).

prototype; arbitrarily named Type I (Supplementary Table S5, **Figure 4** and 5). The *pbp5* or other genes (e.g., *psr*, *ftsW*, ion efflux genes) were flanked by one or two insertions sequences of the IS256, ISL3 or IS982 families (ISEf1, ISEfm1, ISEfm2, IS1542, IS256-like, ISEfa11, or IS1251-like) in isolates carrying types II to XXI, with the exception of type VIII that had CTn5382 (*vanB2*) inserted downstream of *pbp5*. The identifiable boundaries of ISs detected within the 8–10 kb boundaries of *pbp5*, as well as the common nucleotide positions at which insertions occurred, suggested recent acquisition events at some hot-spots on an ancestral sequence (**Figure 4**). Despite of predominance of type I, some types seem to be more associated with specific hosts as the case of type V, predominant in strains from pigs and type II/II-like, III or type XIV-like which are frequent among clinical isolates. Epidemiological distribution of isolates appears in Supplementary Table S5.

Diversity of PBP5 Sequences Reflects the Phylogenomic Diversification of *E. faecium*

We identified 75 PBP5 protein variants (Supplementary Tables S5 and S6) corresponding both to AmpR and AmpS strains which comprise 20 of the previously described variants C4, C7, C9, C11, C15–C18, C21, C24, C46–C48, C50, C51, C61, C63, C65, C67, C71 (Rybkin et al., 1998; López et al., 2009; Galloway-Peña et al., 2011). The other 55 variants were firstly detected in this study either in strains from Portugal ($n = 11$) or from available genomes at NCBI database ($n = 44$). The C4, C7, C8, C9, C12, C19, and C20 sequences were identified in isolates able to transfer *pbp5* (**Table 1**, Supplementary Table S6; **Figure 5**), which belonged mostly to ST18, its SLV ST125, ST280 and ST670 (BAPS 3.3a, 3.1 and 2.1a, respectively) (**Table 1**).

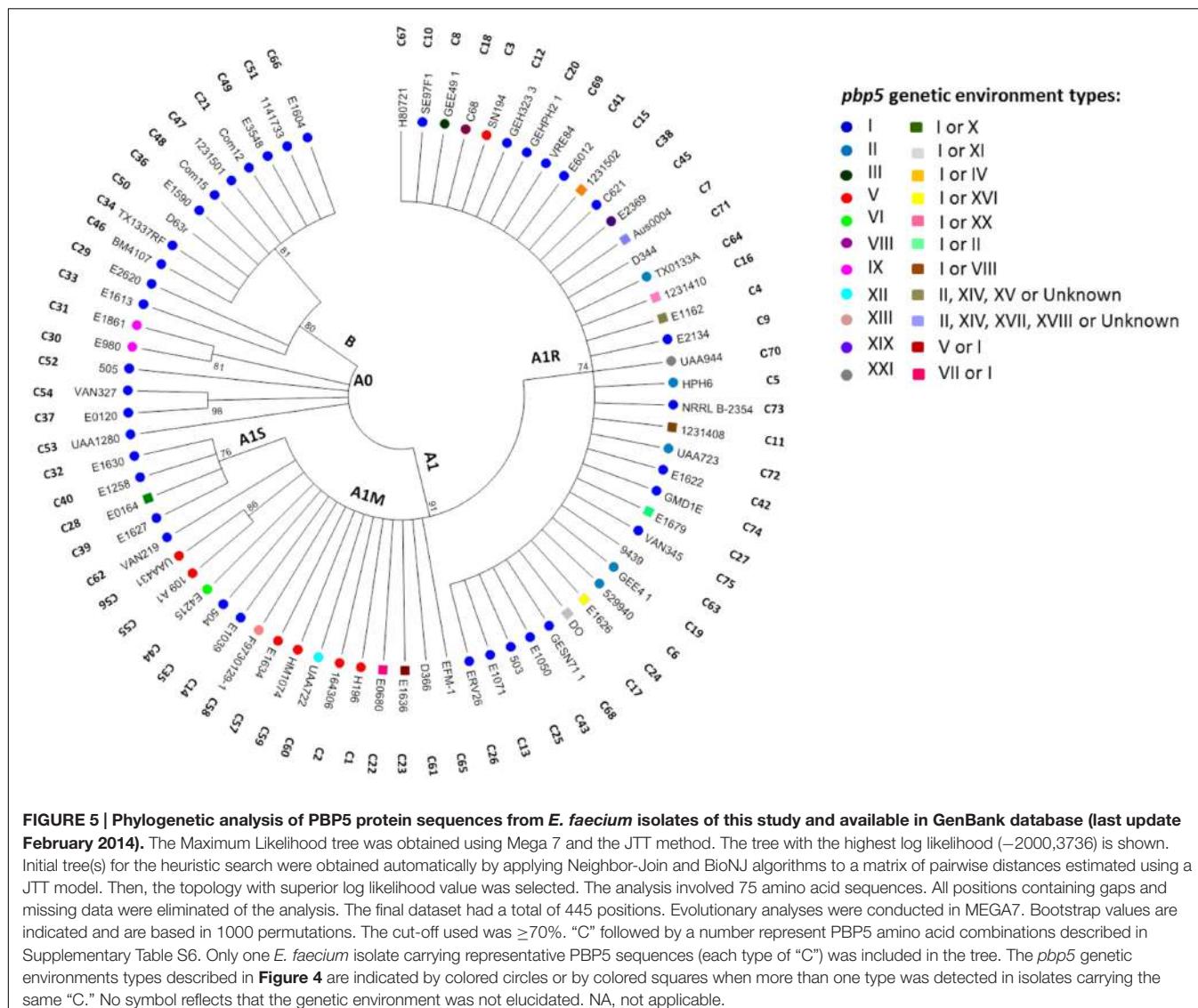
Figure 5 shows the phylogenetic tree constructed with all PBP5 protein sequences from this study and those available at GenBank databases. The tree is split into two major clades arbitrarily named B and A, mirroring the clades associated with populations of non-hospitalized persons and hospital isolates respectively, which were previously inferred from phylogenomic studies of *E. faecium* (Lebreton et al., 2013). Clade B comprises PBP5 of AmpS isolates (PBP5-S), which mainly correspond to strains of BAPS subgroups 1.2 and 1.5. Some are similar to the prototype PBP5-S C46 sequence of *E. faecium* BM4107 strain (Sifaoui et al., 2001) but most of them also exhibit mutations at positions T25A, S39T and D644N, which are also common to PBP5 sequences of clade A isolates (Supplementary Table S6). Two strains, isolated in 1964 and 2006, further showed changes

at S27G + T324A and S27G respectively, such mutations corresponding to the PBP5-R consensus sequence (Pietta et al., 2014). The type I chromosomal region mentioned above was observed in all available isolates from this group (**Figure 5**; Supplementary Table S5).

Clade A includes PBP5 variants grouped in two main clusters arbitrarily designed here A0 and A1, with most isolates sharing mutations at positions V24A, S27G, E100Q, K144Q, T172A, T324A, N496K, A499T and E525D (7 of the 21 positions used to establish sequence diversity of this protein) (Pietta et al., 2014). The Clade A0, represented by C30, C31, C37, C52, C53, and C54 PBP5 sequences, corresponds to AmpS isolates of different BAPS groups (BAPS 1.2, BAPS 2.3a and BAPS 3.3b), recovered from different hosts (animals and humans), different countries and collected from 1995 to 2001 (**Figure 5**, Supplementary Table S6). Most isolates within clade A0 carried *pbp5* within a type I fragment. Sequences of Clade A1 were classified in three subclusters that parallels *E. faecium* populations of BAPS groups 2 and 3 (Willems et al., 2012; Tedim et al., 2015). They were designated as A1S, comprising a subset of PBP5-AmpS variants (mainly associated with human and animal isolates of BAPS subgroup 2.1b); A1M, comprising a subset of PBP5-AmpS and PBP5-AmpR (mostly associated with animal isolates of diverse BAPS subgroups 2.1b, 2.3a, 2.3b, 3.1, 3.2, and 3.3b); and A1R, including almost all PBP5-AmpR (mostly associated with the clinical setting and BAPS subgroups 3.1, 3.3a and 2.1a, the latter two only observed in this group).

All isolates from Clades A1S, A1M and A1R share mutations R34Q, G66E, L177I and A216S but also present some differences. Mutations S39N and A401S plus A499I were specific for subclades A1S and A1M, respectively. The last two polymorphisms were previously documented in two AmpR strains (Pietta et al., 2014) but this study suggests that they were fixed in certain populations despite having been previously discarded as relevant changes. One of the A1M strains had also variations at specific positions linked to the A1R group (A68T, E85D, M485T, V586L, E629V and P667S). Of note is the PBP5 variant C23 within this cluster, which was overrepresented ($n = 44$).

PBP5 variants within the A1R group exhibited six mutations (A68T, E85D, S204G, 466'S/D, M485A/T, E629V, P667S) predominant in this group, some of them located in the active site of the protein (466'S/466'D, M485A/T) and in the end of a turn between the β 1 and β 2 strands (E629V; P667S) (Fontana et al., 1996; Rice et al., 2004). It is of note that some AmpS isolates (BAPS subgroups 3.1, 2.3a and 3.2) with PBP5 sequences



that cluster in the A1R subgroup (including the PBP5 of the recipient *E. faecium* 64/3) lack the mutation M485A and E629V, which confirmed that such mutations might be necessary for AmpR phenotype, as reported (Rice et al., 2004). Changes A68T, E85D and S204G were shared by AmpS and AmpR isolates of A1R group (Supplementary Table S6). Some strains exhibited the particular pattern of mutations Q408H, A558T, G582S, K632Q and, eventually, V462A, N546T and P642L. They had not previously been associated with AmpR. Distribution of different variants of the 8–10 kb boundaries of *pbp5* gene is shown in Figure 5 and Supplementary Table S5. Of note, is the detection of type I in isolates of different groups of clade A1 as well as the association of types V and XI with isolates of group A1M and of the similar types II, III, XIV, XV, and XVII with isolates of group A1R.

We also analyzed the diversity of other genes previously associated with AmpR (*ddcP*, *ddcY*, *ldtEfm*, *pgtA*, *lytG*) in available genomes, (protein sequence per UniRef100 available at

Uniprot using as reference *E. faecium* strain Aus0004). Single-locus phylogenetic tree of PBP5 was congruent with those of proteins codified by *ldtEfm* and *ddcY* but was non-congruent with those of *ddcP*, *pgtA* and *lytG* (data not shown).

Analysis of gene tree topologies (*E. faecium* core genomes vs. *E. faecium* *pbp5* genes) revealed discrepancies that indicate transfer of *pbp5* between *E. faecium* populations (Figure 6) and explains the occurrence of both the same PBP5 variants in isolates of different clonal lineages, and different PBP5 sequences in strains of the same phylogenomic groups (Figure 5, Supplementary Table S5).

Ampicillin Resistance Was Maintained Without Selective Pressure

AmpR was maintained in the wild-type strains and respective *E. faecium* GE1 transconjugants over 200 generations in antibiotic free BHI indicating that the acquired genetic platforms

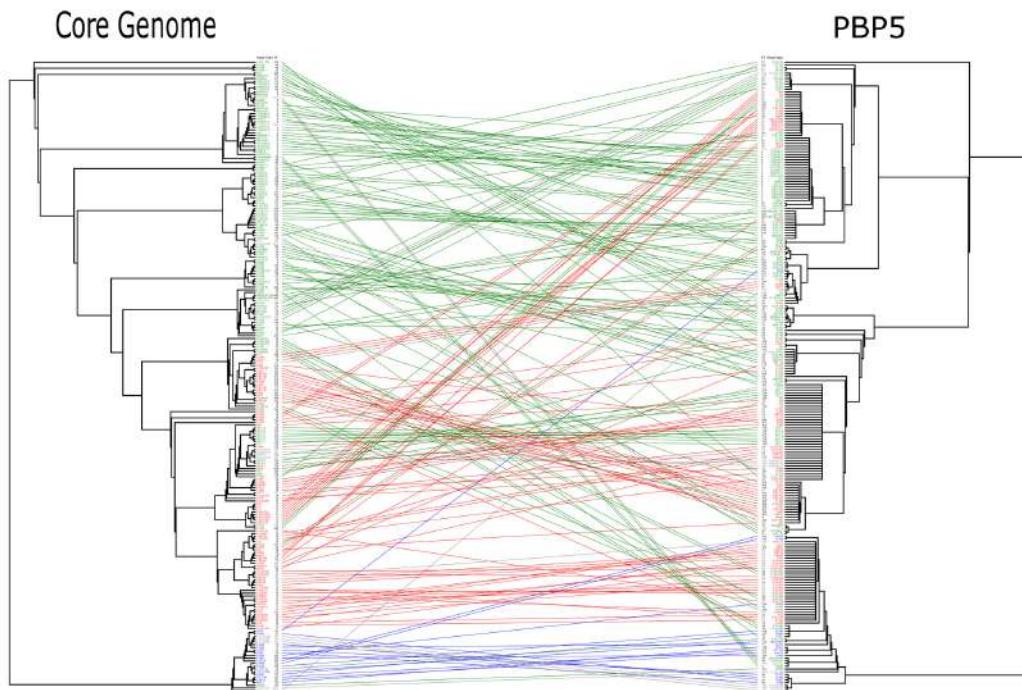


FIGURE 6 | Comparison of gene tree topologies (Core Genome phylogeny vs. *pbp5* phylogeny). Strains of clade B are represented in blue, those of Clade A1 in red and those Clade A2 in green. The edges join the core genome and the corresponding *pbp5* of each strain. Both trees were made by ML using GTR-CAT. Trees and edges were built using APE packages. The results of Mantel test ($r = -0.03$ and significance of 0.761) show the rearrangement of the *pbp5* in comparison with the core genome. The Mantel test (APE package) was made using the distance matrix calculated from nucleotide alignments.

containing *pbp5* may persist in absence of selective pressure in different genetic backgrounds, suggesting no-fitness cost imposed by the acquired *pbp5* platforms. The morphology and growth rate of all evolved transconjugants was similar among each other and to the normal growth of the *E. faecium* GE1 recipient strain.

DISCUSSION

This study documents the occurrence and diversity of a large chromosomal region containing *pbp5* in almost all *E. faecium* genomes and a parallel diversification between the PBP5 and the *E. faecium* core genome. The transferability of this region observed under laboratory conditions using different clonal backgrounds would greatly enhance the possibilities of *E. faecium* adaptation to changing environments.

Polymorphisms in the PBP5 protein sequences allowed grouping of PBP5 variants in clusters that mimic the phylogenomic diversification of *E. faecium* (Galloway-Peña et al., 2012; Lebreton et al., 2013). Lebreton et al. (2013) suggested a model for evolvability of this enterococcal species consisting of a first split of “clade B” and “clade A” coincidental with human and animal co-habitation occurring 30,000 y ago, and a further split of “clade A” in subclades “A1” and “A2” after the introduction of antibiotics in the late 1940s. Two main clusters of PBP5 variants were also identified in the present study, designated as “B” (associated with the *E. faecium* “clade

B”), and “A” that further split in subgroups A0 (including only AmpS isolates) and A1 comprising three small groups linked to different *E. faecium* populations differing in the susceptibility to ampicillin: A1S (AmpS from healthy humans of different BAPS groups), A1M (AmpS and AmpR from humans and animals), and A1R (AmpR from clinical isolates). Based on the apparent universal presence of *pbp5* in *E. faecium* populations, we could speculate that *pbp5* might have predated the *E. faecium* evolutionary split among different hosts. Although some authors have suggested a sequential acquisition of amino acid changes in PBP5 sequence (Galloway-Peña et al., 2011; Pietta et al., 2014), such diversification may indicate different evolutionary routes for AmpR in response to distinct selective pressures in different hosts, similarly to what has been observed for different β -lactamase enzymes of Gram negative organisms (Novais et al., 2010).

The full characterization of the transferable 153 kb chromosomal region that contains not only *pbp5* but also genes contributing to survival in the gastrointestinal tract (e.g., resistance to acids and bile) in almost all available *E. faecium* genomes suggest a contribution of this genetic platform to the adaptation to the mammalian intestine and persistence in abiotic environments. Due to the scarcity of *E. faecium* isolates from non-mammals and non-human hosts, it would result highly speculative to hypothesize about the origin of the PBP5 and other genes located in this genetic platform. The PBP5 is a member of the Mec family, which comprises

proteins with natural low affinity for β -lactams as PBP2a (*mecA*) of *Staphylococcus aureus* and PBP3r of *Enterococcus hirae* (99% identical to PBP5, suggesting a common origin for PBP5 and PBP3r) (Raze et al., 1998; Hiramatsu et al., 2013). For staphylococci, MecA would have been essential for the survival of ancestral members of staphylococci in the presence of β -lactam antibiotics produced by fungi and Actinobacteria and lost in the Devonian period with the emergence of mammals (more than 400 million of years ago). This would be coincidental with the separation and adaptation of staphylococcal species to different hosts (Hiramatsu et al., 2013), in any case isolating *Staphylococcus* populations from old β -lactam producers. Recent antibiotic pressure would have influenced the transference of PBP2a from environmental to human strains of staphylococci. It is of note that the 153 kb transferred genetic platform characterized in this study has a variable G + C content, reflecting HGT events from different sources and suggesting that a similar evolutionary route as that of PBP2a of staphylococci might have also occurred for *E. faecium* PBP5.

The transfer of *pbp5* in *E. faecium* resulting of the interaction between transposons as Tn5382 or Tn916 plus Tn5386 was previously demonstrated for a few isolates (Rice et al., 2005a). During the revision process of this manuscript, a work by García-Solache et al. (2016) was published. This work suggested that transference of chromosomal regions carrying *pbp5* occurs by homologous recombination between similar DNA regions of donor and recipient strains enriched in AT nucleotides with a possible involvement of plasmids (García-Solache et al., 2016). Tn916-like elements were not detected in our strains but the presence of a band of similar size in both donors and recipients and a sequence enriched in AT in the boundaries of the chromosomal transferred region (data not shown) suggested that homologous recombination might occur between *E. faecium* donor and recipient DNA from our study. Although the presence of plasmid genes within this acquired *pbp5* genetic platform was not observed in the transconjugant we sequenced as well as in the case of some transconjugants described in the study of García-Solache et al. (2016), we cannot completely discard this possibility due to the common presence of plasmids of certain families in donors analyzed (data not shown) (Manson et al., 2010; Freitas et al., 2016).

Despite the relevance of elucidation of the mechanism of *pbp5* transfer at molecular level in future studies, it is of note that disparate topologies of the phylogenetic trees of *E. faecium* core genomes and PBP5 sequences obtained in our study reflect how frequent the transfer of *pbp5* genetic platforms under natural circumstances may occur (Tripathi and Sowdhamini, 2008). Similarly, atypical phylogenetic associations have been signaled as evidence of lateral transfer of various types of serine-proteases (PBP5 belongs to this family of enzymes) throughout the prokaryotic world (Tripathi and Sowdhamini, 2008). In fact, we present evidence of inter-chromosomal transfer for different PBP5 C-types. However, in our case, *pbp5* horizontal gene transfer (HGT) events seems to preferentially happen in particular populations, which being

more prone to mutation (the subclade A1 that is characterized by a higher mutation rate than clade B) would further facilitate the *pbp5* fixation and evolution followed by their clonal expansion under antibiotic selective pressure, reflecting the “*ex unibus plurum*” evolutionary dynamics (Baquero, 2011). Heterogeneity of populations that colonize humans, designated as “clouds” elsewhere (Stanczak-Mrozek et al., 2015), leads to a possible global adaptive benefit for certain clones and finally for the overall species, that is enhanced by HGT.

The necessary contribution of different genes for the full expression of AmpR phenotype is suggested by the lower MIC values of the transconjugants in comparison with the wild type strains in this and other studies (Rice et al., 2005b; Zhang et al., 2012). The transferable genetic platform identified here contained *pbp5* and *ponA* but also other genes (*ciaRH* operon) previously associated with β -lactam resistance and virulence in *S. pneumoniae*, but still unexplored in enterococci (Guenzi et al., 1994; Jordan et al., 2008; Krawczyk-Balska and Markiewicz, 2016). Outside this *pbp5*, only *ldtEfm* and *ddcY* showed a similar non-congruent topology with that of *pbp5* indicating that these genes are also under accelerated evolution, which could explain eventual AmpR phenotypes with a lack of correlation with PBP5 sequences (Zhang et al., 2012). The variable ampicillin susceptibility phenotypes observed in different transconjugants even when using the same recipient strain, suggest either a partial transfer of the *pbp5* genetic platform or occurrence of recombination events leading to variation in MICs, which seem to occur frequently in commensal bacteria (Ingle et al., 2016).

CONCLUSION

The arsenal of adaptive traits located on the chromosomal region containing *pbp5* characterized in this study suggests its involvement in the adaptation of *E. faecium* to the gastrointestinal tract of mammals, and its possible contribution to the spread of *pbp5* by horizontal gene transfer, even in the absence of specific β -lactam exposure. However, the apparent frequent transfer events of such genetic platform among “clouds” of closely related *E. faecium* populations, would indicate bacterial shifts in the evolution of pathogenicity (including colonization) and antibiotic resistance that occurred in response to changes in patients’ demographics, medical strategies and interventions, within the paradigm of the “Hamiltonian medicine” (Fraser et al., 2005). The data further highlight the increasing need for evolutionary biology to be aligned with medical challenges (Nesse et al., 2010).

NUCLEOTIDE SEQUENCE GENBANK ACCESSION NUMBERS

The sequences corresponding to representative *pbp5* genetic platforms (Types I-IV) were assigned GenBank accession numbers

JN208885, JN208888, JN208884 and JN208886, respectively. PBP5 amino acid were analyzed and designated by a “C” followed by a number (Table S6). New sequences correspond to GenBank accession numbers JN208883 (C6 amino acid combination), JN208889 (C19 amino acid combination); JN208887 (C8 amino acid combination); JN208886 (C3 amino acid combination), JN208882 (C2 amino acid combination), KC479673 (C1 amino acid combination), KC479675 (C5 amino acid combination), KC479676 (C10 amino acid combination) and KC479674 (C12 amino acid combination). The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MBRG00000000. The version described in this paper is version MBRG01000000.

AUTHOR CONTRIBUTIONS

CN, LP, and TMC designed the study. CN, ARF, APR, MA-H, ES, and RE performed wet lab experiments and participated in the analysis of the data. APT and VFL performed bioinformatic analysis. FB, APR, and LP provided expertise, participated in the analysis of data, and in the revision of the manuscript. CN, APT, and TMC performed the analysis of data and wrote the manuscript. All authors approved the final version 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.2016.01581>

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Fluoroquinolone-Resistant Enteric Bacteria in Sub-Saharan Africa: Clones, Implications and Research Needs

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Fluoroquinolones came into widespread use in African countries in the early 2000s, after patents for the first generation of these drugs expired. By that time, quinolone antibacterial agents had been used intensively worldwide and resistant lineages of many bacterial species had evolved. We sought to understand which Gram negative enteric pandemic lineages have been reported from Africa, as well as the nature and transmission of any indigenous resistant clones. A systematic review of articles indexed in the Medline and AJOL literature databases was conducted. We report on the findings of 43 eligible studies documenting local or pandemic fluoroquinolone-resistant enteric clones in sub-Saharan African countries. Most reports are of invasive non-typhoidal *Salmonella* and *Escherichia coli* lineages and there have been three reports of cholera outbreaks caused by fluoroquinolone-resistant *Vibrio cholerae* O1. Fluoroquinolone-resistant clones have also been reported from commensals and animal isolates but there are few data for non-Enterobacteriaceae and almost none for difficult-to-culture *Campylobacter* spp. Fluoroquinolone-resistant lineages identified in African countries were universally resistant to multiple other classes of antibacterial agents. Although as many as 972 non-duplicate articles refer to fluoroquinolone resistance in enteric bacteria from Africa, most do not report on subtypes and therefore information on the epidemiology of fluoroquinolone-resistant clones is available from only a handful of countries in the subcontinent. When resistance is reported, resistance mechanisms and lineage information is rarely investigated. Insufficient attention has been given to molecular and sequence-based methods necessary for identifying and tracking resistant clones in Africa and more research is needed in this area.

Keywords: fluoroquinolone resistance, quinolone resistance, antimicrobial resistance, Africa, *Escherichia coli*, *Salmonella*, *Vibrio cholerae*, *Campylobacter*

INTRODUCTION

Recent Trends in Fluoroquinolone Use and Resistance in Africa

The antibacterial activity of nalidixic acid, a derived by-product from the synthesis of the antimicrobial chloroquine, was discovered in the 1960s. This first quinolone was the base molecule used to synthesize the even more active fluoroquinolones, which also had more favorable pharmacokinetics (Mitscher, 2005). First generation fluoroquinolones (second generation quinolones), like ciprofloxacin, became clinically available in the 1980s and were patent protected until the 2000s (Paton and Reeves, 1988). Until recently, ciprofloxacin was the most potent fluoroquinolone and highly active against virtually all bacterial enteropathogens. It was therefore widely considered to be the drug of choice for patients with enteric fever, severe gastroenteritis, cholera and salmonellosis (Brown and Reeves, 1997). These and other enteric infections are common in the sub-Saharan African sub-continent where an estimated 960–980 million people, most of them young, share the highest risk of infectious disease. Within a year of introduction of ciprofloxacin, resistance appeared and soon became commonplace (Brown and Reeves, 1997). However, 30 years since its introduction, ciprofloxacin is still the most commonly prescribed fluoroquinolone along with levofloxacin, ofloxacin, and moxifloxacin (Redgrave et al., 2014).

Being orally active, broad-spectrum, and heat-stable, the potential for fluoroquinolone application as well as misuse was high. When the efficacy of earlier orally active antibiotics such as the tetracyclines, amino penicillins, and trimethoprim-sulphonamide combinations declined due to rapidly rising antimicrobial resistance rates, fluoroquinolones were recognized as effective alternatives for common and life-threatening infections (Green and Tillotson, 1997). They quickly became among the most widely applied antimicrobials worldwide. The initial high cost of these drugs prohibited their use in the least affluent African settings. The situation changed upon ciprofloxacin patent expiry in 2003, when cheap generics entered African markets. Other fluoroquinolones, such as pefloxacin, met similar fates, and the fluoroquinolones rapidly became first-line drugs for the empiric treatment of many life-threatening infections (Orogade and Akuse, 2004).

The WHO recently highlighted fluoroquinolone resistance in *Escherichia coli* and related organisms as a principal public health threat (WHO, 2014). Fluoroquinolone resistance was rare worldwide in the 1980s. In Nigeria, as elsewhere, rapid escalation of fluoroquinolone use has been paralleled by a similarly rapid increase in the prevalence of resistant bacteria, including enterics (Omigie et al., 2009; Lamikanra et al., 2011; Namboodiri et al., 2011). In part because quinolone resistance evolved later but also perhaps because mechanisms conferring resistance to a wide range of drugs may have predominated before quinolone-specific mechanisms evolved, fluoroquinolone-resistant strains are typically resistant to multiple antimicrobials (Lamikanra et al., 2011; Namboodiri et al., 2011). Other factors may account for association of fluoroquinolone resistance and multiple resistance in Africa in particular. Quinolones were

widely used for decades elsewhere in the world so that resistance genes and resistant clones had already evolved by the time fluoroquinolones were significantly employed in Africa. It has been hypothesized that chloroquine contributed to selective pressure for fluoroquinolone-resistant bacteria in malaria endemic settings but available data do not support this hypothesis for Nigeria (Davidson et al., 2008; Lamikanra et al., 2011). Irrespective of other factors driving fluoroquinolone resistance in enterics across Africa, introduction of fluoroquinolones into routine care in African clinics rapidly enhanced their selection and transmission in a paradigm that is instructive for future antibacterial introductions. In this paper, we review what is known about fluoroquinolone resistant enteric clones in Africa.

Fluoroquinolone Resistant Enteric Organisms and Overview of Clonal Expansion Problem

Among enteric bacteria, fluoroquinolone resistance has predominantly been reported in the literature on *E. coli* (Robicsek et al., 2006), *Enterobacter* sp. (Robicsek et al., 2006; Cattoir et al., 2007), *Citrobacter freundii* (Cattoir et al., 2007) *Klebsiella pneumoniae* (Robicsek et al., 2006), *Salmonella Typhi* (Dimitrov et al., 2010; Baker et al., 2013), *Salmonella enterica* (Yanagi et al., 2009), *Shigella flexneri* (Hata et al., 2005), and *Vibrio cholerae* (Ismail et al., 2011). There are multiple mechanisms by which these species acquire resistance to fluoroquinolones. Resistance was initially recognized to be due to point mutations in quinolone resistance determining regions (QRDRs) of the *gyrA* and *parC* quinolone target genes and may also be attributable to other chromosomal genes that promote quinolone efflux. Secondly, plasmid mediated quinolone resistance (PMQR) can be mediated through QRDR-protective *qnr* alleles, the ciprofloxacin-acetylating *aac(6)-Ib-cr* allele or quinolone-efflux genes *qepA* and *oqxAB* (Redgrave et al., 2014; Blair et al., 2015). In general fluoroquinolone-resistant strains could have multiple genetic bases for resistance. Some accumulate multiple QRDR mutations and others carry one or more PMQR genes with one or more QRDR mutations. Sequential transition of a strain to higher level resistance is generally attributable to multiple but independent genetic events. Selective pressure from widespread fluoroquinolone use appears to have allowed clones that have independently acquired a combination of natural mutation and recombination events to expand internationally (Hooper, 2003; Strahilevitz et al., 2009; Redgrave et al., 2014).

Fluoroquinolone use is an important selector of evolutionary successful resistant lineages but unlikely to be the only one (Baker et al., 2013). More research is needed to promote in-depth understanding of the drivers of emergence and persistence of resistant lineages. Bacterial clones are, by definition, descended from a common ancestor. Evolution within clones and horizontal gene transfer among them subsequently leads to diversification so that organisms of clonal origin may not always be easily discernible and clonal expansion could be difficult to distinguish from evolutionary convergence. Clone descriptions vary and only a subset of resistance mechanisms receive sufficient focus.

Examples of well-known clonal lineages include lineages of *E. coli* ST131, the fluoroquinolone insensitive and multi-drug resistant (MDR) *Salmonella* Typhimurium DT104, and the ciprofloxacin-resistant *S. enterica* serotype Kentucky ST198-X1-SGI1 clone. These clonal groups are marked by rapid and extensive dissemination and have been extensively tracked in many parts of the world (Threlfall, 2000; Le et al., 2013; Petty et al., 2014). However, “worldwide” or “global” studies of these and other clones frequently under-represent African isolates, and in some cases omit them altogether, pointing to the need for a systematic review of published literature on clones in Africa.

The very definition of a “resistant clone” can vary among studies causing problems in understanding the true scope of clonal emergence and subsequent expansion. Genetic studies, preferably sequence-based, are the gold standard for defining clones. Such studies include phylogenetic studies of resistant bacteria that have been characterized via multilocus sequence typing/analysis or whole genome sequencing (WGS) (Roumagnac et al., 2006; Dahya et al., 2013; Wong et al., 2015). Sequence analysis of resistance genes or plasmids in the context of well-characterized strains can also indicate clonality but, in the absence of host background sequence, cannot robustly differentiate genetically distinct clones. Other studies characterize resistant clones by phenotypic methods such as serogrouping and resistant profiles or pulse-field gel electrophoresis (PFGE; Ke et al., 2014), which looks at genetic profile based on restriction digestion sites. Africa does have an international network to compare PFGE profiles across countries (<http://www.pulsenetinternational.org/networks/africa/>). However, it is known that PFGE profiles of a single clone may not be identical or chromosomal rearrangements may produce significantly different profiles within a clone (Ismail et al., 2011; Price et al., 2013). Mobile elements can be acquired in different lineages and identical phenotypic characteristics do not necessarily imply genetic relatedness (Chattaway et al., 2014).

A clone definition is as good as the discovery methodology. Thus clones are often redefined when more and better information become available. Phenotypic traits—such as serogrouping, virulence, or biochemical profiling that can be acquired convergently in different lineages—cannot be accurately used to track clonal expansion. This has important implications for much of the literature from Africa where sequence-based studies are few. However, the so-called next generation and indeed third-generation sequencing technologies offer an affordable solution to sequence-based clone identification that is increasingly adopted in African studies with great potential for the future. Our review of global fluoroquinolone resistance and clonal expansion in Africa included genetic methodologies such as WGS, detection, or sequencing of fluoroquinolone resistance genes with well characterized strains as the eligible criteria.

SYSTEMATIC SEARCH METHODS

This systematic review was conducted in accordance with the preferred reporting items of systematic reviews and meta-analyses (PRISMA) guidelines (Moher et al., 2009). Relevant

English articles available in the Medline (Pubmed) and African Journals Online (AJOL) databases were retrieved by two authors using predefined search terms (Table S1). The literature search was conducted until October 2015.

Figure 1 summarizes the study selection process. All duplicate articles were removed. Fluoroquinolone resistant organisms are defined as those having minimum inhibitory concentrations above the CLSI breakpoints (2 µg/ml for ciprofloxacin) or zone sizes below the CLSI breakpoints in standardized disk tests. Strains with MICs above the susceptible breakpoint (0.06 µg/ml and 1 µg/ml for ciprofloxacin in *Salmonella* and other enterics respectively) are described as fluoroquinolone non-susceptible in this review. The eligibility of published reports in this review was based primarily on polymerase chain reaction (PCR) or sequence-based detection of fluoroquinolone resistance genes and the use of at least one molecular tool for assessing clonality of the enteric pathogen including multi-locus sequencing typing (MLST), multi-locus sequencing analysis (MLSA), clone-specific PCR, or PFGE, subsequent sequencing of fluoroquinolone resistance genes, PCR amplicons, and WGS of the enteric organism. Studies where only phenotypic resistance screening methods were used were excluded.

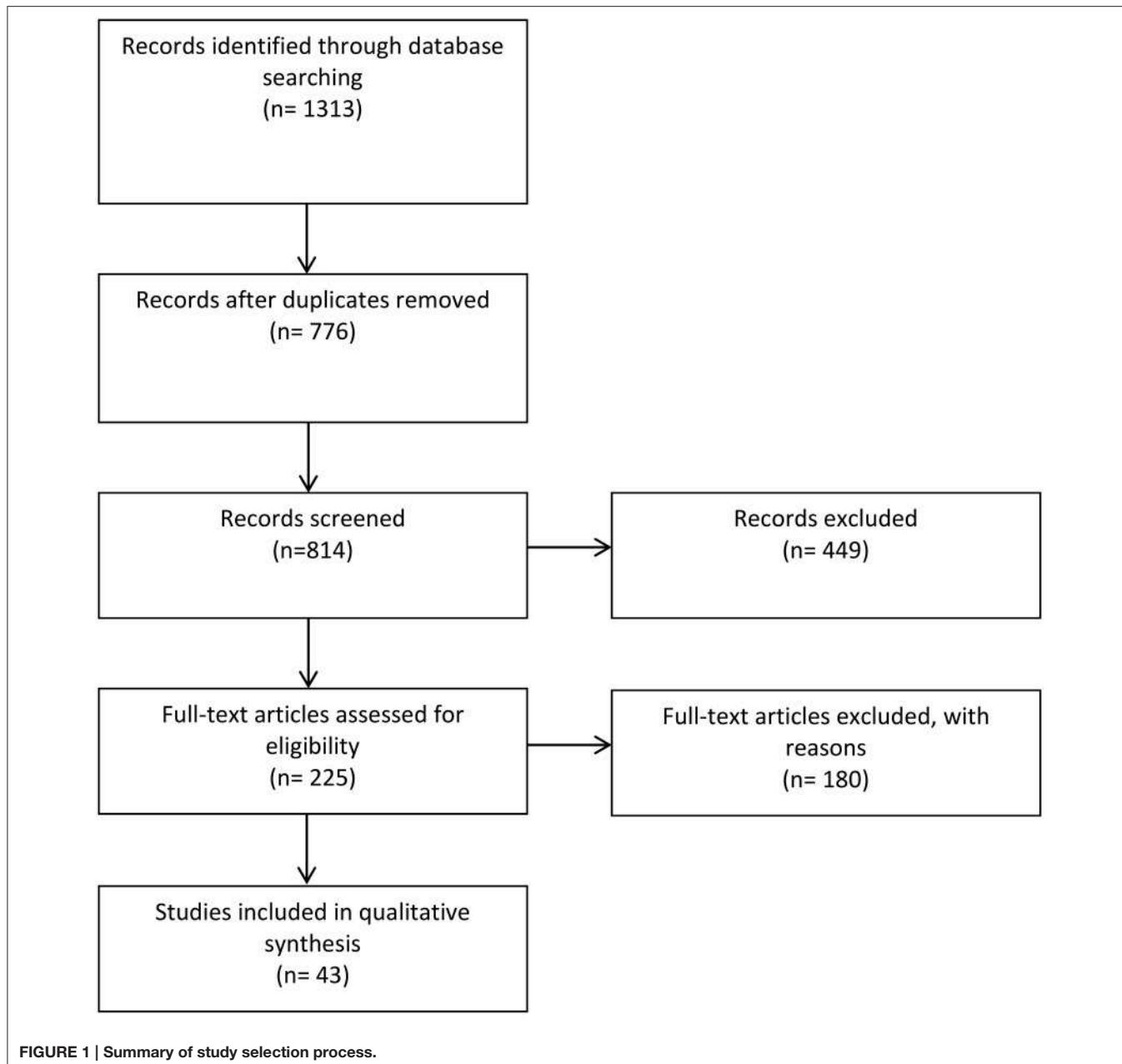
RESULTS

Literature Search and Characteristics of the Studies Included in the Systematic Review

The systematic search of the two electronic databases yielded 1313 articles (**Figure 1**). After the removal of duplicate studies and assessments of titles and abstracts, 776 full-text articles were screened of which 43 studies were considered eligible for the qualitative analysis according to our inclusion criteria (three articles were not included due to inaccessibility). The majority of the articles retrieved were single center studies. Altogether, eligible studies were identified from 17 African countries, less than half of those south of the Sahara. Furthermore, 25 of these studies—almost two-thirds—were performed in just four countries: CAR, Kenya, Nigeria, and South Africa.

The search retrieved articles focused on *V. cholerae*, *E. coli*, and other Enterobacteriaceae and *Campylobacter* spp. but overall, published work on fluoroquinolone-resistant enteric clones in Africa is predominantly focused on *Salmonella* and whole genome sequence data-based studies are almost entirely limited to this genus. One possibility is that the identification of hypervirulent invasive non-typhoidal *Salmonella* lineages on the continent (Okoro et al., 2012) is fueling this research. However, our reading of the literature appears to point to the enhanced focus on the molecular epidemiology of *Salmonella* in East and Southern Africa as being responsible for identifying this clonal expansion problem in the first place and it is probable that expanded research on other pathogens will uncover other local clones.

Many studies seeking fluoroquinolone-resistant strains found strains with intermediate susceptibility and/or strains that were resistant to the quinolones but not the fluoroquinolones (Table



S2). As resistance to the fluoroquinolones typically evolves in a stepwise fashion, the wide prevalence of these intermediate strains is worrisome. While there are very few studies reporting fluoroquinolone-resistant *E. coli* clones, reports are increasingly using sequence-based methods to identify clones, specifically, MLST. The predominance of MLST has been helpful in placing the findings from African countries into global context and demonstrates the extension of knowledge that can come from using sequence-based tools in small studies (Tables 2, 3). There are two *E. coli* MLST schemes in the literature and whilst the Lacher et al. scheme (Lacher et al., 2007) has been used in African studies (Feglo et al., 2013), the more widely used (Wirth et al., 2006) scheme is more frequently applied, making comparisons

among these many small studies possible. Where MLST was not applied *per se*, PCR markers for MLST-defined ST131 clades were used to identify members of those clones (Peirano et al., 2011, 2014; Albrechtova et al., 2012; Gqunta and Govender, 2015). Methodology of this type could be developed for and applied to other clones that are later found to be of interest in Africa (Doumith et al., 2015). A few African studies, generally older ones, have used PCR-based techniques and PFGE (Lavollay et al., 2006; Kariuki et al., 2007; Peirano et al., 2011). While these studies are the principal sources of information on distribution of FQREC soon after these strains evolved in Africa, it is difficult to connect the clones identified therein to those reported more recently.

Data from *Salmonella* are predominantly available from Eastern and Southern Africa, and there are two multicountry studies focused on *Salmonella* Kentucky ST198 (Le et al., 2011, 2013). *V. cholerae* studies reported from outbreaks in Western (Nigeria, Cameroon), Eastern (Kenya), and Southern Africa with one pair of studies focused on the same outbreak on either side of the Nigeria-Cameroon border (Quilici et al., 2010; Marin et al., 2013). Most *E. coli* data studied in this review came from single country studies, limited to CAR (Janatova et al., 2014), Ghana (Namboodiri et al., 2011; Feglo et al., 2013) Kenya (Kariuki et al., 2007; Albrechtova et al., 2012), Nigeria (Lamikanra et al., 2011; Aibinu et al., 2012a), and South Africa (Gqunta and Govender, 2015). Intercontinental studies generally included isolates from only one sub-Saharan African country (CAR or South Africa) and other non-African countries (Lavollay et al., 2006; Peirano et al., 2011, 2014). Therefore there are no data comparing FQREC isolates from different countries in sub-Saharan Africa. Additionally, multicountry or “global” studies are typically including only one country from within sub-Saharan Africa, even though it is well known that biological diversity on the African continent exceeds that in most other parts of the world.

Vibrio cholerae

Cholera, the pandemic diarrheal disease caused by toxigenic *V. cholerae* strains, has become increasingly problematic across Africa in the last two decades but fluoroquinolone resistance has only been recently studied (Scarscia et al., 2006, 2009; Mintz and Guerrant, 2009). Quilici et al. (2010) described the microbiology of a cholera outbreak that spanned the borders of Nigeria and Cameroon in 2009 and (Quilici et al., 2010) was due to a single *V. cholerae* O1 Ogawa El-tor clone. The *ctxB* DNA sequence from this clone was similar (only 3 SNPs) to a strain identified in India in 2007 but different from variants isolated in southern Africa, indicating a possible transmission from Asia to Africa. This clone was resistant to nalidixic acid and MICs to ciprofloxacin were 0.25–0.5 µg/ml, placing them in the susceptible category (based on general enterobacterial breakpoints), although these MICs approach the reduced susceptibility breakpoint of 1 µg/ml. Strains belonging to this clone contained mutations in the QRDR of *gyrA* (Ser83-Ile) and *parC* (Ser85-Leu) but no mutations in *gyrB* or *parE*. A more comprehensive Nigeria study looking at *V. cholerae* O1 strains isolated in the early 1970’s and comparing them with strains of *V. cholerae* O1 and non-O139 strains isolated in 2009–2010 indicated that *gyrA* (Ser83-Ile) and *parC* (Ser85-Leu) mutations were not present in the 1970’s or in non-O1 clinical isolates (Marin et al., 2013). Interestingly, MLVA analysis clustered the 1970’s O1 El-tor strain strains with epidemic O1 El-tor strains but the strains had different *ctxB* types and vibrio 17th pandemic island (VSP-II) profiles indicating that they do not represent one clone (Marin et al., 2013). This can be explained by the housekeeping genes used in this analysis (*pyrH*, *recA*, and *mdh*) being highly conserved in *V. cholerae* serogroup strains. Approaches using whole genome sequence may be required to further define the inter-relatedness of these O1 strains.

The most in-depth assessment of fluoroquinolone resistant mechanisms was a South African study focused on a *V. cholerae*

O1 Ogawa El-tor clone outbreak (Ismail et al., 2011). A selection of strains was assessed for the presence of plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qnc*, and *qepA*) as well as *Vibrio* quinolone resistance determinant (*qnrVC3*). Nalidixic acid resistance was investigated in the presence and absence of two efflux pump inhibitors and QRDR mutations were identified by sequencing. The South Africa strains were negative for any PMQR genes and there was no evidence of active efflux conferring quinolone resistance. Nalidixic acid resistance was attributable to *gyrA* (Ser83-Ile) and *parC* (Ser85-Leu) mutations (Ismail et al., 2011). All of the recent outbreak strains described in these three studies were *V. cholerae* O1 El-tor (two studies also indicated serogroup Ogawa) which showed resistance to nalidixic acid but only a reduced susceptibility to ciprofloxacin (Table 1). It is highly likely that these strains are the same clone and that the same QRDR mutations are responsible for fluoroquinolone resistance. High levels of resistance to nalidixic acid or reduced susceptibility to ciprofloxacin in likely similar *V. cholerae* O1 clones causing epidemics in DRC and Kenya have also been shown (Mercy et al., 2014; Miwanda et al., 2015; Table 1), but a lack of standard methodology for clonal analysis prevents an understanding of clonal spread across Africa. Although ciprofloxacin has only a reduced susceptibility in these strains and continues to be used for cholera management, if additional mutations occur in these circulating clones, resistance to ciprofloxacin may develop.

Campylobacter

Campylobacter jejuni is a leading enteric bacterial pathogen in developing countries particularly among children below the age of 5 years. In spite of numerous reports of *C. jejuni* as well as its resistance to fluoroquinolones (Coker et al., 2002) data on clones of the organisms causing human infections in Africa are non-existent. Characterization of 46 isolates from chickens in Senegal by MLST revealed seven clonal complexes which have all been associated with human disease elsewhere (Kinana et al., 2006). Clonal complex 353 was the most common and Thr-86-Ile substitution in *gyrA* was the major mechanism of quinolone resistance. Rather than diffusion of a single or a few clones, acquisition of resistance appears to be occurring in different lineages in response to selective pressure. Similarly, another study from Senegal, strain typing of 99 chicken and 10 human isolates of *C. jejuni* by PFGE that uncovered common patterns among strains circulating in human and poultry, though were no particular predominant pulsotypes (Cardinale et al., 2006). Altogether, although the data are few, there is yet to be robust evidence for expanded fluoroquinolone resistant *Campylobacter* clones.

Enteric and Commensal *Escherichia coli* and *Shigella*

Diarrhea was the third leading cause of death in children under five in sub-Saharan Africa in 2013 (after malaria and pneumonia), accounting for 12% of the estimated 3.6 million deaths (Liu et al., 2015). In sub-Saharan Africa and south Asia, most attributable cases of moderate-to-severe childhood diarrhea in under-fives are due to the enteric bacteria *E. coli* and *Shigella*

TABLE 1 | Characterization of eligible articles that sought fluoroquinolone resistant clones in humans and animals.**

Country	Organism	No. resistant*/ No total	ID method	Study period	Study population	Genotyping tools					Resistance testing				References
						WGS	MLST	ERIC-PCR	PFGE	Rep PCR	Other*	PMQR	QRDR	Efflux pump up-regulation	aac(6')-lb-cr
Angola	<i>E. coli</i>	9/19	B, S	2009	Stray dogs	–	–	–	✓	–	–	✓	–	–	–
Cameroun	<i>V. cholerae</i> O1 Ogawa El-tor	0/9	B, S, M	2009	Outbreak	–	–	–	–	–	–	✓	–	–	Albrechtova et al., 2014a
Central African Republic	<i>Enterobacteriaceae</i>	33/121	B, S	2011	Human/wildlife	–	–	✓	–	–	✓	–	–	–	Janatova et al., 2014
	<i>Enterobacteriaceae</i>	65/65	B	2011–2012	Clinical isolates (surgical site infections)	–	✓	✓	–	✓	–	✓	–	–	✓
	<i>E. coli</i>	10/121	B, M	2011	Gorillas and other wildlife	–	✓	–	–	–	✓	–	–	–	✓
	<i>E. coli</i>	10/10	B, M	2000–2004	Hospital clinical isolates	–	–	–	✓	✓	–	–	–	–	Lavollay et al., 2006
	<i>S. Typhi</i>	0/19	B, S	1916–2004	Various	✓	–	–	–	–	–	–	–	–	Holt et al., 2008
Cote d'Ivoire	<i>E. coli</i> , <i>Enterobacteriaceae</i>	44/101	B, S	2012	Human, dog, wild life	–	–	–	✓	–	–	✓	–	–	✓
DRC	<i>Salmonella</i> (NTS)	0/233	B, S	2007–2011	Community and Clinical isolates	–	–	–	✓	–	–	✓	✓	–	Lunguya et al., 2013
	<i>V. cholerae</i>	0/1093	B, S	1997–2012	Clinical isolates	✓	–	–	–	–	–	MLVA	–	–	Mwanda et al., 2015
Ghana	<i>E. coli</i>	23/293	B, M	2006–2008	Clinical isolates from tertiary care hospitals	–	✓	–	–	–	–	✓	✓	–	Nambodiri et al., 2011
	<i>E. coli</i>	28/29	B, M	2008–2009	Clinical isolates: Blood, urine, sputum and wound	–	✓	–	–	–	–	–	–	–	Feglo et al., 2013
Ethiopia	<i>Salmonella</i>	19/98	B, S	2000–2005	Animal/Animal products	–	–	–	✓	–	–	–	–	–	Molla et al., 2007
Kenya	<i>E. coli</i>	66/289	B, M	2009	Dogs (mainly), cats and humans	–	–	✓	–	–	✓	–	–	–	✓
	<i>E. coli</i>	12/17	B, M	2004–2005	Clinical isolates: Urine	–	–	✓	–	–	✓	–	–	–	Albrechtova et al., 2012
	<i>V. cholerae</i>	1/76	B, S	2007–2010	Clinical isolates	–	–	✓	–	–	Ribotyping	–	–	–	Karuki et al., 2007
	<i>S. Kentucky</i>	3/3	B, S	2000–2006	Clinical isolates	–	–	–	✓	–	✓	–	–	–	Mercy et al., 2014
	<i>S. Typhi</i>	0/94	B, S	1988–2008	Clinical isolates	✓	–	–	–	–	–	–	–	–	Weill et al., 2006
															Karuki et al., 2010

(Continued)

TABLE 1 | Continued

Country	Organism	No. resistant**/ No total	ID method	Study period	Study population	Genotyping tools						Resistance testing			References	
						WGS	MLST	ERIC-PCR	PFGE	Rep PCR	Other*	PMQR	QRDR	Efflux pump up-regulation	aac(6')-Ib-cr	
	<i>S. Typhi</i>	0/102	B, S	2001–2002	Clinical isolates	–	–	–	✓	–	–	–	✓	–	–	Karukki et al., 2004
	<i>S. Typhi</i>	NS/55	NS	2001–2009	Clinical isolates	✓	–	–	–	–	–	–	–	–	–	Wong et al., 2015
Malawi	<i>S. Typhi</i>	0/112	B, BC	1998–2013	Clinical isolates (blood)	✓	✓	–	–	–	–	–	–	–	–	Feasey et al., 2015
	<i>S. Typhi</i>	NS/112	NS	2004–2013	Clinical isolates	✓	–	–	–	–	–	–	–	–	–	Wong et al., 2015
Nigeria	<i>E. coli</i>	6/162	B, S	2006	Poultry/Pigs	–	–	✓	–	–	✓	–	–	–	✓	Fontini et al., 2011
	<i>E. coli</i>	57/114	B, M	2010–2011	Human clinical isolates, bovine isolates from farms	–	✓	–	–	✓	–	✓	–	–	✓	Inwezerua et al., 2014
	<i>E. coli</i>	26/32	B, M	2011	Humans (pregnant women)	–	✓	✓	–	–	✓	–	–	–	✓	Fontini et al., 2015
	<i>E. coli</i>	9/109	B, M	2008–2009	Clinical isolates from tertiary care hospitals	–	ST131 and ST10	✓	–	–	✓	–	–	–	✓	Albinu et al., 2012a
	<i>E. coli</i>	21/121	B, M	2005	Healthy volunteers	–	✓	–	–	–	#IC-REIP	–	✓	✓	–	Lamikanra et al., 2011
	<i>Salmonella</i> (NTS)	11/149	B, S	2009–2011	Human/poultry/cattle/fish/vegetable	–	–	–	–	–	✓	–	–	–	–	Raufu et al., 2013
	<i>Salmonella</i> (NTS)	2/229	B, S	2010–2011	Pigs	–	–	–	–	–	✓	–	–	–	–	Fashae and Hendriksen, 2014
	<i>S. Hiliuddiy</i>	24/130	B, S	2001	Chickens/poultry meat	–	–	–	✓	–	–	–	–	–	–	Raufu et al., 2009
	<i>S. Kentucky</i>	197/197	B, S	1959–2008	Human/Poultry/sea food/river/environment	–	✓	–	✓	–	✓	✓	–	–	✓	Le et al., 2011
	<i>S. Kentucky</i>	55/55	B, S	2007–2011	Poultry/poultry sources	–	–	✓	–	–	–	–	–	–	–	Raufu et al., 2014
	<i>S. Kentucky</i>	54/70	B, S	1937–2013	Humans/animals/environment	✓	–	✓	–	–	✓	✓	–	–	✓	Le et al., 2013
	<i>V. cholerae</i> O1 and non O1	0/20	B, S, M	1971–2010	Outbreak, historical, environmental	–	–	✓	–	–	MLS A	–	✓	–	–	Marin et al., 2013
	<i>V. cholerae</i> O1 Ogawa El-tor	0/10	B, S, M	2009	Outbreak	–	–	–	–	–	–	✓	–	–	–	Quilici et al., 2010
	<i>E. coli</i>	NS/12	B, M	2000–2010	Clinical isolates	–	✓	–	✓	–	Lineage PCR	–	✓	–	✓	Pereira et al., 2014
	<i>E. coli</i>	0/22	B, M	2008–2009	Clinical isolates (Urine and pus)	–	–	✓	–	–	✓	–	–	✓	–	Pereira et al., 2011

(Continued)

TABLE 1 | Continued

Country	Organism	No. resistant*/ No total	ID method	Study period	Study population	Genotyping tools							Resistance testing	References	
						WGS	MLST	ERIC-PCR	PFGE	Rep PCR	Other*	PMQR	QRDR	Efflux pump up-regulation	
Senegal	<i>C. coli</i>	14/36	B, M	2000–2002	Chicken	–	✓	–	–	–	–	✓	–	–	Khanna et al., 2007
	Salmonella (NTS)	4/1623	B, S, M	1999–2009	Clinical isolates	–	–	–	–	–	✓	✓	–	–	Harois et al., 2014
S. Typhi															
<i>C. jejuni</i>		19/46	B, M	2000–2002	Chicken	–	✓	–	–	–	–	✓	–	–	Khanna et al., 2006
<i>C. jejuni</i> , <i>C. coli</i>		81/181	B, M	2000–2003	Poultry, Human	–	–	–	✓	–	–	✓	–	–	Cardinale et al., 2006
<i>V. cholerae</i> O1		0/15	B, M, S	2012	Clinical isolates	–	–	✓	–	–	–	–	–	–	Mahmud et al., 2014
Sierra Leone															
South Africa	<i>E. coli</i>	14/21	B, M	2012–2013	Clinical isolates	–	✓	–	✓	–	–	✓	–	–	Gurata and Goverder, 2015
	S. Typhi	1/1	B, S	2010	Clinical isolate	–	–	–	✓	–	–	✓	✓	–	Keddy et al., 2010
S. Typhi		0/510	B, S	2003–2007	Clinical isolates	–	–	–	✓	–	MLVA	✓	✓	–	Smith et al., 2010
S. Typhi		NS/41	NS	2004–2012	Clinical isolates	✓	–	–	–	–	–	–	–	–	Wong et al., 2015
S. Kentucky		1/1	B, S	2000–2005	Clinical isolates	–	–	–	✓	–	–	✓	✓	–	Weill et al., 2006
<i>V. cholerae</i> O1 Ogawa El-tor		0/31	B, S, M	2008	Outbreak	–	–	–	✓	–	–	–	–	–	Ismail et al., 2011
Tanzania	S. Typhi	NS/52	NS	2006–2010	Clinical isolates	✓	–	–	–	–	–	–	–	–	Wong et al., 2015
S. Kentucky		3/3	B, S	2000–2005	Clinical isolates	–	–	–	✓	–	✓	✓	–	–	Weill et al., 2006

KEY: ID method: B, Biochemistry; S, Serotyping; M, detection of target genes; BC, Blood culture; ✓, test was conducted; –, test not conducted; NS, Not stated (data not included in manuscript).

*Other, where specific genotypic methods are used to identify clones of that enteric pathogen.

**In some cases where fluoroquinolone-resistant isolates were sought but only fluoroquinolone-resistant or non-susceptible isolates were identified. Details of these studies are provided in Table S2.

WGS, Whole genome sequencing; MLST, Multi-locus sequence typing; MLSA, Multi-locus variable-number tandem repeat analysis; MLVA, multi-locus variable-number tandem repeat analysis; PFGE, pulse field gel electrophoresis; ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus – PCR; REP-PCR, repetitive element palindromic PCR; RFLP, restriction fragment length polymorphism; NTS, Nontyphoidal *Salmonella*, Clinical isolates – from humans; PMQR, plasmid mediated quinolone resistance (including *qnrA*, *qnrB*, *qns*, *qnrC*, *qepA*, and *qnrV/C3*); QRDR, quinolone resistance-determining region [including sequencing the QRDR of DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) genes]; efflux up regulation testing; aac(6')-lb-cr, aminoglycoside acetyltransferase associated with ciprofloxacin resistance; DRC, Democratic Republic of the Congo.

(Kotloff et al., 2013). While there was a report of a single quinolone-resistant *Shigella* isolate from Zaire in 1985 (Frost et al., 1985) and *Shigella* epidemics caused by quinolone resistant bacteria in the last century (Goma Epidemiology Group, 1995; Karas and Pillay, 1996; Vlieghe et al., 2009) we did not identify reports of fluoroquinolone-resistant clones of enteric *E. coli* (FQREC) in the literature before 2000. Available reports appear to suggest that most isolates were fluoroquinolone susceptible until the end of the 1990s (Wistrom et al., 1987; Felmingham and Robbins, 1992; Thornton et al., 1992; Bourgeois et al., 1993; Oyofo et al., 1997; Decousser et al., 1999; Vila et al., 1999; Ahmed et al., 2000; Okeke et al., 2000a,b; Jiang et al., 2002; Orogade and Akuse, 2004). Our findings are corroborated by other recent systematic reviews (Vlieghe et al., 2009; Leopold et al., 2014).

Reports of fluoroquinolone-resistant *E. coli* from cases of diarrhea and commensal reservoirs in Africa are limited to the current millennium (Aibinu et al., 2004; Lamikanra et al., 2011; Namboodiri et al., 2011; Fortini et al., 2015). One study that examined human commensal isolates from Ghana (2006–2008) found a wide range of STs associated with fluoroquinolone resistance (Namboodiri et al., 2011). However, strains belonging to the multilocus sequence type (ST) complex (specifically STs 10, 227, and 617) and the ST 156 sequence type complex (STs 156 and 1473) were detected 8 and 3 times respectively representing half of the total number (22) of FQREC detected in the study. An additional five ST10 isolates were resistant to nalidixic acid and overall, ST10 strains were computed to be overrepresented among quinolone-resistant *E. coli* (QREC) from Ghana ($p = 0.02$, Fishers exact test; Namboodiri et al., 2011). Five ST10 QREC (including four FQREC) harbored *qnrS1*, whilst other strains carried *qepA* or *qnrB2* genes. The ST156 strains harbored three or more QRDR mutations, in contrast to most other strains that had 0–2 and one of the ST156 isolates also carried *qepA*. Interestingly, multiple *qepA*-bearing FQREC ST156 strains have also been reported as human commensals in the Central African Republic and Nigeria (Table 2; Janatova et al., 2014; Fortini et al., 2015). These and other lineages could be more broadly disseminated but remain unflagged because data are insufficient or unavailable. For example, *qnrS1*-bearing clones that have not been multilocus sequence typed (clonality was inferred by ERIC-PCR) have also been isolated from humans and pigs in Nigeria (Fortini et al., 2011, 2015).

Invasive *Escherichia coli*

Fluoroquinolone-resistant *E. coli* are a rising cause of invasive infections in Africa. The majority of reports of fluoroquinolone-resistant enterobacterial lineages only seek lineage information among ESBL-producers (Table 3; Aibinu et al., 2012a; Isendahl et al., 2012; Breurec et al., 2013; Gqunta and Govender, 2015; Rafai et al., 2015) so that the true prevalence of fluoroquinolone-resistant strains is unknown as is the existence of ESBL-negative fluoroquinolone-resistant lineages of importance. Interestingly, while the data are few and biased toward ESBL-positive lineages, there is some overlap between STs of FQREC recovered from clinical infections and those found in the commensal reservoir from humans and wild mammals (Tables 2, 3).

Kariuki et al. examined multiply resistant *E. coli* from urinary tract infections in Kenya (Kariuki et al., 2007). Two PFGE profiles were seen more than once. Strains belonging to these clones had identical *gyrA* sequences, which included Ser83Leu and Asp87Asn mutations in the QRDR regions of this gene. They also carried a plasmid-borne *aac-6(Ib)-cr* gene and some had *qnrA* and *qnrB* genes. Working largely with strains from other areas but including 10 isolates from Bangui, Lavollay et al. found a globally disseminated clone that carried an *aac6-Ib-cr* allele on its CTX-M-15 bearing plasmid and detected two isolates of this clone, as defined by REP-PCR and PFGE, among the Central African Republic isolates (Lavollay et al., 2006).

Available data suggests that pandemic lineages circulate within Africa. For example, fluoroquinolone-resistant strains belonging to the globally disseminated *E. coli* ST131 lineage, which is actually comprised of multiple clones, have rarely been systematically sought but there are sporadic reports that demonstrate their circulation in CAR (Rafai et al., 2015), Nigeria (Aibinu et al., 2012a; Inwezerua et al., 2014), Guinea Bissau (Isendahl et al., 2012), Tanzania (Lupindu et al., 2014), and South Africa (Peirano et al., 2011, 2014; Gqunta and Govender, 2015; Table 3). These data suggest that global dissemination may be dictated by the pandemic potential of the ST131 lineages rather than actual intercontinental transfer of specific clones.

Aibinu et al (Aibinu et al., 2012a) studied 109 *E. coli* isolates obtained from two tertiary care hospitals in Lagos. Of the 14 isolates that were ESBL-producers, three were ST131 *E. coli*. Chromosomal SNPs were not sought but two of the ST131 isolates and six other ESBL-producers (ST 10 complex—two strains, STs 23, 295, 448, and 501) harbored the *aac-(6')-lb-cr* gene (Table 3). These eight strains and the pMQR-negative ST131 strain all carried the ESBL-encoding CTX-M-15 gene. One ST131 strain additionally carried a *qnrB1* gene and a ST 617 strain (ST10 complex) carried *qnrA1*. This study demonstrated the presence of ST131, as well as other notorious *E. coli* clones. Many of the STs reported by Aibinu et al. were also found 2005 in the Nigerian study of Lamikanra et al. (2011) (2009 isolates from that study were not sequenced typed) as well as among 2011 isolates from Fortini et al. (2015). Feglo et al. conducted MLST on a subset of *E. coli* isolates from Kumasi Ghana, hypothesizing that they would find ST131 strains among them. Instead, they found that all of 29 isolates evaluated belonged to the pyelonephritis-associated lineage ST88. Thus, regional or internationally disseminated clones have been successful in African settings and that locally successful clones are not necessarily those with the greatest pandemic reputations.

Salmonella Typhi and Non-Typhoidal *Salmonella*

Salmonella causes an estimated three billion infections in humans and animals yearly (Crump et al., 2004; Coburn et al., 2007). This poses significant socio-economic challenges, especially in resource-limited settings. Classically, *Salmonella* can be divided into two species, *S. bongori* and *S. enterica* (Crosa et al., 1973; Schrire et al., 1987; Reeves et al., 1989). *S. enterica* can be further divided into six sub-groups based on carbohydrate utilization,

TABLE 2 | Multilocus sequence types of quinolone resistant commensals reported from potential reservoirs in sub-Saharan Africa.

City, Country	Host organism	Year	Number of independent isolates	ST	Quinolone resistance mechanisms	References
Accra, Ghana	Human	2006	2	<i>E. coli</i> (ST101)	gyrA (S83L, D87N); <i>qnrB1</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2006	1	<i>E. coli</i> (ST617)	gyrA (S83L, D87N); <i>parC</i> (S80I); <i>qnrS1</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2006	2	<i>E. coli</i> (ST648)	gyrA (S83L, D87N); <i>parC</i> (S80I)	Namboodiri et al., 2011
Accra, Ghana	Human	2006	1	<i>E. coli</i> (ST455)	gyrA (S83L, D87N); <i>parC</i> (S80I, N105S)	Namboodiri et al., 2011
Accra, Ghana	Human	2006, 2007	2	<i>E. coli</i> (ST156)	gyrA (S83L, D87N); <i>parC</i> (S80I, E84K), <i>qepA</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2007	1	<i>E. coli</i> (ST1304)	None detected	Namboodiri et al., 2011
Accra, Ghana	Human	2007	1	<i>E. coli</i> (ST450)	gyrA (S83L, D87N)	Namboodiri et al., 2011
Accra, Ghana	Human	2007, 2008	5	<i>E. coli</i> (ST10)	gyrA (S83L, D87N); <i>parC</i> (S80I), <i>qepA</i> , <i>qnrS1</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2007	1	<i>E. coli</i> (ST354)	gyrA (S83L, D87N); <i>parC</i> (S80I, E84G), <i>qnrB</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST1473)	None detected	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST1496)	None detected	Namboodiri et al., 2011
Accra, Ghana	Human	2008	2	<i>E. coli</i> (ST227)	gyrA (S83A), <i>qnrS1</i>	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST410)	gyrA (S83L, D87N); <i>parC</i> (S80I)	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST1468)	gyrA (S83L, D87N); <i>parC</i> (S80I)	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST131)	gyrA (S83L, D87N); <i>parC</i> (S80I, E84V)	Namboodiri et al., 2011
Accra, Ghana	Human	2008	1	<i>E. coli</i> (ST1470)	gyrA (S83L, D87N); <i>parC</i> (S80I, A108V)	Namboodiri et al., 2011
Dzanga, CAR	African buffalo	2011	1	<i>K. pneumoniae</i> (ST1208)	<i>oqxA</i>	Janatova et al., 2014
Dzanga, CAR	African buffalo	2011	1	<i>K. pneumoniae</i> (ST1209)	<i>oqxA</i>	Janatova et al., 2014
Dzanga, CAR	Peter's Dunker	2011	1	<i>K. pneumoniae</i> (ST1210)	<i>oqxA</i>	Janatova et al., 2014
Dzanga, CAR	Human	2011	4	<i>E. coli</i> (ST3476)	<i>qnrS1</i>	Janatova et al., 2014
Dzanga, CAR	Human	2011	3	<i>E. coli</i> (ST156)	<i>qepA</i>	Janatova et al., 2014
Dzanga, CAR	Human	2011	1	<i>E. coli</i> (ST69)	<i>oqxA</i>	Janatova et al., 2014
Dzanga, CAR	Human	2011	1	<i>E. coli</i> (ST218)	<i>qnrB1</i>	Janatova et al., 2014
Dzanga, CAR	Gorilla	2011	1	<i>E. coli</i> (ST424)	<i>qepA</i>	Janatova et al., 2014
Northern Kenya	Dogs	2012	3	<i>E. coli</i> (ST131*)	<i>aac(6')-lb-cr</i>	Janatova et al., 2014
Ile-Ife, Nigeria	Human	2005	9	<i>E. coli</i> (ST10 complex)	gyrA (S83L), efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	2	<i>E. coli</i> (ST452)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	2	<i>E. coli</i> (ST517)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST494)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST156)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST521)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST448)	gyrA (S83L, D87N); <i>parC</i> (S80I), efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST503)	efflux	Lamikanra et al., 2011
Ile-Ife, Nigeria	Human	2005	1	<i>E. coli</i> (ST504)	gyrA (S83L), efflux	Lamikanra et al., 2011
Ibadan, Nigeria	Human	2011	1	<i>E. coli</i> (ST10)	<i>qnrS1</i> , <i>aac(6')-lb-cr</i>	Fortini et al., 2015
Ibadan, Nigeria	Human	2011	1	<i>E. coli</i> (ST156)	<i>qepA1</i>	Fortini et al., 2015
Ibadan, Nigeria	Human	2011	1	<i>E. coli</i> (ST3147)	<i>qnrS1</i>	Fortini et al., 2015
Senegal	Poultry	2000–2002	11	<i>C. jejuni</i> (ST1036)	gyrA (H81, S119, T86I, G110)	Kinana et al., 2006
Senegal	Poultry	2000–2002	3	<i>C. jejuni</i> (ST1040)	gyrA (H81, S119, T86I)	Kinana et al., 2006
Senegal	Poultry	2000–2002	5	<i>C. jejuni</i> (ST1039)	gyrA (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	3	<i>C. jejuni</i> (ST1041)	gyrA (H81, S119, T86A, G110)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1081)	None detected	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST660)	None detected	Kinana et al., 2006

(Continued)

TABLE 2 | Continued

City, Country	Host organism	Year	Number of independent isolates	ST	Quinolone resistance mechanisms	References
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST22)	None detected	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1037)	<i>gyrA</i> (H81, S119, T86I)	Kinana et al., 2006
Senegal	Poultry	2000–2002	3	<i>C. jejuni</i> (ST1038)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	4	<i>C. jejuni</i> (ST52)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST824)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1359)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1211)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1370)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	1	<i>C. jejuni</i> (ST1358)	<i>gyrA</i> (H81, S119)	Kinana et al., 2006
Senegal	Poultry	2000–2002	8	<i>C. jejuni</i> (ST1035)	<i>gyrA</i> (H81, S119, G110, T86A, T86I)	Kinana et al., 2006

*Determined by marker-based PCR not MLST.

TABLE 3 | Multilocus sequence types of fluoroquinolone-resistant *E. coli* clinical isolates reported from sub-Saharan Africa.

City, Country	Isolation source	Year	Number of independent isolates	ST	Reported quinolone resistance mechanisms	Other resistances	References
Kumasi, Ghana	Blood, Urine, sputum and wounds	2008–2009	28	88 Whittam scheme (Lacher et al., 2007)	None reported	ESBLs, gentamicin, trimethoprim-sulphamethoxazole, chloramphenicol	Feglo et al., 2013
Bangui, CAR	Surgical site infections	2011–2012	12	10	<i>qnrB</i> , <i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Bangui, CAR	Surgical site infections	2011–2012	1	146	<i>qnrB</i> , <i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Bangui, CAR	Surgical site infections	2011–2012	12	131	<i>qnrB</i> , <i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Bangui, CAR	Surgical site infections	2011–2012	5	156	<i>qnrB</i> , <i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Bangui, CAR	Surgical site infections	2011–2012	1	354	<i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Bangui, CAR	Surgical site infections	2011–2012	2	405	<i>qnrB</i> , <i>aac(6')-lb-cr</i>	ESBLs	Rafai et al., 2015
Cape Town, South Africa	Urine and Pus	2008–2009	5	131*	<i>aac(6')-lb-cr</i>	ESBLs	Peirano et al., 2011
Ibadan, Nigeria	Urine	2010–2011	1	2695	<i>qnrB</i> , <i>aac(6')-lb-cr</i>	ESBLs	Inwezerua et al., 2014
Ibadan, Nigeria	Vaginal	2010–2011	1	131	<i>aac(6')-lb-cr</i>	ESBLs	Inwezerua et al., 2014
Lagos, Nigeria	Urine	2011	2	131	<i>qnrB</i> , <i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Lagos, Nigeria	Urine and stool	2011	2	617 (10 complex)	<i>qnrB</i> , <i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Lagos, Nigeria	Urine and stool	2011	1	295	<i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Lagos, Nigeria	Urine and stool	2011	1	23	<i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Lagos, Nigeria	Urine and stool	2011	1	448	<i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Lagos, Nigeria	Urine and stool	2011	1	501	<i>aac(6')-lb-cr</i>		Aibinu et al., 2012b
Port Elizabeth, South Africa	Clinical	2012–2013	12	131*	<i>qnrB</i> <i>qnrS</i> , <i>aac(6')-lb-cr</i>	ESBLs	Gqunta and Govender, 2015

E. coli STs are defined by the Achtman scheme unless otherwise indicated (Wirth et al., 2006).

*Determined by marker-based PCR not MLST.

flagellar, and lipopolysaccharide (LPS) structures (Tindall et al., 2005; Coburn et al., 2007; Grimont and Weill, 2007). Currently there are more than, 2550 serovarieties of *S. enterica*. *S. enterica* is responsible for most cases of salmonellosis in human and

warm-blooded animals (Brenner et al., 2000). Based on the type of disease syndrome and host specificity, *S. enterica* can be broadly divided into two groups (typhoidal and non-typhoidal). “Typhoidal Salmonellae” are generally human-restricted and

cause enteric fever; these include *S. Typhi* and *S. Paratyphi*. The non-typhoidal salmonellae (NTS) encompass several serovars with broad hosts including animals and humans such as *S. Typhimurium*, *S. Enteritidis*, *S. Poona*, and *S. Isangi*. (Dougan et al., 2011). In the WHO Africa sub-region, the burden of non-typhoidal gastroenteritis is estimated at 2.5 million cases and 4100 deaths per year (Majowicz et al., 2010) but this estimate is based on few data. Nontyphoidal *Salmonella* are also risk factors for mortality from childhood diarrhea (O'Reilly et al., 2012). NTS serovars are commonly associated with gastrointestinal disease in hosts but can sometimes cause invasive or systemic disease in humans and animals (Dougan et al., 2011; Langridge et al., 2012).

Emerging epidemiological data from across the subcontinent shows that invasive non-typhoidal *Salmonella* (iNTS) infection has become a major cause of bloodstream infections, overtaking typhoidal enteric fever in many places, especially among malarial and malnourished children and in HIV-positive adults (Reddy et al., 2010). Mortality rates in these vulnerable populations are reported to be as high as 30% in the absence of adequate treatment (Berkley et al., 2005; Reddy et al., 2010). Multidrug resistance of salmonellae to ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole (traditional regimens, cheap, and orally active) is a global phenomenon, presenting particular salmonellosis management challenges in Africa including the epidemic of invasive *S. Typhimurium* ST 313. This clone consists of two closely related lineages that emerged independently ~55 and ~37 years ago, in close temporal association with the HIV pandemic (Kingsley et al., 2009; Okoro et al., 2012). The MDR genes of ST 313 are encoded on a *Tn21*-like element borne on a virulence plasmid pSLT-BT. MDR clones of *S. Typhi* have also emerged and spread intercontinentally and globally involving Africa countries like Kenya (Kariuki et al., 2010; Holt et al., 2011). Prevalence rates of MDR as high as 60.4, 80.7, and 94.7% have been reported from Kenya (Kariuki et al., 2010), DRC (Lunguya et al., 2013), and Malawi (Feasey et al., 2010), respectively. Increasing and dissemination of MDR clones of salmonellae warranted the current use of fluoroquinolones and third generation cephalosporins as the drugs of first choice for treatment (Kariuki and Dougan, 2014; Kariuki et al., 2015a). These newer drugs are expensive and pose significant challenges to resource poor settings. The use of these drugs, together with the use of over-the-counter and substandard antibiotics, has now selected resistance to these drugs in salmonellae (Brenner et al., 2000; Parkhill et al., 2001; McClelland et al., 2004; Berkley et al., 2005; Gordon et al., 2008; Holt et al., 2008; Kingsley et al., 2009; Majowicz et al., 2010; Reddy et al., 2010; Dougan et al., 2011; Langridge et al., 2012; Okoro et al., 2012; O'Reilly et al., 2012; Kariuki and Dougan, 2014; Kariuki et al., 2015a). Initial reports of reduced susceptibility and resistance to quinolones in NTS emerged in the mid 2000's and continue to trickle in from many sub-Saharan African countries including Nigeria (Akinyemi et al., 2007), DRC (Lunguya et al., 2013), Tanzania (associated with travel; Weill et al., 2006), Senegal (Harrois et al., 2014), and Ghana (Nielsen et al., 2012). A Mozambican study identified ~5% of isolates as being resistant to nalidixic acid; resistance to nalidixic acid is regarded as a precursor for 2nd and 3rd generation fluoroquinolone resistance

(Mandomando et al., 2009). Recent reports have shown that strains of MDR *S. Typhimurium* ST313 primarily associated with bloodstream infections have acquired an additional *incH1* plasmid that confers additional resistance to many β -lactams including ceftriaxone. These pathovariants have been identified in Kenya (Kariuki et al., 2015b) and Malawi (Feasey et al., 2014). Other reports have shown an association with the acquisition of extended-spectrum β lactamases (ESBLs) and decreased susceptibility to fluoroquinolones in Salmonellae, the latter attributable to plasmid-borne *qnr* genes, although mechanism underlying the significance of this association has not been fully explored (Akinyemi et al., 2007, 2011; Harrois et al., 2014).

There are more than 20 million cases of human typhoid fever resulting in as many as 200,000 fatalities worldwide each year (Crump et al., 2004; Arjyal et al., 2011). Reports of outbreaks of MDR *Typhi* and *Paratyphi* emerged in the early 1990's in endemic countries in Asia and associated with travel to these regions in non-endemic (Parry et al., 2002; Arjyal et al., 2011). MDR in these instances was characterized by resistance to the first-line drugs of treatment at that time which included chloramphenicol, ampicillin, and cotrimoxazole. Subsequently as was in the case of NTS, fluoroquinolones were introduced as the preferred drug of treatment for enteric fever (Arjyal et al., 2011). Resistance to this drug inevitably emerged with widespread use. Isolates with full resistance or reduced resistance to nalidixic acid and second generation fluoroquinolones such as ciprofloxacin and ofloxacin now dominate in Asia and emerging as the dominant isolates associated with enteric fever in many countries in Africa (Keddy et al., 2010; Kariuki et al., 2015a; Wong et al., 2015).

The epidemiology of fluoroquinolone resistant clones in sub-Saharan Africa is still an emerging story (Kariuki et al., 2004; Feasey et al., 2015; Wong et al., 2015). A travel-associated case of ciprofloxacin resistant *S. Typhi* was reported in South Africa with PFGE pattern 100% identical to pattern JPPX01.0026 in the Global PulseNet *Salmonella* Typhi (Keddy et al., 2010). However, ciprofloxacin-resistant salmonellae appear uncommon in Africa, at least within the available literature. Quinolone resistance and decreased-susceptibility to fluoroquinolones (ciprofloxacin MIC 0.12–1.0 μ g/mL) are more prevalent (Table S2). Such strains are of public health importance because there is correlation between increasing MICs to fluoroquinolones and treatment failure (Parry et al., 2011). The reported prevalence rates of decreased susceptibility to ciprofloxacin from some African countries are 0.3% (Senegal) (Harrois et al., 2014) 1.6% (Ghana) (Nielsen et al., 2012), 4.3% (DRC) (Lunguya et al., 2013), and ~5% (Mozambique) (Mandomando et al., 2009) with reports also from Nigeria (Akinyemi et al., 2007) and Tanzania (Weill et al., 2006), for NTS; and for *S. Typhi*: 5.3% (South Africa) (Smith et al., 2010) and 60.4% (Kenya) (Kariuki et al., 2010). Some of these prevalences are high enough to warrant shifting to other antimicrobials but there are few alternatives in some of the least affluent African settings. There is an indication that different clones of these strains could circulate in Africa (Smith et al., 2010) and herald the emergence and dissemination of fluoroquinolone resistance. With the exception of reports from specific countries most notably Kenya (Kariuki et al., 2010) Malawi (Feasey et al.,

2015), and South Africa (Keddy et al., 2010; Smith et al., 2010), a systematic analyses of lineages associated with drug resistance is yet to be reported. In the absence of a clearer picture, continued first-line usage and misuse of quinolones is more than likely to contribute the acquisition of drug resistance in typhoidal *Salmonellae*.

Fluoroquinolone resistant salmonellae are more common in animals compared to humans. A study in Senegal identified *Salmonella* in almost all bovine carcasses screened. That study reviewed earlier literature demonstrating that *Salmonella* in animals and meat is commonplace in many African settings (Stevens et al., 2006). Recently, the evolution and global (including Africa) dissemination of fluoroquinolone resistant S. Kentucky ST 198 X1-SGI 1 clone, which has poultry as the main reservoir has occurred (Le et al., 2013). This epidemic clone is widespread in poultry in Nigeria and has persisted (Le et al., 2013; Raufu et al., 2014). Using ciprofloxacin resistance as a marker Le et al. (2013) reported that the strain is also probably present in pigs in Nigeria, although the two isolates identified in that study were not sequenced typed (Fashae and Hendriksen, 2014).

DISCUSSION

Assessing Clonality and Tracking Expansion

Methods that have been used to identify fluoroquinolone-resistant bacterial clones in Africa vary across the continent, making between-study and cross-country comparisons difficult (Quilici et al., 2010; Ismail et al., 2011; Marin et al., 2013). For toxicigenic *V. cholerae* for example, serotyping and biotyping—both insufficient to confirm clonality—are only occasionally performed outside clinical reference laboratories (Quilici et al., 2010; Ismail et al., 2011). Genotyping the *ctxB* product (Quilici et al., 2010), MLSA (Marin et al., 2013), and PFGE (Ismail et al., 2011; Marin et al., 2013) have been used to infer clonality in African studies. PFGE has been employed in multiple studies but different protocols were used and the data are therefore non-comparable. Moreover, PFGE identifies different clusters within local clones and would not be a robust method for assessing clonality across Africa (Ismail et al., 2011).

One reason why so many methods have been employed is that so many methods abound. However the robustness, replicability, and inferences that can be made from available methods differ considerably. Methods based on phenotype or PCR amplification patterns have been more widely used in the decades following the evolution of fluoroquinolone resistance but have limited replicability across laboratories and in some cases, limited resolving power (Achtman, 1996). Serotyping is very popular but has the additional downside of being dependent on very labile reagents, a concern that is of particular importance in African laboratories. To ascertain the relatedness of pathogenic bacteria, methods that are specific enough to identify clusters of related isolates and of high resolution to distinguish isolates within these groups are ideal. Many classical molecular typing methods are based on the sequences of a few small loci of

interest or on electrophoretic profiles dependent on particular nucleotide patterns. These principles respectively form the basis of multilocus sequence typing (MLST) and the comparison of PFGE patterns. Assessing MLST types and PFGE patterns are the methods most commonly used to infer clonality of sporadic or epidemic cases of enteric diseases in sub-Saharan Africa. They offer much greater reproducibility and portability than PCR-based or phenotypic methods and while they were initially pricey, set-up, and consumable costs have fallen dramatically in the last two decades. The major disadvantages of these methods are that they analyze a very small portion of the bacterial genome and different parts of a genome accumulate variation at different rates and/or are horizontally inherited. Finally, whilst still utilizable in specific circumstances, these methods lack the level of resolution needed to distinguish between very closely related bacteria.

The arrival of the so-called next-generation, high-throughput sequencing technologies makes it feasible to use WGS to identify and track clones of interest. The major intrinsic challenges so far have been high cost, inadequate infrastructure to maintain sensitive equipment and lack of technical capacity. Overcoming these surmountable challenges will present the opportunity to use the best-fit methods for studying the epidemiology and transmission of infectious diseases in such high burden areas in sub-Saharan Africa. It is increasingly important to situate these resources for WGS and analysis in areas with the most need to maximize gains. In the interim, a transitional advantage of WGS is that it can be used to generate comparative data for sequence based methods with less resolution, such as MLST.

The power and utility of WGS tools is exemplified in a recent study detailing the analyses of a global collection of *S. Typhi* (Wong et al., 2015). The *S. Typhi* H58 haplotype MDR clone has been shown to rapidly expand such as is the case in Malawi in which prior to 2011, sporadic Typhoid was caused by a diversity of clades. In their study, Wong et al. used a combination of WGS and Bayesian analyses to deduce the global population structure, possible intra- and inter-continental transmission pathways and an estimate of time of emergence of the most recent common ancestor of the current H58 global pandemic. These analyses confirmed the highly clonal nature of the H58 isolates, which were very similar to each other genetically (in fact, only ~6 SNPs separates any two H58 strains). They were also able to detect potential on-going epidemics in endemic countries. The H58 clones were estimated to have emerged in the late 1980's and are associated with high levels of drug resistance and reduced susceptibility to fluoroquinolones, due to mutations in DNA gyrase subunit and topoisomerase IV genes (Wirth, 2015; Wong et al., 2015). This is consistent with epidemiological and clinical data from the regions included in the study. This kind of analysis allows identification of genomic changes and acquisitions that underpin many observable phenotypes like increased infectivity, transmissibility or fitness when compared to closely related lineages of the same species. For example, Wong et al provide evidence to show that a chromosomally borne MDR locus and loss of an IncHI1 plasmid in a vast number of examined isolates may have increased the fitness of the H58 clones (Wirth, 2015; Wong et al., 2015).

Exacerbating Factors, Intervention and Research Needs

Clonal expansion of fluoroquinolone resistance in enterics is broadly the consequence of antimicrobial pressure and bacterial and gene dissemination. Antimicrobial resistance is a “Tragedy of the Commons” evolutionary paradigm that is worsened by failure to appreciate the problem; that is the absence of a “recognition of necessity” (Hardin, 1968; Okeke, 2009). Thus, infrastructural, bacterial and human-related factors exacerbate the effects of antimicrobial pressure and bacterial exchange. Sub-par diagnostic infrastructure leads to incorrect estimation of true disease burden and veiling of significant resistant clones. The lack of national, regional and internationally coordinated antimicrobial stewardship leads to inconsistencies and non-uniformity in many reported cases; this makes it difficult to interpret data and to select truly representative studies.

Antimicrobial Pressure

Antimicrobial pressure plays a large, important and potentially controllable role in the expansion of clonal resistance. Vien et al. showed that the prevalence of *qnr* resistance genes in gut Proteobacteria can increase following one course of antibiotic treatment (Vien le et al., 2012). In African countries, the contribution of pressure has not been measured, as antimicrobial use in humans and animals remains largely undocumented. Nonetheless, a high infectious disease burden warranting justifiable antimicrobial use, poor diagnostic test infrastructure and uptake and unregulated use of antibiotics combine to exacerbate the adverse effects that antimicrobial use has on resistance, and its potential to select undesirable clones. While the community impact of antimicrobial use and overuse are often discussed, (Feasey et al., 2014) recently presented a case study illustrating that antimicrobial use can be associated with acquisition of fluoroquinolone resistance by a highly virulent clone of *Salmonella* Typhimurium in a treated patient. In that case study, recrudescence of a bloodstream infection isolate after acquisition of a resistance plasmid is the most likely explanation for death. The effects of antimicrobial pressure on the evolution of fluoroquinolone resistance extend beyond quinolone use. Evidence suggests that fluoroquinolone-resistant clones are selected and maintained by multiple antimicrobial classes, a feature that is easily explained by the fact that the successful clones in question are resistant to multiple antimicrobials. Interventions are required to prevent the over-prescription of antibiotics and preventing unsanctioned use in humans and livestock. Policies that address these issues have long been in existence but are inadequately implemented in most parts of Africa (WHO, 2001).

Antibiotic use strategies such as mixing and cycling have been a proposed tool for reducing antibiotic resistance. Such proposals are based on very few investigations, which give variable results as to their effectiveness (Brown and Nathwani, 2005; Abel zur et al., 2014). Studies with *S. Typhi* have shown that FQ resistance is not typically associated with fitness cost and suggests that FQ resistant strains would be naturally maintained even if fluoroquinolones use was reduced and so antibiotic cycling is unlikely to be beneficial (Baker et al., 2013). Even

if mixing or cycling might be effective under some conditions, Africa currently has too few effective antimicrobial classes to effectively employ such techniques. In particular, withdrawing fluoroquinolones out of rotation limits treatment options for multiple life-threatening infections.

Dissemination

Enteric infections are principally attributable to poor sanitation, hygiene, food safety and access to potable water. Lack of such basic infrastructure contributes to endemicity in resource-poor settings and amplifies the prevalence of resistant strains in humans as well as animal and environmental reservoirs. Opportunities for bacteria to move from one colonized individual to another enhance the dissemination of resistant clones. In Africa, crowded cities and significant numbers of immunocompromised people are potential factors that could enhance the dissemination of clones. Interestingly, mathematical modeling to evaluate the impact of these risk factors found that increased pathogen transmission rates and longer durations of infectivity were even more important factors enabling spread, (Pitzer et al., 2015). Therefore, whilst unjustified antimicrobial use contributes to resistance, inadequate access to appropriate antibiotics when needed could also exacerbate the problem.

Travel has been associated with the dissemination of fluoroquinolone-resistant enteric clones in other parts of the world (Soraas et al., 2013). While there are reports pointing to export of resistant clones from African countries (Soraas et al., 2013), research does not appear to have determined whether and to what extent, resistant clones are imported into Africa. Research by Okoro et al. points to inter-country dissemination of multiply resistant non-typhoidal *Salmonella* in Africa but there are also very few studies documenting regional transmission as well (Okoro et al., 2012).

Clonal expansion of bacteria is often associated with selective advantage conferred by changes in bacterial genomes. These changes can be in the form the acquisition of chromosomal and/or plasmid-borne MDR determinants as well as mutations in genes targeted by antimicrobials, subsequently leading to the emergence and maintenance of fitter clones in animals and humans. This systematic review has shown that pandemic lineages within Africa are sometimes represented by different within-lineage clones than those elsewhere, pointing to bacterial factors that promote clonal expansion in select lineages. Host factors including presence of underlying conditions such immunosuppression (Gordon et al., 2007; MacLennan et al., 2008) and human migratory patterns attributable to social and economic pressures also contribute to the emergence and maintenance of MDR clones in Africa.

Surveillance

Our literature review identified very little research on fluoroquinolone-resistant enteric clones within Africa. There are however a large number of reports of fluoroquinolone resistant clones isolated in Europe and North America (Pitout et al., 2009; Soraas et al., 2013) that associate clone acquisition with travel to African countries (for example Pitout et al., 2009; Soraas et al., 2013). These “eyes of the hippopotamus” (Guerrant et al.,

2005) reports point to under-detection and under reporting in Africa and the need for more local studies to estimate the true magnitude and extent of the problem. Our research also did not uncover significant information about resistance reservoirs, particularly in non-human hosts and the environment. What few animal data we did encounter however points to a large non-human reservoir of enteric organisms, many of them resistant, and the occurrence of these organism through the food chain. Studies reviewed for this paper were almost entirely small research studies on human isolates from a handful of laboratories. The location of these laboratories is the principal determinant of locations on the continent from which data are available. In order to understand resistance and track the emergence of clones, multi-center surveillance is required.

The European Antimicrobial Resistance Surveillance Network (EARS-Net) enables comparison between European countries (<http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx>). Recent analysis of the data has shown that an increase of fluoroquinolone consumption in Europe also increases the rate of resistance (Redgrave et al., 2014). To understand the scale of resistance in Africa, a similar model based on a regulated and coordinated surveillance system across Africa would be ideal. In contrast to the EU, where an obvious framework for coordination exists, it is less clear how to implement such a scheme across Africa. Potential frameworks include public as well as private sub-regional alliances and there are multicountry data from the Francophone Pasteur Institute Network (Breurec et al., 2013). Continental coverage cannot be obtained from either. Looking forward, a regional organization, perhaps the nascent African Society for Laboratory Medicine, or perhaps a purpose-initiated one, might be lead such an initiative. Alternatively, different laboratories and networks could feed data to a repository. As well as putting in proposed minimum reporting guidelines for research on antimicrobial resistance (Omulo et al., 2015), the surveillance system would also be expanded to include detection of clones and mechanisms of resistance that are causing resistance so that the spread can be tracked in real-time. A defined methodology for assessing clones to enable tracking would be needed so that different strains across Africa could be accurately compared and experience from Europe suggests that this is best achieved through sequence-based methods.

A network is only as good as the data it can collate from participating countries. Implementation of such a scheme in some countries would be difficult, given the state of existing infrastructure but existing institutions in many other countries that may be well-positioned to provide a local picture of resistance problems if the necessary coordination were to occur. As has been recently reported from Ghana, it is possible to set up a national surveillance system coordinated from the ministry of health (Opintan et al., 2015). The laudable Ghanaian initiative was not without its challenges, many of them linked to resource limitations, and the problem that most of the usable data came from only a handful of participating institutions, mostly referral hospitals. However inception is the first step in building a robust surveillance system and most African countries are yet to

reach that point. In low-income African settings, it is predicted that susceptibility data will save lives and costs, compared to widespread used empirical therapy without local susceptibility information (Penn et al., 2015).

In addition to microbiological infrastructure and activity, support is needed for molecular studies that will identify the underlying genetic bases for resistance, including the existence and transmission of resistant clones. Comparison of isolates from within sub-Saharan Africa to those from outside the sub-region is important as recent studies have shown that clinical, cultural practices and socio-economic factors impact on the nature of circulating MDR clones in different parts of the world. Better antibiotic stewardship at local, country, and regional levels is needed in order to monitor and standardize protocols used for testing and clinical prescription. Much of the inquiry around antimicrobial resistance in African countries occurs within academic and non-governmental sectors, a likely contributor to fragmentation and limited coverage. Government participation and lead in public health initiatives such as strengthening of existing infrastructure and development of medical and research capacity remain a critical need.

AUTHOR CONTRIBUTIONS

MC: Coordinated and collated systematic searches and references, evaluated eligible papers, performed systematic search, wrote sections of the manuscript. AA: performed systematic search, wrote sections of the manuscript, evaluated eligible papers, contributed to outlining the paper. CO, KF: performed systematic search, evaluated eligible papers, wrote sections of the manuscript. JO: evaluated eligible papers, edited sections of the manuscript and contributed to outlining the paper. IO: Initiated the project, performed systematic search, wrote sections of the manuscript, evaluated eligible papers, coordinated the writing.

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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.2016.00558>

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Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones?

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Enterococci are Gram-positive bacteria that are found in plants, soil and as commensals of the gastrointestinal tract of humans, mammals, and insects. Despite their commensal nature, they have also become globally important nosocomial pathogens. Within the genus *Enterococcus*, *Enterococcus faecium*, and *Enterococcus faecalis* are clinically most relevant. In this review, we will discuss how *E. faecium* and *E. faecalis* have evolved to become a globally disseminated nosocomial pathogen. *E. faecium* has a defined sub-population that is associated with hospitalized patients and is rarely encountered in community settings. These hospital-associated clones are characterized by the acquisition of adaptive genetic elements, including genes involved in metabolism, biofilm formation, and antibiotic resistance. In contrast to *E. faecium*, clones of *E. faecalis* isolated from hospitalized patients, including strains causing clinical infections, are not exclusively found in hospitals but are also present in healthy individuals and animals. This observation suggests that the division between commensals and hospital-adapted lineages is less clear for *E. faecalis* than for *E. faecium*. In addition, genes that are reported to be associated with virulence of *E. faecalis* are often not unique to clinical isolates, but are also found in strains that originate from commensal niches. As a reflection of more ancient association of *E. faecalis* with different hosts, these determinants thus, they may not represent genuine virulence genes but may act as host-adaptive functions that are useful in a variety of intestinal environments. The scope of the review is to summarize recent trends in the emergence of antibiotic resistance and explore recent developments in the molecular epidemiology, population structure and mechanisms of adaptation of *E. faecium* and *E. faecalis*.

Keywords: *E. faecium*, *E. faecalis*, high-risk nosocomial clones, antibiotic resistance, virulence

INTRODUCTION

Enterococci are low-GC Gram-positive ovococci that can form pairs and chains of diverse lengths. Bacteria from the genus *Enterococcus* are facultative anaerobic and grow optimally at 35°C, but can tolerate temperatures ranging from 10°C to 45°C (Byappanahalli et al., 2012). The genus comprises 54 species (Parte, 2014), which are ubiquitously present in nature but the gastrointestinal tract (GIT)

of animals, including mammals, reptiles, birds (Mundt, 1963a) and insects (Martin and Mundt, 1972), is thought to be the largest reservoir of enterococci (Gilmore et al., 2013).

In humans, enterococci are common commensals of the GIT. In addition, enterococci have become ever more prominent as a causative agent of nosocomial infections since the 1970s (Arias and Murray, 2012). Two species, *Enterococcus faecalis* and *Enterococcus faecium*, cause the vast majority of hospital-acquired enterococcal infections in humans (Agudelo Higuera and Huycke, 2014). Of these two, *E. faecium* has rapidly acquired resistance to several classes of antibiotics. First, in the 1970s and 1980s, *E. faecium* gained high-level resistance to ampicillin (Grayson et al., 1991; Galloway-Peña et al., 2009) and since the 1980s it acquired resistance to aminoglycosides, fluoroquinolones, and glycopeptides, particularly vancomycin (Leclercq et al., 1988; Uttley et al., 1988; National Nosocomial Infections Surveillance System, 2004). *E. faecalis* has also acquired resistance to aminoglycosides, but resistance to ampicillin and vancomycin is much rarer than in *E. faecium* (Edelsberg et al., 2014). Worryingly, resistance to antibiotics that are used to treat vancomycin resistant enterococci (VRE), like linezolid, tigecycline, and daptomycin, has already been reported (Aksoy and Unal, 2008; Montero et al., 2008; Scheetz et al., 2008; Niebel et al., 2015).

The intrinsic resistance of enterococci to some antibiotics, including aminoglycosides, and the ability to acquire and disseminate antibiotic resistance determinants, like those involved in vancomycin resistance, only partly explain the recent emergence of these organisms as nosocomial pathogens. In addition, the plasticity of the enterococcal genomes allow enterococci to rapidly respond and adapt to selective constraints by acquiring genetic determinants that increase their ability to colonize or infect the host (Hendrickx et al., 2009; Palmer et al., 2012; Lebreton et al., 2013; Van Tyne and Gilmore, 2014). Other host or environmental factors, most notably exposure to antimicrobial agents, may favor an increase in colonization density of enterococci in the GIT of hospitalized patients (Donskey et al., 2000; Ubeda et al., 2010; Ruiz-Garbajosa et al., 2012). Antibiotic therapy that leads to the depletion of Gram-negatives, can reduce production of the antimicrobial peptide REGIII γ by Paneth cells, and this may promote the outgrowth of VRE (Brandl et al., 2008).

Patients undergoing transplants or with underlying diseases, such as diabetes or renal failure, and patients with long-term catheter usage, are at higher risk of developing infections caused by multi-drug resistant (MDR) enterococci (Arias and Murray, 2012). High-density colonization of the patient GIT facilitates the transmission of MDR enterococci among hospital ward through fecal contamination (Arias and Murray, 2012). Therefore epidemiological surveillance and outbreak investigations, together with infection control policies and interventions, such as the use of protective barriers and proper disinfection, are key for infection control of these organisms in the nosocomial environment (Sydnor and Perl, 2011). High-level enterococcal GIT colonization can also lead to urinary tract infections (UTI) (De Vecchi et al., 2013; Neelakanta et al., 2015), which may progress to bloodstream infections or

endocarditis (Patterson et al., 1995; Fernández-Guerrero et al., 2002). Enterococci from high-density intestinal populations may also directly translocate from the GIT into the bloodstream (Kamboj et al., 2014).

Enterococci have become one of the most common causes of health care-associated infections with *E. faecalis* causing approximately 60% of infections and *E. faecium* the remainder (de Kraker et al., 2013; Sievert et al., 2013). This review will focus on *E. faecalis* and *E. faecium* as both species have emerged as important nosocomial pathogens over the last 30 years and represent a major hub for the dissemination of antibiotic resistance genes.

EMERGENCE OF ANTIBIOTIC RESISTANCE IN ENTEROCOCCI

Enterococcus faecalis and *E. faecium* exhibit intrinsic resistance to a broad range of antibiotics. Below we briefly describe the mechanisms that cause resistance to the classes of antibiotics that are currently used in the treatment of enterococcal infections.

Compared to other low-GC Gram-positive cocci, enterococci exhibit decreased susceptibility to β -lactam antibiotics. The β -lactams act through the inactivation of penicillin-binding proteins (PBPs), thereby interfering with synthesis of peptidoglycan. All enterococci display decreased susceptibility to β -lactam antibiotics due to the expression of PBPs with an intrinsic low affinity for this class of antibiotics. Resistance to β -lactams, most notably to ampicillin, is currently far more widespread in *E. faecium* than in *E. faecalis* (Cattoir and Giard, 2014). The most important determinants for β -lactam resistance in *E. faecium* are mutations in the genes encoding the PBP5 (Zorzi et al., 1996; Rice et al., 2001; Zhang et al., 2012). There are clear indications that *E. faecium* progressed toward high-level ampicillin resistance in the 1970s and 1980s through the acquisition of specific mutations in the *pbp5* gene (Grayson et al., 1991; Galloway-Peña et al., 2009, 2011). It is of importance to note that these chromosomally encoded PBPs can be transferable (Dahl et al., 2000; Hanrahan et al., 2000; Rice et al., 2005), which indicates that dissemination of high-level ampicillin resistance can be the result of both clonal spread of strains with mutated *pbp5* genes and horizontal gene transfer. In addition to mutations in *pbp5*, production of β -lactamase has been described in both *E. faecalis* and *E. faecium* (Tomayko et al., 1996; Sarti et al., 2012). In general, the expression level of beta-lactamase in these species is low and impact on ampicillin susceptibility marginal.

Another group of antibiotics to which *E. faecium* and *E. faecalis* exhibit moderate intrinsic and high-level acquired resistance are the aminoglycosides. In *E. faecalis*, intrinsic resistance is thought to be caused by the inability of the antibiotic to enter the cytoplasm and inhibit ribosomal protein synthesis (Aslangul et al., 2006). In *E. faecium*, two chromosomally encoded genes, a 6'-N-aminoglycoside acetyltransferase (*aac(6')*-II) (Costa et al., 1993) and an rRNA methyltransferase (*efmM*) (Galimand et al., 2011), have been associated with intrinsic resistance to tobramycin and kanamycin. In addition to intrinsic resistance to aminoglycosides, the therapeutic

success of these antibiotics is critically compromised by high-level resistance, due to the gain of aminoglycoside modifying enzymes, such as phosphotransferases, acetyltransferases, and nucleotidyltransferases by *E. faecium* and *E. faecalis* (Chow, 2000; Miller et al., 2014).

The assessment of enterococci, particularly *E. faecium*, as important agents of MDR nosocomial infections was definitively established when they acquired resistance to vancomycin. While vancomycin-resistant enterococci were virtually non-existent in hospitals in the USA before 1990, nowadays 87% of *E. faecium* strains from nosocomial infections are vancomycin-resistant, while this is only 14% for *E. faecalis* (Edelsberg et al., 2014). Vancomycin is a glycopeptide antibiotic, which prevents cross-linking of peptidoglycan by binding to the D-alanine-D-alanine (D-Ala-D-Ala) moiety of the peptide chains that crosslink peptidoglycans. The mechanisms by which enterococci become resistant to vancomycin have been extensively reviewed elsewhere (Courvalin, 2006). In short, enterococci become resistant to vancomycin when the terminal amino acids of peptidoglycan precursors are altered from D-Ala-D-Ala to D-Ala-D-lactate (D-Ala-D-Lac) or to D-Ala-D-Serine (D-Ala-D-Ser), leading to high-level and low-level resistance to vancomycin, respectively. Nine gene clusters are currently known to be involved in vancomycin resistance in enterococci. These vancomycin resistance gene clusters are *vanA*, *vanB*, *vanD*, and *vanM*, causing vancomycin resistance through the formation of D-Ala-D-Lac, and *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* which catalyze the formation of D-Ala-D-Ser (Courvalin, 2006; Boyd et al., 2008; Xu et al., 2010; Lebreton et al., 2011). The most frequently found, and thus clinically most relevant, vancomycin resistance determinants are *vanA* and *vanB*, which are both part of larger gene clusters, which encode a two-component regulatory system and enzymes involved in the recycling of D-Ala-D-Ala peptidoglycan precursors to D-Ala-D-Lac. Both *vanA* and *vanB* are located on transposons that contribute to the dissemination of vancomycin resistance among enterococci (Courvalin, 2006). Recently, *vanM* was found to be the most important vancomycin-resistance determinant among different *E. faecium* lineages in hospitals in Shanghai, China (Chen et al., 2015). Differences in the sequence diversity and prevalence of each *van* operon could be due to the different ecological origins of the *van* clusters. While *vanA* seems to have originated from soil organisms, *vanB*, *vanG*, and *vanD* have been reported in gut commensal microbiota (Guardabassi et al., 2005; Domingo et al., 2007; Howden et al., 2013).

While vancomycin resistance emerged and spread in USA hospitals in the 1990s, carriage of vancomycin-resistant *E. faecium* (VREF) was rare among hospitalized patients in Europe. In contrast, VRE carriage among farm animals, and, to a lesser extent, in healthy humans was higher in Europe than in the USA (Klare et al., 1995; Devriese et al., 1996; Stobberingh et al., 1999). The widespread occurrence of VRE among farm animals was linked to the use of the vancomycin-analog avoparcin as a growth promoter in Europe (Bager et al., 1997). The presence of indistinguishable vancomycin resistance transposons in both animal and human reservoirs provided the first indication that animal-derived enterococci could be a reservoir of antibiotic

resistance genes that could be transmitted to humans (Arthur et al., 1993; Jensen et al., 1998; Woodford et al., 1998; Willems et al., 1999). After the ban on the use of avoparcin in 1997, the prevalence of VRE in animal husbandry declined in Europe (Klare et al., 1999; van den Bogaard et al., 2000; Aarestrup et al., 2001). In several European countries, the prevalence of VRE among hospitalized patients increased in the 21st century, but is still lower than the endemic levels reported in US hospitals (Bourdon et al., 2011; Gastmeier et al., 2014; Pinholt et al., 2015; Simner et al., 2015). Levels of VRE among hospitalized patients are also high in Australia, with 37% of *E. faecium* bacteremia isolates exhibiting resistance to vancomycin. Interestingly, vancomycin resistance in Australian *E. faecium* isolates is almost exclusively caused by *vanB*-type transposons, while *vanA* is the major vancomycin-resistance determinant in Europe and the USA (Coombs et al., 2014).

Due to the emergence and rapid spread of VRE, the antibiotics linezolid, daptomycin and tigecycline are increasingly used for the treatment of VRE infections.

Linezolid, the first oxazolidinone antibiotic, was introduced for clinical use in the USA in 2000. It acts on the ribosome by binding to a universally conserved site on 23S rRNA of the large 50S subunit of the ribosome (Colca et al., 2003), thereby inhibiting elongation of the polypeptide chain. The currently described linezolid resistance mechanisms alter the peptidyl transferase center of 23S rRNA, by a mutation in the 23S rRNA gene, with the G2576T mutation being most prominent (Jones et al., 2008), mutations in the genes encoding ribosomal proteins L3 and L4 (Chen et al., 2013) or methylation of the adenine at position 2503, which is catalyzed by the methyltransferase Cfr. The *cfr* gene is encoded on various conjugative and non-conjugative plasmids in enterococci (Diaz et al., 2012; Shen et al., 2013). Recently, the oxazolidinone resistance gene *optrA* has been identified in *E. faecalis* and *E. faecium* isolates of human and animal origin (Cai et al., 2015; Wang et al., 2015). This gene codes for an ATP-binding cassette transporter, which contributes to reduced susceptibility for oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol). The *optrA* gene was associated with a conjugative plasmid (Wang et al., 2015). Nevertheless, linezolid resistance is still rare. In a study using data from 19 US hospitals in the period 2007–2010, linezolid resistance was reported in 1.1 and 1.8% of *E. faecium* and *E. faecalis* isolates, respectively (Edelsberg et al., 2014).

Daptomycin is a lipopeptide antibiotic that targets the cell membrane through interactions with phospholipids (Humphries et al., 2013). The genetic basis for resistance in *E. faecalis* and *E. faecium* was first studied through genome sequencing of pairs of strains that developed resistance *in vitro* or *in vivo* (Arias et al., 2011; Palmer et al., 2011; Tran et al., 2013a). Additional genetic and biochemical work has established the role of the three-component regulatory system LiaFSR in contributing to daptomycin resistance in enterococci. The LiaFSR system is conserved in low-GC Gram-positive bacteria and governs the cell envelope stress response (Jordan et al., 2006). Mutations in the *liaF* gene of *E. faecalis* lead to a redistribution of cardiolipin-rich microdomains from the division septum to other regions of the

cytoplasmic membrane, which affects the antimicrobial activity of daptomycin. In addition, other mutations in phospholipid biosynthesis genes, most notably in the cardiolipin synthase gene *cls*, are required for full expression of daptomycin resistance in *E. faecalis* (Davlieva et al., 2013; Tran et al., 2013b). Similar mechanisms have been associated with daptomycin resistance in *E. faecium* (Munita et al., 2012, 2014; Davlieva et al., 2013). Notably, emergence of daptomycin resistance in *E. faecium* during daptomycin therapy is not always linked to mutations in *liaFSR* and mutations in *cls* may already be sufficient (Kelesidis et al., 2013; Lellek et al., 2015). Daptomycin resistance is infrequent, but more common in *E. faecium* than in *E. faecalis*. A worldwide study in isolates from hospitalized patients over the period 2005–2012 showed levels of daptomycin resistance of 0.18% for *E. faecium* and 0.02% for *E. faecalis* (Sader et al., 2014). However, in certain settings daptomycin resistance may be considerably more frequent, as illustrated by a study including 4,274 *E. faecium* and 7,007 *E. faecalis* isolates from 19 US hospitals, which found daptomycin resistance among 3.9 and 0.2% of *E. faecium* and *E. faecalis* isolates, respectively (Edelsberg et al., 2014). Similar levels of daptomycin resistance in *E. faecium* were recently reported for a German hospital (Lübbert et al., 2015).

Tigecycline is a semisynthetic derivative of the broad-spectrum tetracycline antibiotic minocycline, which acts on the ribosome by inhibiting its association with aminoacyl-tRNAs. The emergence of resistance during tigecycline therapy was first observed in *E. faecalis* (Werner et al., 2008; Cordina et al., 2012) and has recently been described in *E. faecium* (Niebel et al., 2015). In enterococci, resistance to tigecycline can be mediated through upregulation of tetracycline resistance determinants *tetL* (encoding an efflux pump) and *tetM* (providing ribosomal protection) (Fiedler et al., 2015) and mutations in the ribosomal protein *rpsJ* (Beabout et al., 2015; Cattoir et al., 2015; Niebel et al., 2015). Tigecycline resistance among *E. faecium* and *E. faecalis* is rare at 0.3% for both species (Hoban et al., 2015) and the antibiotic can still be used successfully to treat bacteremias caused by MDR enterococci, especially when it is used in combination with daptomycin (Polidori et al., 2011). The recovery of enterococcal isolates with decreased susceptibility to tigecycline in samples of animal origin is of concern, as extensive use with tetracyclines in the veterinary setting could select for tigecycline tolerant strains (Freitas et al., 2011).

In addition to the antibiotics described above, the antibiotics tedizolid, telavancin, dalbavancin, and oritavancin have recently been approved by the Food and Drug Administration in the USA for the treatment of skin infections by Gram-positive bacteria (Crotty et al., 2016). Tedizolid is an oxazolidinone antibiotic that has improved *in vitro* activity compared to linezolid. Enterococcal strains that have acquired linezolid resistance due to the acquisition of the *cfr* gene or the G2576T mutation in the 23S rRNA gene, may still have relatively low MICs ($\leq 4 \mu\text{g/ml}$) for tedizolid (Barber et al., 2016; Silva-Del Toro et al., 2016). Telavancin, dalbavancin and oritavancin are semi-synthetic glycopeptides antibiotics that, similar to vancomycin, affect peptidoglycan stability by binding to the D-Ala-D-Ala moiety of the peptide chains that crosslink peptidoglycan chains.

Interestingly, oritavancin is also active against *vanA*- and *vanB*-type vancomycin-resistant enterococci, while telavancin and dalbavancin have limited activity against enterococci carrying the *vanA*-type vancomycin resistance transposon but are active against enterococci with *vanB*-type vancomycin resistance (Jones et al., 2013; Karlowsky et al., 2015). Tedizolid, telavancin, dalbavancin, and oritavancin may be useful as alternatives to linezolid and vancomycin, but they do not have radically different modes of action and they may therefore suffer from the same resistance mechanisms that are threatening the efficacy of linezolid and vancomycin. Therefore, antibiotics that target other structures in the enterococcal cell, including teixobactin (Ling et al., 2015) and the acyldepsipeptides (Brötz-Oesterhelt et al., 2005), hold considerable promise as novel compounds for the treatment of infections with MDR enterococci.

MOLECULAR EPIDEMIOLOGY AND POPULATION STRUCTURE OF *E. faecium* AND *E. faecalis*

E. faecium

As a consequence of the global rise of VREF, a large number of molecular epidemiological studies have been performed to obtain insights into the dissemination of VREF clones in and between hospitals, in farm animals and healthy humans. However, methods like pulse-field gel electrophoresis (PFGE), soon proved to be insufficiently reproducible to study genetic relatedness of isolates (Morrison et al., 1999).

A first insight into the existence of particular ecotypes in *E. faecium*, was obtained by using amplified fragment polymorphism (AFLP) to infer the genetic relatedness of strains from diverse hosts and environments (Willems et al., 2000). This study revealed that strains from hospitalized patients grouped in a specific sub-population that was distinct from groups of strains that were isolated from humans in the community and farm animals.

The use of AFLP for global studies showed the limitations of this technique for comparisons of data obtained from different laboratories. Therefore an alternative method, termed multi locus sequence typing (MLST), was used in follow-up studies. In MLST, allelic profiles are based on the sequences of fragments of a number of housekeeping genes (seven in the case of the *E. faecium* and *E. faecalis* MLST schemes) (Homan et al., 2002; Ruiz-Garbajosa et al., 2006). Compared to AFLP, MLST has the distinct advantage that data are easily collated and shared through an on-line database. The first analyses of *E. faecium* MLST data were performed with the algorithm eBURST (Feil et al., 2004) and confirmed a distinct clustering of strains derived from the hospital environment. This cluster was named clonal complex 17 (CC17) and soon CC17 was found to be disseminated throughout the world (Willems et al., 2005; Top et al., 2008). The later use of larger datasets and different algorithms for the analysis of MLST data (Didelot and Falush, 2007; Francisco et al., 2009), showed that eBURST can sometimes fail to correctly assign STs to clonal complexes (CCs), particularly when genetic variation

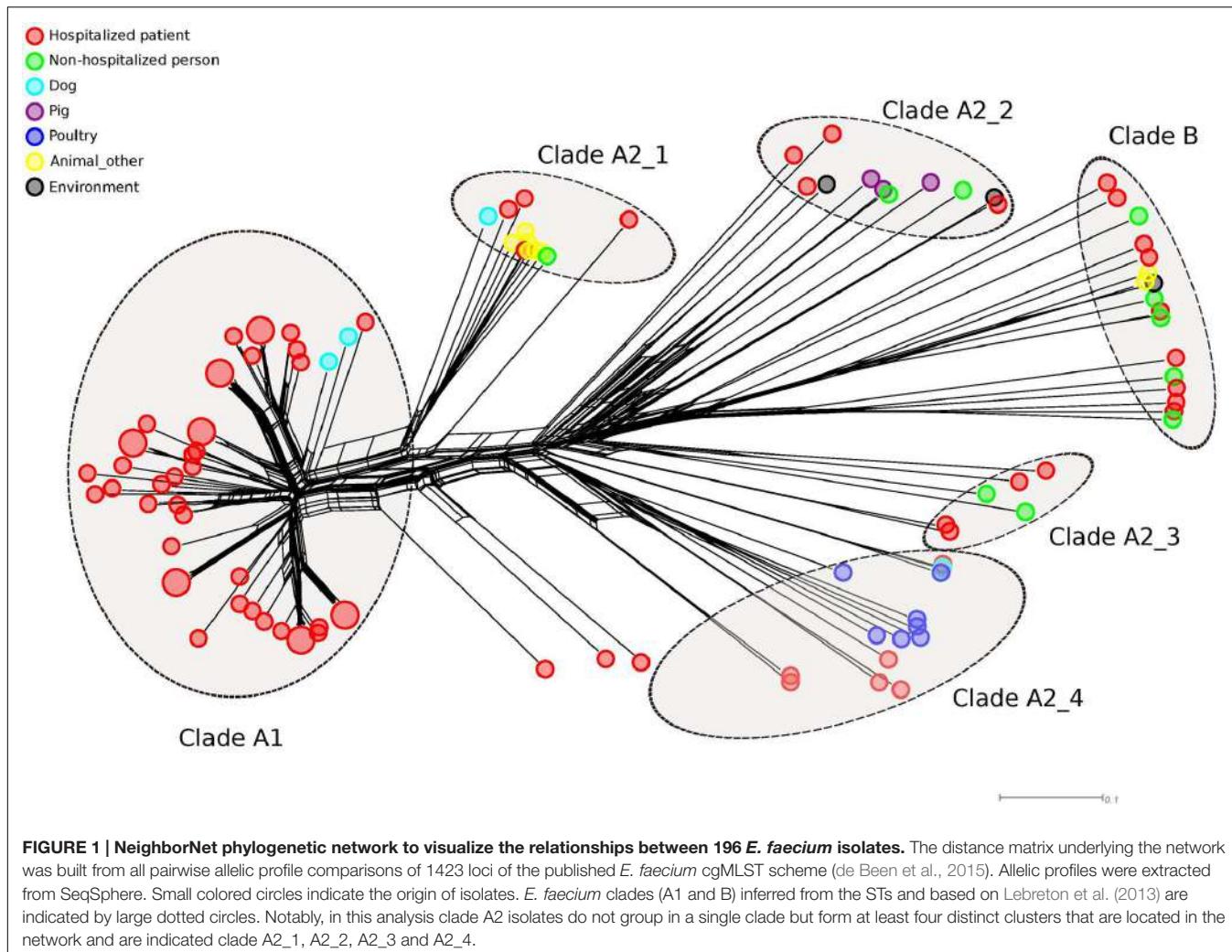
in populations is largely driven by recombination rather than mutations, like is the case in *E. faecium* and *E. faecalis* (Turner et al., 2007).

Bayesian analysis of population structure (BAPS) has successfully been used to probabilistically infer the population structure and levels of recombination of several microbial pathogens (Corander et al., 2012; Thomas et al., 2014). When applied to *E. faecium* MLST data, BAPS allowed the partitioning of 519 STs of 1720 *E. faecium* isolates into 13 non-overlapping groups. Of these groups, BAPS 3–3 was significantly associated with isolates from hospitalized patients, while BAPS 2–1 and 2–4 were significantly associated with farm animals. This observation again confirmed that there exists structure in the *E. faecium* population, with a distinct subpopulation of isolates that are almost exclusively found in hospitalized patients (Willems et al., 2012). One of the important nodes in the previously described hospital-associated CC17, ST78, and its descendant STs, grouped in BAPS 2–1 together with farm animal isolates, while two other important CC17 nodes, ST17 and ST18 with their descendant STs, clustered in another BAPS group (BAPS 3–3). These findings indicate that nosocomial *E. faecium* isolates have not evolved from a single ancestor, like previously postulated, but rather the cumulative acquisition of adaptive elements in nosocomial isolates may have occurred multiple times in different genetic backgrounds. Another conclusion that could be drawn from Bayesian modeling of MLST data is that hospital isolates displayed a relative low-level of admixture, despite the high recombination rates in *E. faecium*, suggesting that once strains have adapted to the distinct hospital niche, they become ecologically isolated (including isolation by dominance) and recombination with other populations declines (Willems et al., 2012).

Despite the overt lack of reproducibility, PFGE long remained the “gold standard” for molecular typing of *E. faecium* until the introduction of whole genome sequence (WGS)-based epidemiology. Howden et al. (2013) published a landmark study in which they used whole genome sequencing to track an outbreak of *vanB*-VREF in a large hospital in Australia. Interestingly, detailed phylogenomic analysis and precise mapping of the *vanB* gene revealed that 18 of the 36 *vanB*-VREF had acquired the *vanB* transposon during the outbreak period. This indicates that for *vanB*-VREF, frequent *de novo* generation of VREF through horizontal gene transfer may contribute to the emergence of VREF, in addition to clonal spread. This study was followed by multiple other studies that used whole genome sequencing to investigate the molecular epidemiology of VREF with high resolution and high accuracy (Reuter et al., 2013; Salipante et al., 2015; Brodrick et al., 2016; van Hal et al., 2016). Bender et al. (2016) performed whole genome sequencing of 49 *vanB*-VREF, primarily of ST192 (39/49), from invasive infections from hospitals all across Germany and found that spread of the Tn1549-*vanB*-type resistance involved exchange of large chromosomal fragments between *vanB*-positive and *vanB*-negative enterococci rather than independent acquisition events of the *vanB* transposon alone.

WGS-based studies also proposed that the *E. faecium* population was divided into two species-level subdivisions, based on phylogenetic analysis and the determination of average nucleotide identity (ANI) between the two sub-populations. The sub-populations were termed clade A or hospital-associated clade, primarily containing isolates from hospitalized patients and clade B or community-associated clade, mostly containing isolates from healthy, non-hospitalized individuals (Galloway-Peña et al., 2012; Palmer et al., 2012). The high level of diversity between these two clades indicates that the clade A-B split is ancient and precedes the modern antibiotic era. Further work provided further evidence for this split and showed that the population of *E. faecium* in clade A had a second split of more recent date (74 ± 30 years), with clade A1 containing the majority of clinical isolates and clade A2 mostly comprising animal-derived strains (Lebreton et al., 2013). WGS also confirmed that *E. faecium* was subject to high rates of recombination, leading to changes in MLST profile in otherwise closely related strains, which invalidates the use of MLST for tracking transmission events (van Hal et al., 2016). Another finding of van Hal et al. (2016) was that other *E. faecium* strains are the most important donors of imported DNA fragments. Specifically, strains from clade B are an important reservoir for donating foreign DNA to clade A strains (de Been et al., 2013). These findings indicate that hospital-acquired clade A1 *E. faecium* strains have recently emerged from a background of human commensal and animal isolates. Tree-like network representations of *E. faecium* genomic relatedness based on cgMLST data also revealed the distinct clustering of human commensal, animal and human clinical strains (Figure 1). Notably, in this analysis the animal isolates do not group in a single clade A2 but form multiple distinct clusters that are located in the network, between the human commensal and clinical isolates, indicating that clade A2 may not be monophyletic, as previously postulated (Lebreton et al., 2013). Interestingly, clade A1 *E. faecium* strains have lower fitness in natural environments, where they are out-competed by other *E. faecium* clones (Leclercq et al., 2013). Similarly, clade B strains outcompete clade A strains in an animal model of gut colonization in the absence of selection by antibiotics (Montealegre et al., 2015). These data highlight the exquisite niche specialization to the hospital environment of clade A1 strains, which may come at a fitness cost in non-hospital environments.

The WGS-based epidemiological studies described above generated phylogenetic trees based on SNPs, by mapping sequencing reads to a reference genome. This approach, although providing high-resolution typing data, complicates comparisons of data between studies and hampers the construction of globally accessible databases. This limitation may be overcome by using a genome-wide gene-by-gene comparison approach, as in classical MLST, but with an important extension of the number of analyzed genes from seven to the entire core genome of the species, i.e., 1423 genes as in the recently published core genome MLST (cgMLST) scheme of *E. faecium* (de Been et al., 2015). The *E. faecium* cgMLST scheme



allowed high-resolution tracing of *E. faecium* outbreaks and performed equally well as an SNP-based phylogenetic approach, but has the advantage that data can be collated in an online database and a standardized nomenclature for clones can be used.

E. faecalis

Enterococcus faecalis appears to be the most widespread and abundant species of *Enterococcus* and can be found in the intestines of humans, farm, companion, and wild animals and in the environment (Mundt, 1963a,b; Devriese et al., 1992a,b; Tannock and Cook, 2002; Ruiz-Garbajosa et al., 2009). Initial molecular epidemiological studies, using MLST, showed the existence of CCs, in which isolates originating from different hosts are contained (Ruiz-Garbajosa et al., 2006; McBride et al., 2007). While many *E. faecalis* clones are shared between hospitalized patients and other reservoirs, some CCs (specifically CC2, CC9, and CC87) and STs (ST6 and ST16) seemed to be enriched among isolates from hospitalized patients (Ruiz-Garbajosa et al., 2006; McBride et al., 2007; Freitas et al., 2009; Kuch et al., 2012). Similarly, in isolates collected from across

the globe, antibiotic resistance is more prevalent in strains that belong to CC2, CC8, CC9, CC16, and CC87 (Kawalec et al., 2007; McBride et al., 2007; Freitas et al., 2009; Kuch et al., 2012).

Analysis of the extent of congruence between the topologies of the seven different MLST gene trees revealed that that all 42 pairwise comparisons of the sequences of the MLST loci were incongruent. This observation suggests that *E. faecalis* is a highly recombinogenic species (Ruiz-Garbajosa et al., 2006). For this reason Tedim et al. (2015) used BAPS to investigate the *E. faecalis* population structure based on MLST data of hospitalized ($n = 133$) and non-hospitalized individuals ($n = 173$) isolated from feces. A hierarchical BAPS clustering analysis partitioned the *E. faecalis* population into 5 BAPS groups. Contrary to what has been described in *E. faecium* and to eBURST based analyses of *E. faecalis* MLST data, no significant association was found between isolates from hospitalized patients and particular *E. faecalis* BAPS groups (Tedim et al., 2015).

The first comprehensive *E. faecalis* phylogenomic study involved 18 *E. faecalis* genomes representing clinical ($n = 10$),

human commensal ($n = 3$), and animal isolates ($n = 3$), and isolates of unknown origin ($n = 2$) (Palmer et al., 2012). The analysis revealed that the phylogenetic diversity of *E. faecalis* was limited compared to *E. faecium*, with variations in the ANI in a narrow range between 97.8 and 99.5%. There was also no clear structure in the phylogenetic core gene tree, with no distinct clustering of isolates according to source. Similarly, clustering of these 18 isolates supplemented with 3 other published *E. faecalis* genomes from a clinical site, human feces and pig based on an *ad hoc* developed *E. faecalis* cgMLST scheme using tree-like network methods also revealed no distinct clustering of isolates according to their source (Figure 2). A recent genome-based study of 515 *E. faecalis* genomes, mainly isolated from clinical settings on the British Isles, revealed three dominant lineages of hospital-associated lineages (L1, L2, and L3). Isolates in L1 and L3 originated from both the UK and USA (Raven et al., 2016). These data clearly indicate independent clonal expansion with subsequent national dissemination. The data may also point toward the existence of specific hospital-associated or hospital-adapted lineages. However, in order to unequivocally infer hospital-adapted lineages it is essential to correct the data for clonal outbreaks, by including outbreak clones only once in the analysis. The fact that only a limited number of STs (3, 2, and 2 STs in lineage 1, 2, and 3, respectively) were represented in these three lineages may suggest that these lineages contain multiple replicates of outbreaks clones. Analyses for recombination frequencies in the core genome of L1, L2 and L3 isolates indicated low levels of recombination, which seems to contrast with previous MLST-based analyses (Ruiz-Garbajosa et al., 2006; Raven et al., 2016). Possibly, recombination drove the initial diversification of *E. faecalis* but contributed less to the relatively recent evolution of the three dominant *E. faecalis* lineages described by Raven and co-workers (Raven et al., 2016).

ADAPTIVE ELEMENTS IN NOSOCOMIAL LINEAGES

Enterococci are ubiquitous in nature, where they act as commensals and opportunistic pathogens. A consequence of these different lifestyles is that enterococci need to adapt to different micro-environments, each one exerting strong selective pressures. A strategy for bacterial species to survive when confronted with a wide range of selective pressures is to specialize in particular fitness peaks. *E. faecium* probably followed such an evolutionary trajectory, resulting in the emergence of specific hospital-adapted lineages. Successful hospital-adapted clones in *E. faecium* have the ability to exchange mobile genetic elements, carrying antimicrobial resistance, and virulence determinants, by horizontal gene transfer (Leavis et al., 2003; Hegstad et al., 2010; Palmer et al., 2010; van Schaik et al., 2010). The cumulative acquisition of adaptive elements has been named “genetic capitalism”, in which the acquisition of a particular adaptive element by a particular clone enhances its fitness, thereby increasing the likelihood of acquiring a second adaptive element

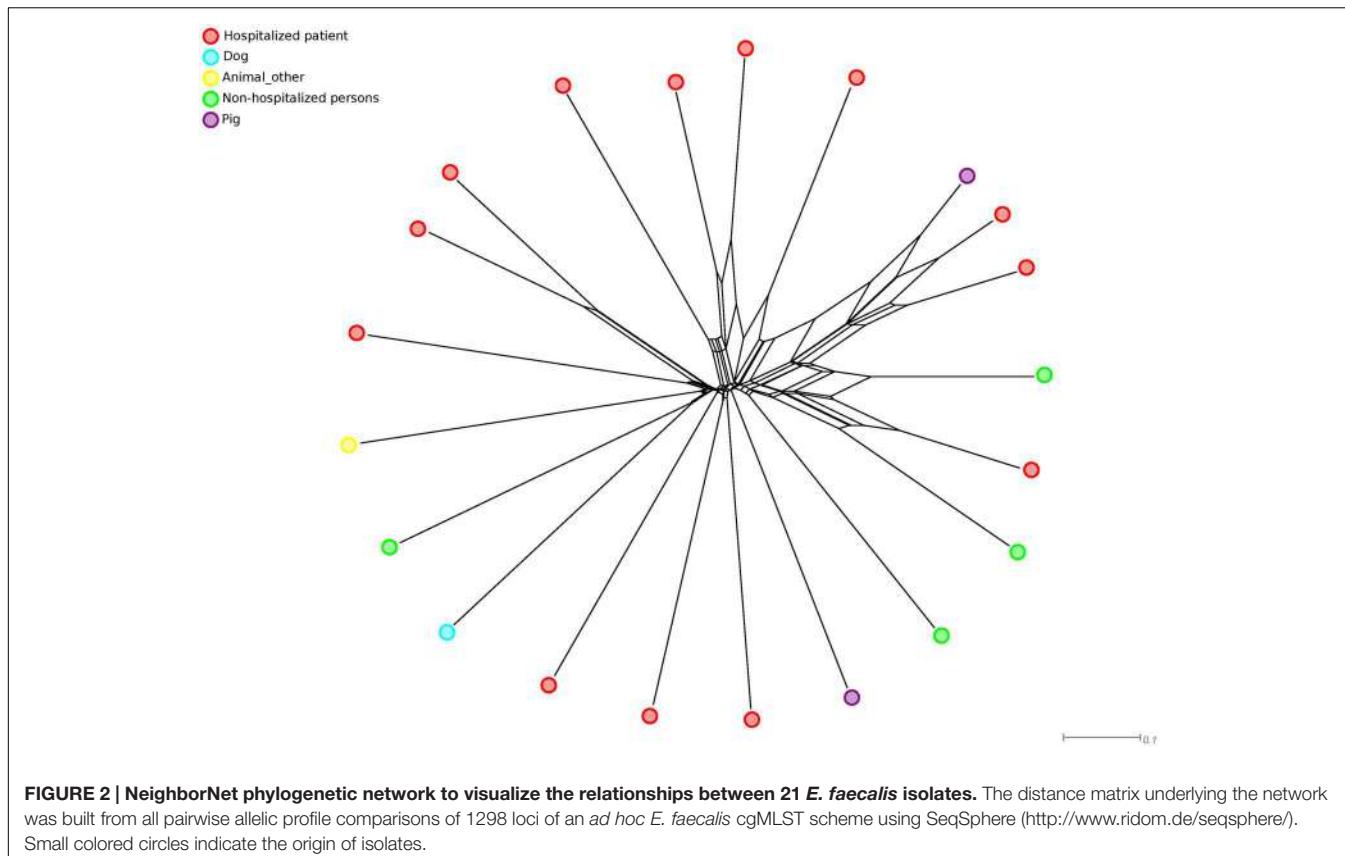
which finally can lead to the emergence of high-risk multidrug-resistant clones (Baquero, 2004). This cumulative acquisition of adaptive elements by the hospital-associated *E. faecium* clade A1 strains is reflected by their larger genome sizes ($2,843 \pm 159$ genes; 2.98 ± 0.15 Mbp), compared to the genomes of strains in clade A2 ($2,597 \pm 153$ genes; 2.75 ± 0.14 Mbp) or clade B ($2,718 \pm 120$ genes; 2.84 ± 0.1 Mbp) (Lebreton et al., 2013).

The first adaptive element that was described as being specific for hospital-associated *E. faecium* strains was Esp (Willems et al., 2001), a surface protein with a signal sequence for transport and a LPxTG-like motif for cell wall anchoring. The *E. faecium esp* gene is located on an integrative conjugative element, called ICEEfjm1, and contributes to biofilm formation, and UTIs and endocarditis in animal models (Leavis et al., 2004; Heikens et al., 2007, 2011; Leendertse et al., 2009; Sava et al., 2010; van Schaik et al., 2010; Top et al., 2011).

In addition to Esp, other determinants were found to be specific or significantly enriched among hospital-associated isolates. Characteristics of these genes have been previously discussed (Leavis et al., 2003; Heikens et al., 2008; Hendrickx et al., 2009, 2013; Zhang et al., 2013; Paganelli et al., 2016). Comparative genomic analyses of 73 *E. faecium* strains revealed major differences in gene content between clinical (clade A1), animal (clade A2) and non-clinical (clade B) strains, particularly through gain and loss of gene clusters with predicted roles in carbohydrate metabolism (Kim and Marco, 2013; Lebreton et al., 2013). Many of the clade B-specific genes have a predicted role in the utilization of complex carbohydrates from dietary sources, which were replaced with genes that were associated with the utilization of amino sugars (e.g., galactosamine), which occur on epithelial cell surfaces and in mucin. This metabolic switch may have contributed to the niche specialization of *E. faecium*.

As mentioned above the population structure of *E. faecalis* seems to be significantly different from *E. faecium* as no clearly defined *E. faecalis* ecotypes appear to exist. Correspondingly, previous studies have reported shared antibiotic resistance and virulence genes, such as the *E. faecalis* pathogenicity island, *esp*, capsule polysaccharide genes, and genes encoding for gelatinase, aggregation substance, cytolysin, and Ace, among *E. faecalis* isolates from a wide variety of different niches like the hospital, animals, food products and the environment (Eaton and Gasson, 2001; Creti et al., 2004; McBride et al., 2007; Freitas et al., 2009; Solheim et al., 2009; La Rosa et al., 2015). Note that the absence of clearly defined ecotypes in *E. faecalis* can be interpreted as a high multiplicity of closely related fine-grained ecotypes, none of them reaching predominance.

Comparative genomic analysis of 18 *E. faecalis* genomes showed that the accretion of mobile genetic elements in multiple *E. faecalis* lineages appears to be a major source of genome diversity (Palmer et al., 2012). Although antibiotic resistance and pathogenicity island traits have converged in some *E. faecalis* lineages, substantial differences in gene content exist, indicating that specialization toward specific ecotypes is not apparent in this species, in contrast to *E. faecium*. In addition, a hierarchical



clustering based on genome content of 38 *E. faecalis* genomes from diverse sources including clinical and non-clinical isolates, unearthed no evidence of distinct lineages in *E. faecalis* and no genes were found to be significantly enriched among clinical or non-clinical strains (Kim and Marco, 2013). A pangenome analysis of 168 *E. faecalis* isolates revealed no genes, or even homoplastic non-synonymous single nucleotide polymorphisms, that were ubiquitous in the three dominant lineages of hospital isolates, while being absent from all other sporadic lineages (Raven et al., 2016). These observations are all in accordance with high levels of genetic exchange between ecologically diverse *E. faecalis* clones.

In bacteria, mechanisms that preclude the acquisition of foreign DNA, include the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system (Horvath and Barrangou, 2010) and restriction and anti-restriction modification (RM-, antiRM-) systems (Clewell et al., 2014). *E. faecalis* strains lacking CRISPR-Cas appear to more readily acquire DNA through horizontal gene transfer and, consequently have a larger genome size than *E. faecalis* strains that carry CRISPR-Cas (Palmer et al., 2010, 2012). This indicates that the CRISPR-Cas status of *E. faecalis* strains may contribute to ecological adaptation. In *E. faecium* CRISPR-Cas is only found in a subset of clade B strains, again highlighting the general lack of barriers to horizontal gene exchange in this species (Lebreton et al., 2013). Restriction and anti-restriction systems located on mobile elements

or enterococcal chromosomes can also determine the flow of adaptive traits among populations of the same and/or different species (Clewell et al., 2014). Anti-RM systems include analogs of ArdA (alleviation of restriction of DNA) that are located on conjugative transposons (CTns) that are found widely spread in enterococci. ArdA proteins act against Type I restriction systems (detected in Tn916 and CTn6000) and other genes presumpitively involved in methylation (CTn6000) (Clewell et al., 2014). Recently, a type IIS restriction-modification (R-M) system SfaNI was described in *E. faecalis* (Furmanek-Blaszk and Sektas, 2015).

Other strategies seem to have exerted positive as well as negative selective pressures on *E. faecalis* clones. *E. faecalis* strain V583 uses phage particles to establish and maintain dominance of its intestinal niche in the presence of closely related competing strains (Duerkop et al., 2012). Furthermore, five of the seven prophages in the same strain (phage01, phage03, phage04, phage05, and phage07) can be excised from the bacterial chromosome and four of them produced infective virions that may promote gene dissemination among isolates and increase pathogenicity (Matos et al., 2013; La Rosa et al., 2015). Conversely, Gaca and Gilmore recently demonstrated how the accretion of mobile genetic elements in *E. faecalis* V583, renders it unable to co-exist with native enterococci in healthy human fecal flora (Gaca and Gilmore, 2016).

CONCLUSION

The ubiquitous nature of enterococci, the flexibility of their genomes and the widespread use of antibiotics in human and veterinary medicine, are important factors that drive the current emergence of *E. faecium* and *E. faecalis* as MDR nosocomial pathogens.

The population structure of *E. faecium* is characterized by a deep phylogenetic split that separates human commensal isolates (clade B) from farm animal and hospital isolates (clade A). It is tempting to speculate that the deep phylogenetic split in *E. faecium* is driven by anthropogenic influences since strains from farm animals (clade A2) and clinical isolates (clade A1), share a common feature as they both originate from environments where mammalian hosts are in close contact with each other and usage of antibiotics is high. The evolutionary trajectory of *E. faecium*, which has led to a clear clade structure, suggests that *E. faecium* is colonizing rugged fitness landscapes, in which characters of particular clones in clade A and B impose strong fitness differences. Under these circumstances, gene exchange between diverging populations is reduced over large genomic regions, as a collateral effect of strong divergent selection on genes involved in local adaptation. This mechanism was named divergence hitchhiking (DH) (Via, 2012). DH facilitates the divergence of genes linked to genomic regions that are most involved in local adaptation, and may explain the evolution of clones in populations with extensive gene flow. In *E. faecium* clade A, DH may have contributed to the fixation of ecologically important genomic traits, like antibiotic resistance or genes linked to pathogenicity. Indeed, using a neutral model incorporating microepidemics and migration, which mimics a situation where ecological factors may limit transmission between subpopulations, showed that *E. faecium* hospital isolates with extensive genotype relatedness markedly deviated from this neutral model compared with other common nosocomial bacteria like *Staphylococcus aureus* and *Staphylococcus epidermidis*, indicating that these hospital isolates represent a subpopulation adapted to the hospital environment (Numminen et al., 2016).

The *E. faecalis* population analyzed in the studies described above included human commensal, farm animal and clinical isolates. It is an open question why this anthropogenic divergence, as observed in *E. faecium*, is not seen in *E. faecalis*. An explanation for this may reside in differences in population size of both species. Indeed, *E. faecalis* seems to be more widespread and abundant in the intestines of cattle, pigs, dogs, horses and poultry (Devriese et al., 1987, 1991, 1992a,b, 1994). *E. faecalis* has also been more frequently isolated from wild mammals, reptiles, birds, insects, and wild plants than *E. faecium* (Mundt, 1963a,b; Martin and Mundt, 1972). Finally, in healthy humans *E. faecalis* is also more frequently found than *E. faecium* (Tannock and Cook, 2002). Moreover, it seems that *E. faecalis* clones are maintained longer in the gut than *E. faecium* clones (Ruiz-Garbajosa et al., 2009). The higher population density of *E. faecalis* relative to

E. faecium, in combination with its broader host-range, should provide *E. faecalis* with more opportunities for genetic exchange and diversification than *E. faecium*. High population densities also assure frequent transmission events, favoring gene flow among strains. This may act as a unifying force preventing the evolution of distinct clades in *E. faecalis*. Using the same neutral model described above for *E. faecium*, showed that the population structure of *E. faecalis* could be reflective of the evolutionary dynamics of a generalist organism which regularly experiences a high level of drift and gene flow between different host species (Numminen et al., 2016). Different strategies influence interactions of *E. faecalis* with populations of the same or of different species. A mechanism of substantial genetic exchange in *E. faecalis* has been postulated by Manson and co-workers, who described conjugative transfer of chromosomal fragments driven by integration of pheromone responsive conjugative plasmids carrying IS256 insertions and recombination across IS256 copies in the genome (Manson et al., 2010). The authors demonstrated that such an event could include transfer and recombination of up to 857 kb (~ 25% of the genome) and show that essentially every chromosomal location can be transferred by this mechanism. Other strategies may include phages, mobile genetic elements and the generation of polymicrobial biofilms (Weigel et al., 2007; La Rosa et al., 2015, 2016; Gaca and Gilmore, 2016).

High-level genetic connectivity of strains between different niches and hosts might favor dissemination of adaptive elements (e.g., virulence and resistance genes) that are acquired by *E. faecalis* populations in one niche (e.g., animals raised for food production) and finally end up being selected for in another environment (e.g., the hospital). In fact, the panmictic structure of *E. faecalis* favors its behavior as a collective evolutionary individual, which has promoted the generalist lifestyle of *E. faecalis*. The high level of genetic connectivity of *E. faecalis* strains in different niches, in combination with a relative small genome size, is in accordance with the 'Black Queen Hypothesis'. According to this hypothesis collective genes shared in a highly recombinogenic structure act as "common goods", and may favor the loss of genes in a particular niche in individual strains, provided they are not lost entirely from the community (Morris et al., 2012). While there is evidence in favor of this hypothesis, there is still a distinct lack of studies using WGS to describe the evolution, genetic diversity and population structure of *E. faecalis* and these are urgently needed to validate or falsify the current view of *E. faecalis* as a generalist.

In conclusion, *E. faecium* and *E. faecalis* are widely distributed among humans, animals and the environment. The social behavior of enterococci, which includes the accretion of plasmids, phages and CTns (Werner et al., 2013; Clewell et al., 2014), but also the ability to maintain microbial communities (Barnes et al., 2012; Paganelli et al., 2013), has allowed them to rapidly acquire antibiotic resistance genes and genetic elements that increase their ability to colonize and infect patients. *E. faecium* and *E. faecalis* differ in their population structure. *E. faecium* has clearly defined sub-populations, one of which contains

the majority of strains that are currently causing nosocomial infections. In contrast, *E. faecalis* is a generalist, with a highly panmictic population where genes involved in patient colonization or virulence are widely spread across niches.

AUTHOR CONTRIBUTIONS

Most of the writing for this manuscript was done by AG, WS, and RW, with MR contributing analyses for both figures and TC, FB, and JC contributing concepts and text in sections 3 and 4. All authors have contributed to and revised the manuscript prior to submission. In our opinion, all authors meet the

requirement of authorship as outlined in the Frontiers author guidelines.

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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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Characterization of carbapenem-resistant *Acinetobacter baumannii* isolates in a Chinese teaching hospital

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Carbapenem-resistant *Acinetobacter baumannii* (CRAB) presents a serious therapeutic and infection control challenge. In this study, we investigated the epidemiological and molecular differences of CRAB and the threatening factors for contributing to increased CRAB infections at a hospital in western China. A total of 110 clinical isolates of *A. baumannii*, collected in a recent 2-year period, were tested for carbapenem antibiotic susceptibility, followed by a molecular analysis of carbapenemase genes. Genetic relatedness of the isolates was characterized by multilocus sequence typing. Sixty-seven of the 110 isolates (60.9%) were resistant to carbapenems, 80.60% (54/67) of which carried the *blaOXA-23* gene. Most of these CRAB isolates (77.62%) were classified as clone complex 92 (CC92), and sequence type (ST) 92 was the most prevalent STs, followed by ST195, ST136, ST843, and ST75. One CRAB isolate of ST195 harbored plasmid pAB52 from a Chinese patient without travel history. This plasmid contains toxin-antitoxin elements related to adaptation for growth, which might have emerged as a common vehicle indirectly mediating the spread of OXA-23 in CRAB. Thus, CC92 *A. baumannii* carrying OXA-23 is a major drug-resistant strain spreading in China. Our findings indicate that rational application of antibiotics is indispensable for minimizing widespread of drug resistance.

Keywords: *Acinetobacter baumannii*, carbapenem resistance, OXA-23, plasmid, CC92

Introduction

Acinetobacter baumannii is an opportunistic Gram-negative pathogen, which has recently been successfully spreading worldwide nosocomial infections and causing outbreaks of hospital-acquired infections, primarily due to its prominent ability to acquire antibiotic resistance (Peleg et al., 2008; Kempf and Rolain, 2012). *A. baumannii* is developing into multidrug resistant (MDR), extensively drug resistant (XDR), and pandrug resistant (PDR) bacteria, and its adaptation to the environment with drug resistance has previously been reported (Durante-Mangoni and Zarrilli, 2011). Carbapenems were regarded as the most powerful antibiotics because of its extremely effective antibacterial activity and low toxicity, but the emergence of carbapenem resistance in *A. baumannii* has become a global concern recently (Peleg et al., 2008; Tiwari et al., 2012a,b).

Resistance of *A. baumannii* to expanded-spectrum cephalosporins and carbapenems is rapidly growing over the years (Bradford, 2001; Peleg et al., 2008). For example, surveillance by the CHINET project in China revealed that the rate of resistance of *A. baumannii* to the carbapenem, such as imipenem, doubled from 30.1% in 2006 to 62.8% in 2013 (Wang, 2008; Fupin et al., 2014).

Various types of β -lactam antibiotics, for example carbapenems, contain β -lactam rings in their structures and can be inactivated by β -lactamase enzymes. The β -lactamases are classified into four different molecular groups, Ambler classes A through D, according to amino acid sequence identities (Bush et al., 1995). Class A, C, and D (OXA enzymes) of β -lactamases contain a catalytically active serine residue that cleaves the lactam ring of antibiotics (Bush et al., 1995; Tiwari and Moganty, 2014). Class B of β -lactamases is a metallo-enzyme that requires zinc for their catalytic activity, and therefore, has a completely different mechanism for enzyme activity (Tiwari and Moganty, 2013). Carbapenems, such as imipenem and meropenem, have an exceedingly broad spectrum of activity and are able to resist hydrolysis by most of the β -lactamases, including extended-spectrum and derepressed class C chromosomal AmpC β -lactamases (Bush et al., 1995). However, most of the metallo- β -lactamases and a number of class A and D β -lactamases are able to hydrolyze broad spectrum carbapenems, such as imipenem and meropenem (Walther-Rasmussen and Hoiby, 2006; Evans and Amyes, 2014). Thus, outbreaks of OXA-23-producing *A. baumannii* have been reported from various regions of the world (Carvalho et al., 2009). It is generally believed that OXA-23 is responsible for carbapenem antibiotic resistance. Previously, Liu et al. (2015) reported the dissemination of MDR OXA-23-producing *A. baumannii* clones throughout multiple cities in China, but little is known about the molecular mechanisms of resistance to carbapenems in western China.

The multilocus sequence typing (MLST) has been widely applied in genotyping of bacteria, including *A. baumannii* (Bartual et al., 2005). Molecular epidemiological research of *A. baumannii* indicates that CC92 has played an important role in nosocomial infection outbreak and spread nationwide (Runnegar et al., 2010).

This study aimed to report the dissemination of *A. baumannii* harboring carbapenemase genes throughout a university hospital in western China, then identify the risk factors for carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections, and finally perform a comprehensive evaluation and comparison of their genetic diversity.

Materials and Methods

Bacteria Isolates

A total of 110 consecutive and non-duplicated *A. baumannii* clinical isolates were collected from different departments [intensive care unit (ICU), gastroenterology, respiratory, neurosurgery and other wards] at the First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan, China from 2012

to 2013. Isolates were identified by standard laboratory methods and ATB New (bioMérieux, France). *A. baumannii* was further verified when two PCR products were yielded as reported: a 425-bp internal control amplicon corresponding to the *recA* gene of *Acinetobacter* spp. and the 208-bp fragment of the 16S rRNA (Chiang et al., 2011) intergenic spacer region of *A. baumannii* (Table 1). All strains were stored at -80°C , and bacteria were grown on tryptose agar or Mueller–Hinton broth or agar (Oxoid, England).

Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) of carbapenems including imipenem and meropenem for *A. baumannii* were determined by the agar dilution method as previously described in the guidelines from the Clinical and Laboratory Standards Institute (CLSI, 2014). *Escherichia coli* ATCC25922 and *A. baumannii* ATCC19606 were used as quality control strain. The results were interpreted according to the CLSI guidelines (CLSI, 2014), i.e., CRAB was defined as an *A. baumannii* isolate that was resistant to both imipenem and meropenem (i.e., $\geq 8 \mu\text{g/ml}$ as resistant), whereas carbapenem-susceptible *A. baumannii* (CSAB) possessed carbapenem MIC of $\leq 2 \mu\text{g/ml}$ and carbapenem-intermediate *A. baumannii* (CIAB) has MIC of $4 \mu\text{g/ml}$.

PCR Experiments

The genes encoding carbapenemases class A [e.g., *Klebsiella pneumoniae* carbapenemase gene, *bla*_{KPC} (Li et al., 2014)], class B [e.g., the metallo- β -lactamases, *bla*_{IMP} (Valenzuela et al., 2007), *bla*_{VIM-1} (Tsakris et al., 2000), *bla*_{SIM} (Ellington et al., 2007), and *bla*_{NDM-1} (Yong et al., 2009)], class C [e.g., *bla*_{AmpC} (Bou and Martinez-Beltran, 2000)], and class D [e.g., *bla*_{OXA-23} (Woodford et al., 2006), *bla*_{OXA-24} (Woodford et al., 2006), *bla*_{OXA-51} (Fu et al., 2010), *bla*_{OXA-58} (Netsvyetayeva et al., 2011), and *bla*_{OXA-235} (Higgins et al., 2013)] were investigated by polymerase chain reaction (PCR).

To prepare DNA templates, each isolate was grown on tryptose agar (Oxoid, England) plate overnight. Two to three colonies were suspended in 100 μl of sterile distilled water in a 1.5-ml Eppendorf tube, and the suspension was heated at 100°C for 15 min, followed by centrifugation at 12,000 g for 10 min to pellet the debris. The resultant supernatant was used as the DNA template in the PCRs, which were carried out in a 50- μl volume containing 0.2 mM each deoxynucleotide, 0.5 μM each primer, 1.25 U of Taq polymerase, and 5 μl of 10 \times buffer (Thermo, China). Primers (Table 1) were synthesized by Sangon Company (Sangon, China). Reaction conditions of PCR were 94°C for 5 min and 30 cycles of 94°C for 30 s, 55°C for 30 s (57°C for *bla*_{AmpC} amplification), and 72°C for 30 s, followed by a final extension at 72°C for 5 min.

Multilocus Sequence Typing

MLST was used to describe the genetic backgrounds of CRAB and CSAB in all clinical isolates (Diancourt et al., 2010; Hamouda et al., 2010; Pournaras et al., 2014). eBURST was performed to cluster sequence types (STs) into clonal complexes (CCs) and infer evolutionary descent. MLST was carried out as described

TABLE 1 | Primers used in this study.

Locus	Primer	Oligonucleotides (5' → 3')	Expected size (bp)	Source
16S	F	CAT TAT CAC GGT AAT TAG TG	208	Chiang et al. (2011)
	R	AGA GCA CTG TGC ACT TAA G		
RecA	F	CCT GAA TCT TCT GGT AAA AC	425	Chiang et al. (2011)
	R	GTT TCT GGG CTG CCA AAC ATT AC		
KPC	F	GCT CAG GCG CAA CTG TAA GT	823	Li et al. (2014)
	R	GTC CAG ACG GAA CGT GGT AT		
IMP	F	CTA CCG CAG AGT CTT TG	587	Valenzuela et al. (2007)
	R	AAC CAG TTT TGC CTT ACC AT		
VIM-1	F	AGT GGT GAG TAT CCG ACA G	261	Tsakris et al. (2000)
	R	ATG AAA GTG CGT GGA GAC		
17SIM	F	TAC AAG GGA TTC GGC ATC G	570	Ellington et al. (2007)
	R	TAA TGG CCT GTT CCC ATG TG		
NDM-1	F	TCT CGA CAT GCC GGG TTT CGG	475	Yong et al. (2009)
	R	ACC GAG ATT GCC GAG CGA CTT		
AmpC	F	ACT TAC TTC AAC TCG CGA CG	663	Bou and Martinez-Beltran (2000)
	R	TAA ACA CCA CAT ATG TTC CG		
OXA-23	F	GAT CGG ATT GGA GAA CCA GA	501	Woodford et al. (2006)
	R	ATT TCT GAC CGC ATT TCC AT		
OXA-24	F	CAA GAG CTT GCA AGA CGG ACT	420	Woodford et al. (2006)
	R	TCC AAG ATT TTC TAG CRA CTT ATA		
OXA-51	F	TAA TGC TTT GAT CGG CCT TG	353	Fu et al. (2010)
	R	TGG ATT GCA CTT CAT CTT GG		
OXA-58	F	TCG ATC AGA ATG TTC AAG CGC	530	Netsvyetayeva et al. (2011)
	R	ACG ATT CTC CCC TCT GCG C		
OXA-235	F	TTG TTG CCT TTA CTT AGT TGC	831	Higgins et al. (2013)
	R	CAA AAT TTT AAG ACG GAT CG		

by Bartual et al. (2005) for all isolates. In brief, internal fragments of seven housekeeping genes, *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*, were amplified, purified, and sequenced (Fu et al., 2010; Ruan et al., 2013). And eBURST (version 3, <http://eburst.mlst.net/>) was used to assign STs to CCs and define the genetic relatedness of STs with the most stringent definition of the groups by sharing the same alleles at ≥6 of 7 loci (Feil et al., 2004).

Plasmid Conjugation, Extraction, Sequencing, and Analysis

Plasmid conjugations were performed by using meropenem-resistant *A. baumannii* as donors and an azide-resistant *E. coli* J53 as the recipient ($\text{MIC} > 200 \mu\text{g/ml}$; Bogaerts et al., 2006). Matings were performed as described previously (Wang et al., 2003), and transconjugants were recovered on L-agar plates containing meropenem (8 $\mu\text{g/ml}$) and azide (150 $\mu\text{g/ml}$).

Plasmid DNA was extracted using a Plasmid Midi Kit (Omega, USA) according to the manufacturer's instructions. The plasmid was cut by EcoRI, then the fragments were ligated to the vector pET21a. The complete sequencing work was performed by the ABI-3730 XL system (Biosune Biotechnology Company, Shanghai, China) and sequences were assembled by Life Technologies (BigDye V3.1, four fluorescence reagent kit). The sequenced plasmid was annotated by the RAST server (Aziz et al., 2008), then all of the predicted proteins were further compared against the NCBI non-redundant protein database

using the BLASTP program. In addition, the DNAMAN software (Lynnon Corporation, USA) was used to generate a circular map of plasmid pAB52.

Nucleotide Sequence Accession Numbers

The complete nucleotide sequences (Supplementary Table S1) of plasmid pAB52 in this study were submitted to GenBank with assigned accession number KR030046.

Results

Characterization of Clinical Isolates

According to our previous study, 67 of the total 110 *A. baumannii* isolates exhibited resistance to carbapenems, imipenem and meropenem, with MIC values of ≥8 $\mu\text{g/ml}$. These CRAB isolates were from different types of specimens including sputum ($n = 49$), throat swabs ($n = 5$), blood ($n = 1$), douche ($n = 4$), and others ($n = 8$). Epidemiological analysis of the 67 patients with CRAB revealed that 27 were ≥70 years old, 15 were between 60 and 70 years old, 24 were between 21 and 60 years old, and one was ≤20 years old. Among 40 males (59.7%) and 27 females (40.3%), 24 isolates were collected from the ICU, and 17 isolates were collected from the respiratory department. Clinical characteristics of the 67 patients with CRAB are summarized in Table 2.

TABLE 2 | Clinical characteristics of 67 patients with Carbapenem-resistant *Acinetobacter baumannii* (CRAB).

Gender	Total number	Source	Total number	Department	Total number
Male	40 (59.70%)	Blood	1 (1.49%)	Intensive care unit	24 (35.82%)
Female	27 (40.30%)	Sputum	49 (73.13%)	Respiratory	17 (25.37%)
Age (years)	Total number	Swab	5 (7.46%)	Neurosurgery	9 (13.43%)
0–20	1	Secretion	5 (7.46%)	Infectious diseases department	2 (2.99%)
21–50	11	Douche	4 (5.98%)	Other wards	15 (22.39%)
51–60	13	Cerebrospinal fluid	2 (2.99%)		
70+more	27	Other	2 (2.99%)		

Expression of Carbapenemase Genes in *A. baumannii* Isolates

All isolates were screened for the presence of β -lactamases genes (Table 3). In the 67 CRAB isolates, all of them were positive to *recA*, 16S rRNA conservative region and *blaOXA-51*. The major carbapenemase gene *blaOXA-23* was detected in 80.60% of all isolates. In addition, 65 isolates (97.10%) were found to carry *blaAmpC*. Other β -lactamase genes including *blaKPC*, *blaIMP*, *blaVIM-1*, *blaSIM*, *blaNDM-1*, *blaOXA-24*, *blaOXA-58*, and *blaOXA-235* were undetectable in all isolates. Thus, the expression of *blaOXA-23* is still the dominant carbapenem resistance mechanism in *A. baumannii* among the isolates investigated aside from the inherent *blaOXA-51* gene. One susceptible isolate carried *blaOXA-72*.

Genetic Analysis of STs

To investigate whether the isolates were genetically related, MLST was performed to characterize the CRAB, CIAB and CSAB (Figure 1). Each ST was represented by a dot that was proportionally sized to the number of *A. baumannii* containing that ST. The MLST analysis revealed a total of 32 different STs in 67 CRAB, including 6 existing STs and 26 novel STs. 77.62% of the 67 CRAB isolates were classified to CC92, ST92 and its 4 novel single-locus variants (SLVs), ST195, ST136, ST843, and ST75, were the predominant STs, found in 34.33, 7.46, 5.97, 4.48, and 2.99% of isolates, respectively. Among these five predominant STs, 82.35% of carbapenem-resistant isolates carried the *blaOXA-23* gene, in which ST92 is one of the most widespread STs across the world and belongs to the CC92 in *A. baumannii* MLST databases (Wang et al., 2013). Seventeen of the 32 CRAB STs belong to CC92 (Figure 1A). In this study, the number of polymorphic sites in *gpi* ($n = 8$), *rpoD* ($n = 4$), *cpn60* ($n = 3$), *recA* ($n = 2$), and the other three loci (*gdhB*, *gyrB*, and *gltA*) indicated that all were the same in CC92. Seven of the eight CIAB were CC92 (Figure 1B), but only 3 of the 35 CSAB were CC92 (Figure 1C). All these suggested that CC92 represents the most widely distributed carbapenem-resistant CC identified in the hospital.

Plasmid Analysis

To detect the nosocomial infection mediated by *A. baumannii* plasmid DNA, a plasmid conjugation assay was performed. However, none of the carbapenem-resistant isolates transferred plasmids from *A. baumannii* isolates to *E. coli* J53 successfully. This could be due to multiple reasons. Carbapenem resistance genes were located in the genome, and plasmids were not conjugative or unable to replicate in the *E. coli* host. Of note, with no. 52 CRAB isolate (which harbored pAB52 plasmid) as the donor strain, a few colonies with azide resistance and displaying a similar morphology as the *E. coli* J53 recipient were obtained from the conjugation. Yet, these colonies were found to be arisen from *A. baumannii* based on the amplification and sequence analysis of 16S rRNA and seven housekeeping genes *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*. Likely, the generation of azide-resistant *A. baumannii* was a result of spontaneous mutation(s). In addition, plasmid extraction assay confirmed no plasmid-containing *E. coli* J53 colonies, indicating that pAB52 could not be transferred from *A. baumannii* to *E. coli* J53.

RAST analysis showed that the plasmid pAB52 was 8,893 bp in size and contained 10 open reading frames (ORFs; Figure 2A), with an average G+C content of 34.5%. Three genes with a putative role in virulence were detected in pAB52: a septicolytic-like gene coding for a pore-forming toxin (Rosado et al., 2008; Gan et al., 2012), a TonB-dependent receptor gene coding for an outer membrane protein involved in iron uptake and virulence (Acosta et al., 2011; Gan et al., 2012), and a toxin-antitoxin (TA) gene coding for a functional TA system and that its toxin, SpfT (Gan et al., 2012; Jurenaite et al., 2013), inhibits translation (Figure 2A). One ORF (orf 9) codes for proteins that share similarity to hypothetical proteins encoded by plasmid genes found in other bacteria (Gan et al., 2012), while the predicted products of three others (orf 1, orf 2, and orf 3) do not match any known sequences. orf 9, which is adjacent to the replication initiation protein, is similar to replication protein of other genomes (Figure 2B). Therefore, it might contribute to a replication ability. Further analysis indicated that this plasmid

TABLE 3 | Positive rates of β -lactamase genes in CRAB.

No. of strains	Rate of gene (%)										
	KPC	IMP	VIM-1	SIM	NDM-1	AmpC	OXA-23	OXA-24	OXA-51	OXA-58	OXA-235
67	0	0	0	0	0	97.10	80.60	0	100	0	0

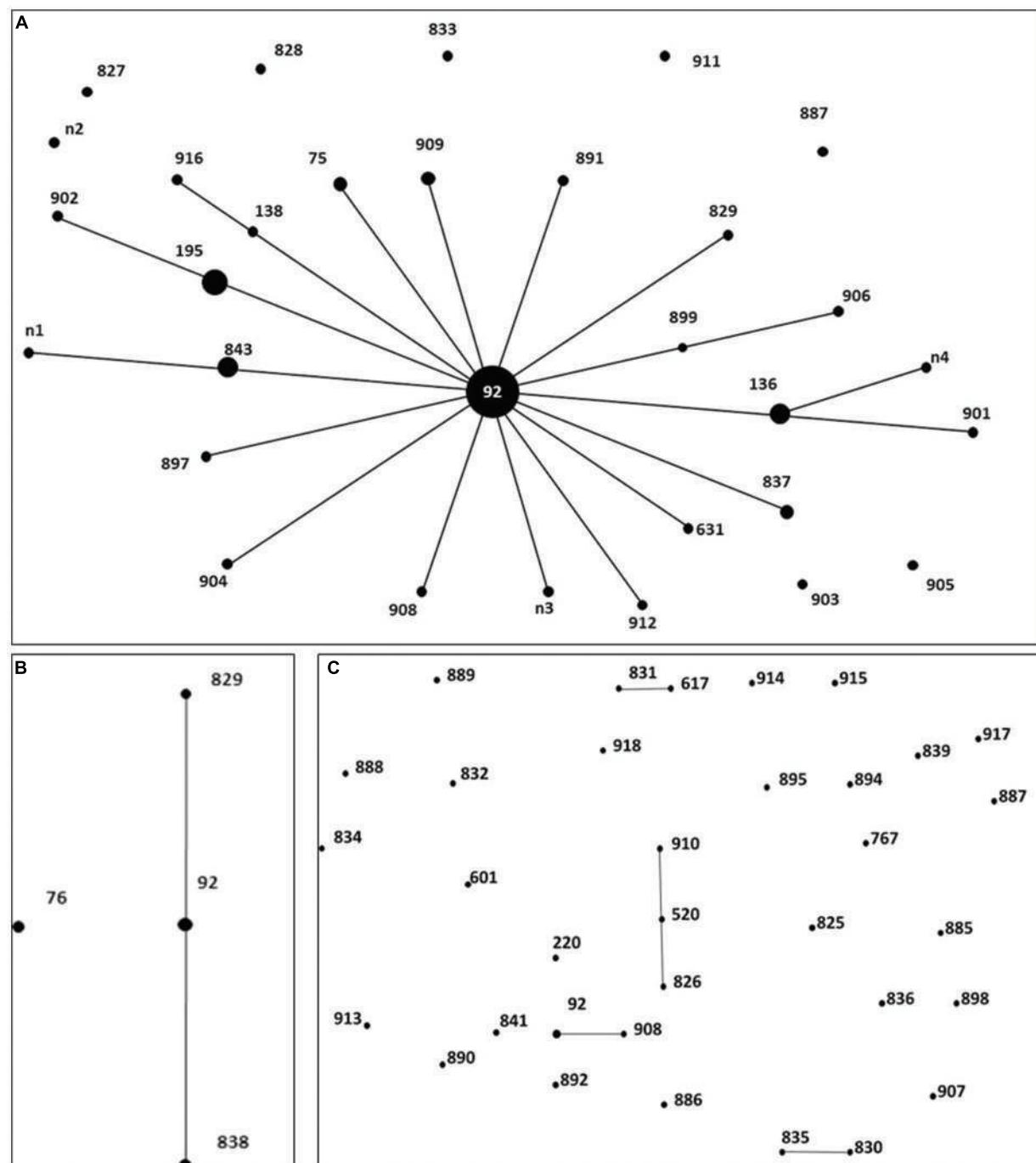


FIGURE 1 | Distribution of genes in 67 Carbapenem-resistant *Acinetobacter baumannii* (CRAB), 8 carbapenem-intermediate *A. baumannii* (CIAB), and 35 carbapenem-susceptible *A. baumannii* (CSAB). Minimum spanning tree analysis by eBURST algorithm of CRAB (A), CIAB (B) and CSAB (C) isolates based on MLST data. Each circle represents a specific sequence type (ST). The size of each circle homologizes to a different number of isolates, with larger sizes representing higher frequency of occurrence. The solid lines connecting the circles indicate the relationship between different STs.

shared most nucleotide identity with previously described isolates of *A. baumannii*, WM99C, TCDC-AB0715, AB210, AB0057, 1656-2, AYE, UMB001, and ATCC17978, *Acinetobacter* sp. 6013150 and 6013113 (**Figure 2B**).

Discussion

Clinical characterization of 67 patients with CRAB indicated that compromised immunity (e.g., elderly patients suffering

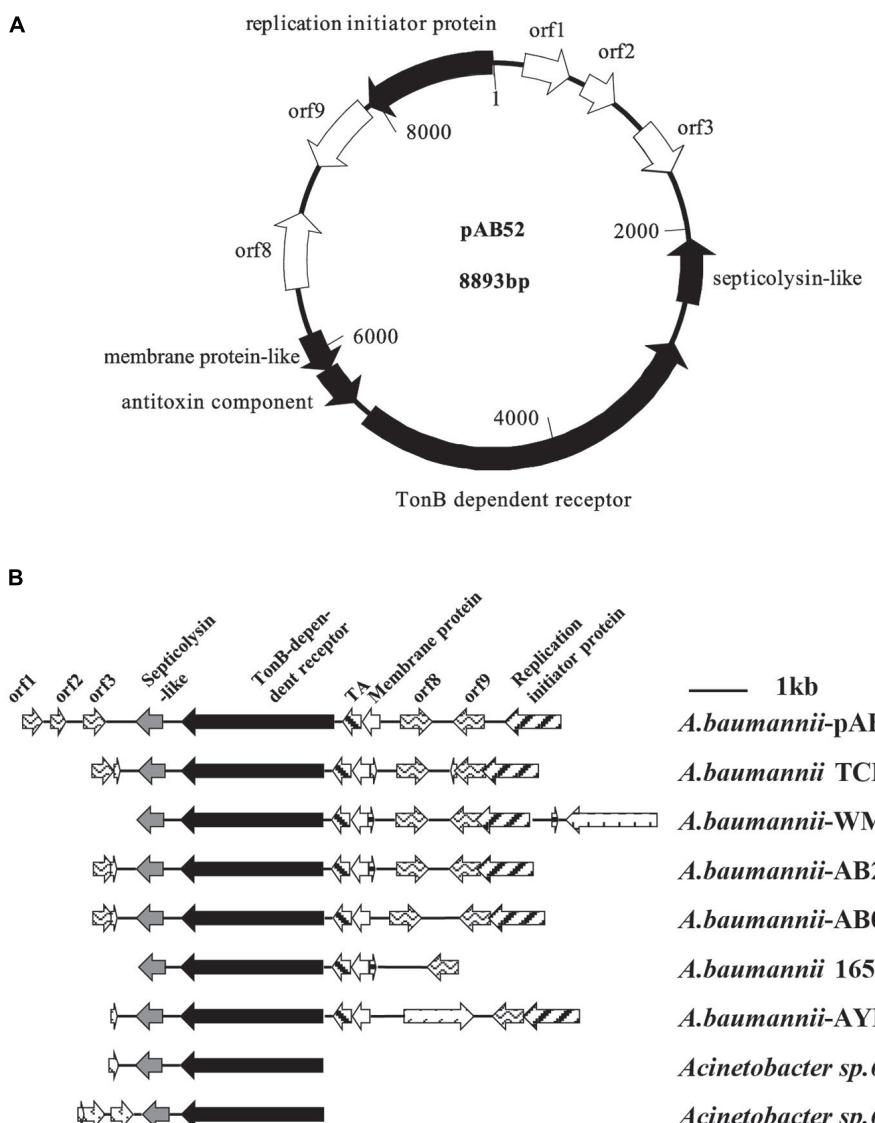


FIGURE 2 | (A) Diagram of plasmid pAB52 (GenBank accession number KR03004). The open reading frames (ORFs), represented by arrows pointing in the direction of transcription, are white, except for that for the reported genes, which is black. **(B)** Comparative analysis of the genetic environment of the TonB-dependent receptor in plasmid DNA by RAST software. Regions with 100% nucleotide sequence identity or similar biological function are filled with the same pattern.

from respiratory failure, encephalorrhagia, extensive burns, and chronic obstructive pulmonary disease) increases the risk of *A. baumannii* infection. In addition, males appear to have a greater risk of infection than females.

Surveillance by the CHINET project demonstrated that the *A. baumannii* rate of imipenem resistance increased over twofold between 2006 and 2013 to 62.8% in China (Fupin et al., 2014). In contrast, the resistance rate of *Acinetobacter* spp. to cefoperazone/sulbactam was only 36.4% (Fupin et al., 2014), far below the rates of imipenem resistance. Mirroring the national trend, the *A. baumannii* resistance rate of imipenem and cefoperazone/sulbactam in our hospital was 60.9 and 14.75%, respectively (Ying et al., 2013).

We observed that OXA-23 was also the major carbapenemase mechanism responsible for the resistance phenotype (Table 3), as the gene was expressed in most CRAB isolates. This result is consistent with previous reports in which the CRAB is often caused due to the expression of OXA enzymes, particularly OXA-23 (Fu et al., 2010; Merino et al., 2014; Zowawi et al., 2015).

In our experiments, we unexpectedly found one particular carbapenem-susceptible isolate, which belonged to ST220 and carried the *bla*_{OXA-72} gene, a member of the *bla*_{OXA-24} subgroup, but this isolate did not carry any other resistance genes. This is the first time that *bla*_{OXA-24} has been detected in western China. Of note, this isolate was not only susceptible

to meropenem and imipenem, but also susceptible to other β -lactams, aminoglycosides, tetracyclines, fluoroquinolone, rifampicin, and trimethoprim-sulfamethoxazole (data not shown). Our results are in contrast with previous reports that nosocomial infections were triggered by CRAB-carrying plasmids encoding OXA-72 carbapenemase via plasmid DNA from Lithuanian (Povilonis et al., 2013) and Taiwan (Lu et al., 2009). There are several possible reasons for the different results. For instance, the resistance genes may not get expressed because of different or changed genetic environment. Further investigation is needed why the presence of *blaOXA-72* does not produce β -lactam resistance.

MLST is an unambiguous and utility technique that identifies accurate and portable nucleotide sequences of internal fragments from multiple loci housekeeping genes and was used to assess the genetic background of populations in an ocean of bacteria (Maiden et al., 1998). Based on our existing epidemiological data and the eBURST arithmetic, it was shown that CC92 was widespread and this major clone was resistant to carbapenem antibiotics in our hospital. Ruan et al. (2013) explained that CC92 is a widespread variant that has advantages with respect to acquiring resistance determinants and surviving in the nosocomial environment, which renders it preferentially selected under antibiotic pressure. This may be the primary reason for the current epidemiological situation of CC92 in our study. In addition, 82.35% of CRAB from the five predominant STs in CC92 carried the *blaOXA-23* gene. We propose that the *blaOXA-23* gene is tied to the genetic background of CC92, representing an important setting in which to study the emergence of carbapenem resistance. A novel SLVs of CC92, ST843 was identified in three isolates, and sharing six same alleles with ST92 and n1 (Figure 1A). We could infer that it may be one of the evolutions of *A. baumannii* to adapt nosocomial environment.

We used eBURST to analyze the distribution of the STs of CSAB, and found it was more dispersive than the distribution of CRAB (Figure 1). This may confirm that CSAB has a more diverse genetic background compared to CRAB. The high genetic diversity in CSAB might be attributed to the decreased survival rate of antibiotic treatment. None of them emerge more than once among 35 carbapenem-susceptible isolates from May 2012 to October 2013 except for 3 isolates belonging to CC92. These data also imply that CSAB may have a more diverse genetic source compared to the CRAB, and suggest CC92 is an evolution for *A. baumannii* to survive while antibiotics existed.

We also observed that a few *A. baumannii* isolates could acquire azide resistance after the bacteria were mixed and incubated with *E. coli* J53 together overnight. This can be totally expected since spontaneous mutation(s) conferring azide resistance could arise and be selected in the presence of azide, similar to those *in vitro* selections of drug-resistant mutants. This can be considered as a process of adaptation to a selection pressure. Indeed, mutations conferring resistance to azide have been reported in both *E. coli*

(Fortin et al., 1990) and *Acinetobacter* spp. (Elkeles et al., 1994).

The cryptic plasmid pAB52 from CRAB isolate no. 52 does not carry any carbapenem resistance genes. It matches best with pAC12 which was reported in 2012 in Malaysia (Gan et al., 2012), and the ST of this pAB52 CRAB is ST195 (1-3-3-2-2-96-3) in parallel with *A. baumannii* AC12. According to the results of pAB52 sequence blast in NCBI (date not shown), all plasmids carry the same genes with over 99.9% identity over the full length, and belong to the *A. baumannii* plasmid group (GenBank accession number CP008850, CP007535, CP007550, CP001183, CP007578, KJ586856, KJ477078, and CP006964). Thus, plasmid pAB52 is larger with an additional segment of TonB gene (Figure 2B), but these plasmids may have the same function. For example, the septicolysin-like protein encoded in these plasmids suggests that it may be involved in survival of bacteria in the lungs and blood (Acosta et al., 2011). The TA systems have been suggested to mediate bacterial persistence by generating slowly growing cells tolerant to antibiotics and environmental changes (Jurenaite et al., 2013). In particular, the pAB52-containing strain was isolated from the sputum of a 74-year-old male who lived in a rural district in western China and had not traveled to any areas before admission to the hospital's ICU. This individual was found to have acquired an XDR *A. baumannii* infection during his hospital stay and eventually, he died from respiratory failure. The observation suggests that probably plasmid-borne *A. baumannii* TA systems contribute for the evolution of antibiotic resistance in this opportunistic pathogen.

In summary, this investigation confirms that the CC92 producing *blaOXA-23* was the leading reason for the dramatic increase in the carbapenem resistance rates in China. Awareness of carbapenem-resistant organisms and their development in hospitals has crucial implications in optimizing infection control practices, establishing antimicrobial stewardship programs within the hospital, and finally establishing active regional surveillance systems (Zowawi et al., 2015).

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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.2015.00910>

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Wide distribution of carbapenem resistant *Acinetobacter baumannii* in burns patients in Iran

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Antimicrobial resistance in carbapenem non-susceptible *Acinetobacter baumannii* (CNSAb) is a major public health concern globally. This study determined the antibiotic resistance and molecular epidemiology of CNSAb isolates from a referral burn center in Tehran, Iran. Sixty-nine CNSAb isolates were tested for susceptibility to antimicrobial agents using the E test methodology. Multiple locus variable number tandem repeat analysis (MLVA), Multilocus sequence typing (MLST) and multiplex PCR were performed. PCR assays tested for ambler classes A, B, and D β -lactamases. Detection of ISAb1, characterization of integrons, and biofilm formation were investigated. Fifty-three (77%) isolates revealed XDR phenotypes. High prevalence of *bla*_{OXA-23}-like (88%) and *bla*_{PER-1} (54%) were detected. ISAb1 was detected upstream of *bla*_{ADC}, *bla*_{OXA-23}-like and *bla*_{OXA51}-like genes in 97, 42, and 26% of isolates, respectively. Thirty-one (45%) isolates were assigned to international clone (IC) variants. MLVA identified 56 distinct types with six clusters and 53 singleton genotypes. Forty previously known MLST sequence types forming 5 clonal complexes were identified. The Class 1 integron (class 1 integrons) gene was identified in 84% of the isolates. The most prevalent (33%) cassette combination was *aacA4-catB8-aadA1*. The IC variants were predominant in the *A. baumannii* lineage with the ability to form strong biofilms. The XDR-CNSAb from burned patients in Iran is resistant to various antimicrobials, including tigecycline. This study shows wide genetic diversity in CNSAb. Integrating the new Iranian *A. baumannii* IC variants into the epidemiologic clonal and susceptibility profile databases can help effective global control measures against the XDR-CNSAb pandemic.

Keywords: antimicrobial resistance, MLST, MLVA, international clone, integron

INTRODUCTION

Acinetobacter baumannii is an important healthcare-associated pathogen that can cause of life threatening infections (Öncül et al., 2014; Girerd-Genessay et al., 2015). *A. baumannii* is the second most common multidrug-resistant cause of nosocomial infection in burn patients in Iran (Alaghehbandan et al., 2012; Azimi et al., 2015). Its infections have become critical challenge to

health care systems due to increasing levels of resistance to antimicrobial agents in nosocomial isolates of *A. baumannii* (Cai et al., 2012; Moradi et al., 2015). In developing countries, including Iran, the occurrence of carbapenem non-susceptible *A. baumannii* (CNSAb) infections is a growing problem in hospitalized burn patients (Mahdian et al., 2015; Zowawi et al., 2015). Inadequate management of the antibiotic therapy of CNSAb infections often leads to the emergence of extensive and pandrug resistant (XDR and PDR) CNSAb strains, which present significant health challenges by prolonging hospitalization, treatment failures, and increased mortality (Liu et al., 2014b). The most important mechanism of carbapenem resistance in *A. baumannii* is the production of carbapenem-hydrolyzing β -lactamases of Ambler classes A, B, and D. In addition to being resistant to all β -lactams available, carbapenemases have a high capacity to spread, since their genes have been commonly found in transferable plasmids containing integrons and insertion sequence (IS) elements (Karah et al., 2012).

Worldwide surveillance has shown that the global population structure of CNSAb isolates is diverse, but a small number of widespread clones, including the international clonal (IC) lineage I–III, also defined based on multilocus sequence typing (MLST) as clonal complexes (CC), may be predominant in healthcare settings (Jeannot et al., 2014). Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has been considered as a rapid and cost-effective method for fine-scale typing of *A. baumannii*, enabling inter-laboratory comparison of typing data (Karah et al., 2012). A recent study found the high discriminatory power of MLVA, sufficient to detect diversity among isolates showing identical MLST types (Almeida and Araujo, 2013).

Up-to-date surveillance information regarding genotypic spread, plus local antimicrobial susceptibility patterns of clinical isolates of *A. baumannii*, are necessary for effective drug therapy and control of XDR and PDR *A. baumannii* hospital outbreaks (Fishbain and Peleg, 2010). Considering the points mentioned above, the aim of this study was to determine molecular epidemiology, to identify the dissemination of the most common resistance genes, to investigate the prevalence of integrons and arrangement of integron gene cassettes among CNSAb isolates from a referral burn center in Tehran, Iran.

MATERIALS AND METHODS

Bacterial Isolates

A total of 92 non-repetitive *A. baumannii* strains were collected between January 2012 to May 2013, from the burn wound infections of hospitalized patients in Shahid Motahari hospital, the only referral burn center in Tehran, Iran. Species of these isolates were initially characterized using the API20NE system (bioMérieux, Marcy-l'Etoile, France) and then final identification of isolates were performed by multiplex PCR using *gyrB*-directed primers according to the study of Higgins et al. (2010b).

Antimicrobial Susceptibility Tests and Biofilm Formation Assay

The Clinical and Laboratory Standards Institute (CLSI) guideline (Clinical and Laboratory Standards Institute [CLSI], 2014) for minimum inhibitory concentrations (MICs) using the E test was used to assess the susceptibility of 92 *A. baumannii* isolates to imipenem (Ezy MICTM strips, Himedia, India). All isolates with MICs of imipenem >4 mg/L were defined as CNSAb isolates and were recruited for further screening (Clinical and Laboratory Standards Institute [CLSI], 2014). The susceptibility of CNSAb isolates to 17 other antimicrobial agents such as amikacin, ampicillin-sulbactam, cefepime, ceftazidime, ciprofloxacin, colistin, gentamicin, levofloxacin, meropenem, minocycline, piperacillin, piperacillin-tazobactam, rifampicin, tetracycline, tigecycline, tobramycin, and trimethoprim-sulfamethoxazole were also carried out using the E test (Ezy MICTM strips, Himedia, India). The phenotypic presence of Metallo- β -lactamase (MBL) enzymes was detected by imipenem/imipenem + EDTA strips (Ezy MICTM strips, Himedia, India). The MIC ratio of MBL-E test of ≥ 8 mg/L was interpreted as indicative of MBL activity, according to the manufacturer's instructions. Since there is no breakpoint for tigecycline and rifampicin against *A. baumannii* strains in the CLSI guidelines; therefore, the criteria for interpretation of the MIC values of tigecycline (MIC of ≤ 1 mg/L defined as susceptible and >2 mg/L as resistant) were determined according to the European committee on antimicrobial susceptibility testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing, 2014) for members of the *Enterobacteriaceae* sp. and CLSI criteria, using breakpoint values suggested for *Staphylococcus aureus* applied to rifampicin (susceptible defined as ≤ 1 mg/L and resistant defined as ≥ 4 mg/L). The MIC₅₀ and MIC₉₀ of each antibiotic were calculated. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* ATCC 35218 were used as quality control organisms. The phenotype of *A. baumannii* is defined as MDR and XDR according to the International Expert proposal for Interim Standards Guidelines (Magiorakos et al., 2012). The formation assay and quantitative analysis of biofilm were performed according to a previous study (Zhang et al., 2014).

Molecular Detection of β -lactamases-encoding Genes and Characterization of Integrons

A series of PCR amplifications were done for detection of different Ambler class *bla* gene groups: class A; *bla*_{PER-1}, *bla*_{VEB-1}, *bla*_{CTX-M}, *bla*_{KPC}, *bla*_{GES}, *bla*_{TEM} and *bla*_{SHV-1} (Poirel et al., 2003; Ellington et al., 2007; Bonnin et al., 2011a,b; Potron et al., 2011; Robledo et al., 2011; Climaco et al., 2013); Class B, *bla*_{IMP-1}, *bla*_{VIM-2}, *bla*_{GIM-1}, *bla*_{SPM-1} *bla*_{SIM-1} and *bla*_{NDM-1} (Chen et al., 2011); Class C, *bla*_{ADC} (Bou and Martinez-Beltran, 2000) and Class D, *bla*_{OXA-23}, *bla*_{OXA-40}, *bla*_{OXA-51}, *bla*_{OXA-58} (Woodford et al., 2006) and *bla*_{OXA-143}-like (Higgins et al., 2010a) encoding genes were detected. To determine whether IS*Aba*1 was present upstream of

*bla*_{OXA-23}-like, *bla*_{OXA-51}-like (Turton et al., 2006) and *bla*_{ADC} (Heritier et al., 2006) genes, PCR mapping experiments were performed. Detection and characterization of class 1, 2, and 3 integrons, as well as gene cassette mapping and sequencing of class 1 integrons were carried out using previously described PCR assay (Dillon et al., 2005).

Genetic Diversity and Population Structure

The IC lineage so-called PCR-based group were identified using three-locus dual assay multiplex PCR as described previously (Turton et al., 2007). Isolates pertaining to the novel variant of the PCR-based group, according to the new combination of amplified products obtained from the two separate multiplex PCRs, did not correspond to those previously defined. Moreover, the CNSAb isolates were genotyped using the MLVA-8 scheme method developed by Pourcel et al. (2011). For clustering analysis, the allele strings were entered into the BioNumerics software v.7.0 (Applied Maths, Sint-Martens-Latem, Belgium) as character values. A cut-off value of 90% similarity was applied to define clusters (Nhu et al., 2014). MLST was performed according to the Bartual method described previously (Bartual et al., 2005). Allele sequences, sequence types (STs), primer sequences and other details are available from the MLST website at <http://pubmlst.org>. The minimum spanning tree (MSTree) algorithm was used to predict the clonal relationship between all CNSAb isolates. MSTree algorithm was constructed with the BioNumerics software v.7.0 (Applied Maths, Sint-Martens-Latem, Belgium) using a categorical coefficient according to a previous report (Ruan et al., 2013).

Statistical Analysis

Statistical analyses were performed by Student's *t*-test, Fisher's exact test and the chi-square test with the SPSS software package (version 16). *P*-values <0.05 in all experiments were considered as significant.

RESULTS

Antibiotic Susceptibility Testing and Biofilm Assay

In this study, from the 92 *A. baumannii* isolates, 69 isolates were confirmed as CNSAb. The MIC determination of all CNSAb isolates, exhibited non-susceptibility rates ≥95% to cefepime, ceftazidime, levofloxacin, meropenem, piperacillin, piperacilllin-tazobactam, rifampicin and trimethoprim-sulfamethoxazole (Table 1). CNSAb isolates showed high susceptibility to colistin (100%), tigecycline (87%) and minocycline (69%) with MIC₅₀ values of 0.01, 0.25, and 5 mg/L, respectively and MIC₉₀ values of 1, 3, and 10 mg/L, respectively (Table 1). Fifty-three (77%) CNSAb isolates revealed XDR phenotypes. In all XDR isolates following colistin with 100% sensitivity, tigecycline (83%) and minocycline (67%) were the most effective antibiotics. Fifty-six (81%) of all CNSAb isolates formed strong biofilm whereas 13 (19%) of these isolates were considered as weak biofilm forming

strains. Our study revealed a significant association of strong biofilm formation with antimicrobial resistance (*P* = 0.0001).

Detection of Antibiotic Resistance Determinants

Phenotypic detection of MBL showed that none of the isolates were positive for MBL. The presence of *bla*_{OXA51}-like and *bla*_{ADC} were confirmed in all CNSAb isolates. The high prevalence of *bla*_{OXA-23}-like (53; 77%) and *bla*_{PER-1} (37; 54%) were seen in CNSAb isolates. Co-existence of *bla*_{OXA-23}-like/*bla*_{OXA-40}-like and *bla*_{OXA-23}-like/*bla*_{OXA-58}-like genes were detected in 6 (9%) and 2 (3%) of the isolates, respectively. The PCR results were negative for the other β-lactamase encoding genes including *bla*_{OXA-143}-like, *bla* MBL (*bla*_{IMP-1}, *bla*_{VIM-2}, *bla*_{GIM-1}, *bla*_{SPM-1} *bla*_{SIM-1} and *bla*_{NDM-1}), *bla*_{CTX-M}, *bla*_{KPC}, *bla*_{GES} *bla*_{TEM} and *bla*_{SHV-1}. ISAb1 was detected upstream of *bla*_{ADC}, *bla*_{OXA-23}-like, and *bla*_{OXA51}-like genes in 67 (97%), 29(42%), and 18 (26%) of CNSAb isolates, respectively (Figure 1). A significant correlation was observed between the presence of ISAb1 in upstream of *bla*_{OXA-23}-like and increasing MIC of imipenem (*MIC* ≥ 16 mg/L) (*P* = 0.042). In the presence of ISAb1 in upstream of *bla*_{OXA-51}-like and increasing imipenem MICs, there was no significant correlation (*P* = 0.347).

Class 1 integron gene cassettes were identified in 58 (84%) CNSAb isolates while class 2 and 3 integrons were not found. Six different cassette combinations were detected within the

TABLE 1 | The minimum inhibitory concentration (MIC) distribution of 18 antimicrobial agents for the 69 CNSAb isolates as determined by E test.

Antimicrobial agents ^a	MIC (μg/mL)			Non-susceptible (%)
	Range	MIC ₅₀	MIC ₉₀	
CST	0.016 to 2	0.01	1	0
IPM	≤ 8 to ≥ 128	12	32	100
MEM	≤ 8 to ≥ 256	24	48	97
PIP	5 to ≥ 240	≥240	≥240	99
TZP	0.01 to ≥ 240	120	≥240	96
SAM	2/1 to ≥ 256/128	32/16	≥256/128	65
CAZ	4 to ≥ 256	128	≥256	96
FEP	4 to ≥ 256	128	≥256	97
CIP	0.1 to ≥ 240	30	60	95
LVX	0.5 to ≥ 240	10	60	95
TET	1 to 120	5	30	39
MIN	0.1 to 60	5	10	31
TGC	0.023 to 32	0.25	3	13
TOB	1 to ≥ 240	30	120	66
GEN	2 to ≥ 240	30	120	93
AMK	8 to ≥ 256	64	256	86
SXT	5 to ≥ 240	30	60	99
RIF	0.1 to 120	10	30	97

^aAMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colisitin; FEP, cefepime; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TOB, tobramycin; TZP, piperacillin-tazobactam.

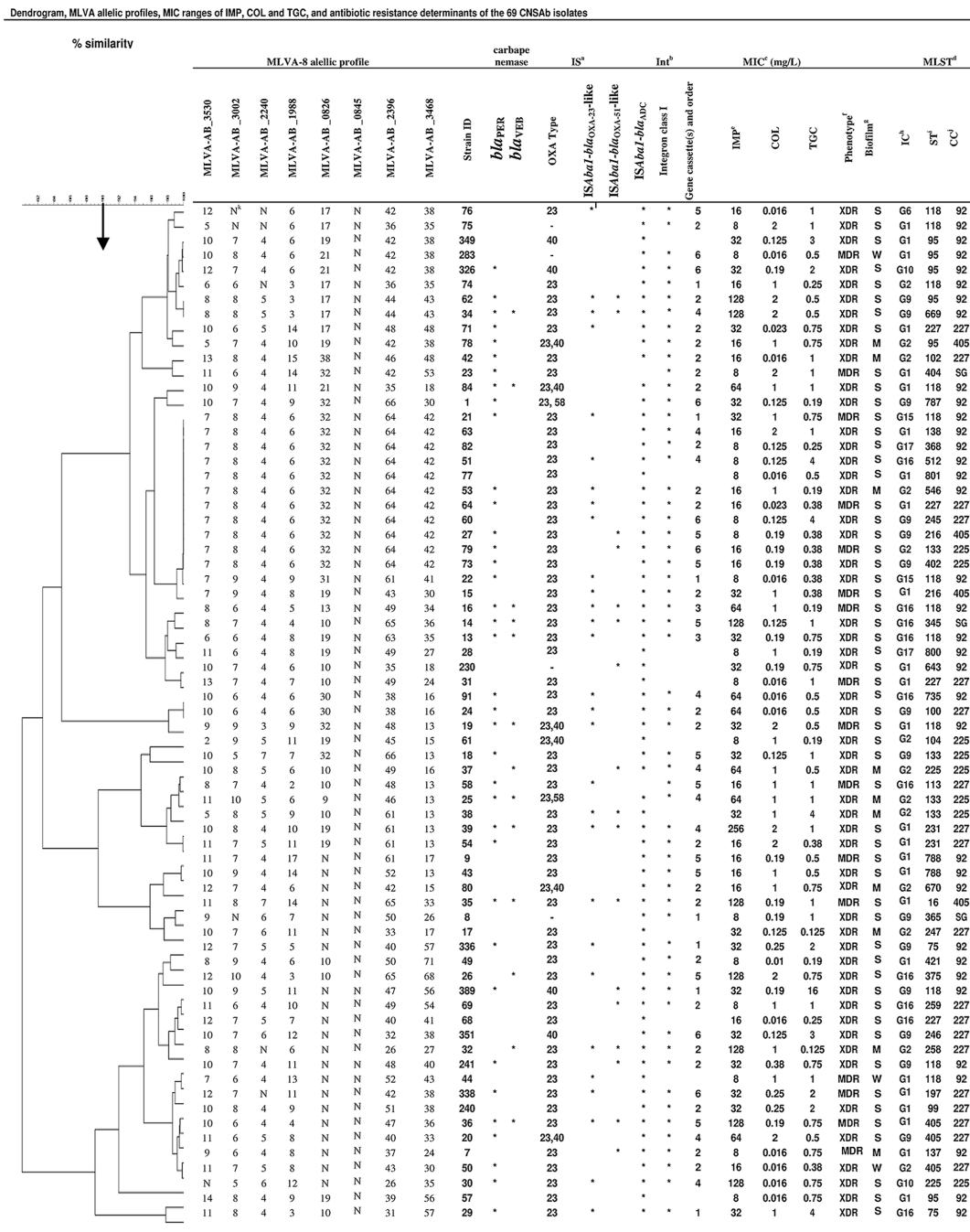
^aIS, insertion sequences^bInt, Integrons^cMIC, minimal inhibitory concentration^dMLST, multilocus sequence typing^eIPM, imipenem; CST,colistin; TGC,tigecycline^fMDR, multidrug-resistant; XDR, extensively drug-resistant^gBiofilm formation: S,strong; M, moderate, W, weak^hIC, international clonal lineageⁱST, sequence type^jCC, clonal complex^kN, not amplified^{l*}, sign of positive test

FIGURE 1 | Dendrogram shows the genetic diversity of 69 carbapenem non-susceptible *Acinetobacter baumannii* isolates by MLVA and MLST; carbapenemase encoding genes; MIC ranges of colisitin, imipenem, and tigecycline; resistance phenotype; biofilm formation and international clonal lineage.

class 1 integrons. The most prevalent cassette combination, *aacA4-cattB8-aadA1* (2.3 Kb), were detected in 23 (39%) of class 1 integrons. The other cassette combinations including *arr3-aacA4* (1.3 Kb), *aacC1-orfX-orfX'-aadA1* (2.5 Kb), *orfI-aadA1* (1.5 Kb), *dfrVII* (0.7 Kb) and *dfrXII-orfF-aadA2* (1.7 Kb) were detected in 10 (14.5%), 9 (13%), 7 (10%), 7 (10%), and 2 (3%) CNSAb isolates, respectively. The gene cassette arrangement showed that the class 1 integrons harbor genes encoding resistance to aminoglycosides, chloramphenicol, rifampicin and trimethoprim/sulfamethoxazole. Also, the results of this study show that integron-associated β -lactamases encoding genes were not detected in class 1 integrons-harboring CNSAb isolates.

Genetic Diversity and Population Structure

Three-locus dual assay multiplex PCR showed eight different PCR-based groups (G1, G2, G6, G9, G10, and G15–G17) among the 69 CNSAb isolates. Twenty-five (36%) and 13 (19%) CNSAb isolates belonged to G1 (IC II) and G2 (IC I), respectively. Based on the variations in combination of PCR amplicons of the *blaOXA-51*-like, *csuE* and *ompA* genes, 31 (45%) of the CNSAb isolates belonged to 6 IC variants PCR-based groups (**Table 2**). G9 (14; 45%) was the most common IC variant (**Table 2**).

The MSTree algorithm of the MLST data of this study suggests that 40 previously known STs combined into four CC (**Figure 2**). Thirty-six (52%) and 13 (19%) of all 69 CNSAb isolates belonging to CC-92 and CC-405, respectively. According to the MSTree algorithm, only one singleton ST (ST345) was identified (**Figure 2**). ST118 was the predominant ST, comprising 17.3% of isolates.

By the MLVA typing method, the 69 CNSAb isolates were grouped into 56 distinct MLVA types with 6 clusters and 53 singleton genotypes. Loci MLVA-AB_0845 was absent (N) in all studied isolates. In this study, the VNTR loci MLVA-AB_3002 and MLVA-AB_2240 displayed lower diversity, whereas VNTR loci MLVA-AB_2396 and MLVA-AB_3468 showed higher level of diversity.

Thirty STs corresponded to 30 MLVA types; other STs (118, 95, 75, 227, 405, 231, 788, 225, 227, and 133) included heterogeneous MLVA profiles (**Figure 1**).

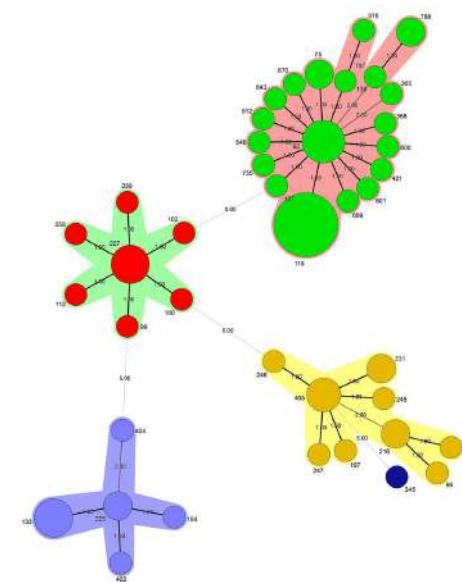


FIGURE 2 | The minimum spanning tree (MSTree) was constructed using a categorical coefficient based on multilocus sequence typing (MLST) data. Each circle stands for a different sequence type (ST). The color of a circle and the line clustering the ST with the same color represents an identical ST. Size of the circle corresponds to the number of isolates. The width of the line reflects the genetic distance between ST (heavy and thin lines represent a difference in one allele and two or more alleles, respectively).

In this study, IC variants were classified into 23 STs belonging to CC92, CC405, CC227, CC225 and one singleton (**Figure 1**).

Relationship between IC Lineages with Antibiotic Resistance Determinants

Table 3 shows the frequency of antibiotics resistance in two major epidemic lineages and IC variants. Susceptibility to all antimicrobial agents, except tobramycin, in isolates belonged to IC II was higher than the IC I and IC variants (**Table 3**). It is noteworthy that the IC variants had the highest resistance rate to tigecycline (7; 22.5%) with MIC₅₀ and MIC₉₀ of 0.25 and 3 mg/L, respectively (**Tables 1** and **3**). Among the 13 carbapenem

TABLE 2 | Combinations of amplicons obtained in the dual multiplex PCRs used to describe 31 *A. baumannii* IC variants.

Variant type (No.)	PCR group 1			PCR group 2		
	<i>csuE</i> 702 bp	<i>blaOXA-51</i> -like 559 bp	<i>ompA</i> 355 bp	<i>csuE</i> 580 bp	<i>ompA</i> 343 bp	<i>blaOXA-51</i> -like 162 bp
G 6 ^a (1)	–	–	–	+	–	–
G 9 (14)	–	–	+	+	–	+
G 10 (2)	–	+	–	+	+	–
G 15 (2)	+	–	–	–	–	+
G 16 (10)	–	+	–	+	–	–
G 17 (2)	+	–	+	–	–	+

^aG; PCR-based group.

TABLE 3 | Frequency of antimicrobial non-susceptibility in three major epidemic lineages in the 69 CNSAb isolates.

IC ^b (No.)	% Non-susceptibility to CLSI antimicrobial groups ^a																	
	A									B								
	IPM	MEM	CAZ	SAM	AMK	TOB	GEN	CIP	PIP	TZP	FEP	MIN	TET	TGC	LVX	SXT	CST	RIF
IC1 (13)	100	100	100	85	92	62	92	100	100	100	100	48	46	9	100	100	0	100
IC2 (25)	100	92	88	48	84	76	88	88	96	92	96	19	32	5	88	96	0	92
V (31)	100	100	100	61	84	61	100	97	100	97	100	25	39	24	97	100	0	97
Total (69)	100	97	96	65	86	66	93	95	99	96	97	31	39	13	95	99	0	97

^aConsiderations in the assignment of agents to Groups A, B, and C include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA clinical indications for usage, and current consensus recommendations for first-choice and alternative drugs. **Group A** are considered appropriate for inclusion in a routine, primary testing panel, as well as for routine reporting of results for the specific organism. **Group B** comprises agents that may warrant primary testing. **Group O (Other)** includes agents that have a clinical indication for the organism. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used for quality control of antimicrobial susceptibility testing and included in each run.

^bIC, international clonal lineage; V, IC variants; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colisitin; FEP, ceferipime; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TOB, tobramycin; TZP, piperacillin-tazobactam.

non-susceptible IC I, 12 (92%) were XDR while this rate was 14 (56%) and 27 (87%) in the isolates belonging to the IC variants and IC II, respectively. The resistance to beta lactam antibiotics in IC I and IC variant isolates was higher than that in IC II. Thirty-one (100%) IC variants and 22 (88%) of the IC II population formed strong biofilm but only 3 (23%) of IC I had this characteristic. In the present study, resistance to antimicrobials was comparatively higher among IC variant than IC I and IC II CNSAb isolates ($P = 0.0012$ and $P = 0.0001$, respectively). A slightly higher susceptibility to antimicrobials was observed in IC II than IC variants and IC I ($P > 0.05$). Our analysis revealed a significant correlation ($P = 0.003$) between the presence of *bla*_{PER1} and biofilm formation in the CNSAb isolates. The CNSAb isolates belonging to all clones had the *bla*_{OXA-23}-like gene. All CNSAb isolates classified in IC I, were positive for the *bla*_{OXA-23}-like gene (Table 4). According to Table 4, the frequency of *ISAbal*/*bla*_{OXA-23}-like and *bla*_{PER-1} genes in a population belonging to IC variants were significantly higher than IC I and IC II ($P = 0.027$ and $P = 0.003$, respectively). Table 4 shows that the distribution of different types of class 1 integrons is related to major epidemic lineages with predominance of class 1 integrons belonging to type 2.

DISCUSSION

Nosocomial infections caused by CNSAb are currently among the most difficult to treat. The control of CNSAb infections among hospitalized patients continues to present serious challenges to patient management in developing countries like Iran (Diancourt et al., 2010; Giannouli et al., 2010; Bahador et al., 2013). The main challenge remains choosing the most likely effective antibiotics as determined by *in vitro* testing of antibiotics, based on the local susceptibility patterns (Fishbain and Peleg, 2010). Overall, the findings of this study are consistent with recent studies from Iran that show an alarming trend of CNSAb increase and in MDR *A. baumannii* resistance against a wide spectrum of antimicrobial agents (Moradi et al., 2015). However, in the present study

colistin, tigecycline, and tetracyclines exhibited a potent activity against CNSAb isolates. Our data revealed a decrease (10%) of resistance in CNSAb isolates to tigecycline (Bahador et al., 2013), likely due to the implementation of restriction policy on the empirical prescription of tigecycline. Decrease in antibiotic resistance after implementation of restriction policies have been shown in several reports (Altunsoy et al., 2011; Sistanizad et al., 2013). Our finding of positive association in strong biofilm formation and resistance to antimicrobial is consistent with a recent report (Badave and Kulkarni, 2015). This could be due to inadequate penetration of the antimicrobials into the biofilms, and the emergence of persister cells in the biofilms (Penesyan et al., 2015).

The findings of our study on *ISAbal* elements upstream of *bla*_{OXA} genes and their role as a promoter to enhance OXA-enzymes expression and MIC of imipenem confirms previous reports (Liu et al., 2014a); however, this finding are in contrast to a recent report by Peymani et al. (2012b) from Iran. Not only did our research find that 42% of CNSAb isolates were the *ISAbal*-*bla*_{OXA-23}-like gene; but also, a significant correlation was observed between the presence of *ISAbal* upstream of *bla*_{OXA-23}-like gene and increasing imipenem MICs (≥ 16 mg/L). This dissimilarity might be due to differences in the *A. baumannii* strains available for study. Additionally, our finding of high prevalence of *ISAbal* sequences upstream of the *bla*_{ADC} among 97% of CNSAb isolates is consistent with previous report from Iran; and indicate that presence of *ISAbal* sequences upstream of *bla*_{ADC} played an important role in increasing resistance to cephalosporins ($MIC_{50} = 128$ mg/L).

However, IC II is the most common strain in the world (Liu et al., 2014a); and majority of the clinical strains (45%) in this study belong to IC variants. The incidence rate of IC variants observed in this study is similar to previously reported rates of 46% from Sweden and 51% from Iran, and is considerably higher than that in other recent reports, including Iran (3%), Latvia (6%), Romania (15%), Italy (23%) and Norway (28%) (Fishbain and Peleg, 2010; Hojabri et al., 2014; Liu et al., 2014a). With regard to a previous study (Fishbain and Peleg, 2010) and the

TABLE 4 | Frequency of antimicrobials resistance determinants in epidemic clonal lineages of the CNSAb isolates.

IC ^a (No.)	Amber classification No. (%)	Class 1 integron types ^b No. (%)					
		Class A	Class D	Co-existence of Class D	IS _{ABA} upstream beta-lactamase encoding genes No. (%)	Class 1 integron No. (%)	Class 1 integron types ^b No. (%)
bla _{PER}							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	3 (23)	1 (8)	9 (69)
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	1 (4)	0	7 (28)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	1 (3)	19 (76)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	2 (3)	30 (97)
bla _{VEB}							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	4 (31)	5 (39)
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	7 (28)	24 (96)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	18 (58)	6 (19)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	29 (42)	58 (84)
bla _{OXA} -40-like							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	—	—
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	1 (4)	8 (32)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	6 (19)	9 (29)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	7 (10)	23 (33)
bla _{OXA} -23-58-like							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	—	—
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	1 (4)	3 (12)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	6 (19)	5 (16)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	7 (10)	10 (14)
bla _{OXA} -23-like							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	—	—
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	1 (4)	3 (12)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	6 (19)	5 (16)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	7 (10)	10 (14)
bla _{OXA} -51-like							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	—	—
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	1 (4)	3 (12)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	6 (19)	5 (16)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	7 (10)	10 (14)
IS _{ABA} -bla _{ADD}							
IC-I (13)	4 (31)	3 (23)	13 (100)	2 (15)	1 (8)	—	—
IC-II (25)	10 (40)	4 (16)	17 (68)	2 (8)	0	1 (4)	3 (12)
V (31)	24 (77)	6 (19)	24 (77)	5 (16)	2 (6)	6 (19)	5 (16)
Total (69)	48 (69)	13 (19)	54 (78)	9 (13)	6 (9)	7 (10)	10 (14)

^aAbbreviations: IC, international clone; V, variants of IC.
^bType 1, orf-aadA1; Type 2, aacA4-catE8-aadA1; Type 3, dfrXII-orfX-aadA2; Type 4, aacC1-orfX-orfX'-aadA1; Type 5, aacC1-orfX'-orfX-aadA1; Type 6, dfrVII.

study of Karah et al. (2012), 14 CNSAb isolates showed a new combination of amplicons and were considered in this article as the PCR-based group (G) 15–17. Three of the 6 PCR-based groups in the present study, namely G6, G9, and G10, have been documented in previous studies (Karah et al., 2012; Bahador et al., 2014). Although there are differences among the IC variant types in this study and previous studies in Iran (Peymani et al., 2012b; Rezaee et al., 2013; Hojabri et al., 2014), the IC variants are highly resistant to antibiotics, which suggests that it is conceivable that the MDR phenotype has substantially contributed to their spread in Iran (Bahador et al., 2014).

The results of this study indicate that, in spite of the high rate of antibiotic resistance determinants in the IC I stains in comparison with the IC variant strains, the IC I strains had lower prevalence. This dissimilarity might be due to the higher frequency of strong biofilm formers in the IC variant strains than in the IC I strains. Several studies proved that, cell adhesiveness and ability to form biofilm were higher in the bla_{PER-1} producing isolates (Longo et al., 2014), and this is in agreement with the result of this study. The capability to form biofilm and presence of the bla_{PER} gene were significantly higher in IC variants. In this study, the IC II had slightly higher susceptibility to antimicrobial agents than other clonal lineage population. This might be due to low-frequency of carbapenem-hydrolyzing class D β-lactamase (CHDL) genes and IS_{ABA}1 elements, as well as low ability of biofilm formation in IC II strain as compared to IC I and IC variant stains.

It has been clearly shown that, the high prevalence of class 1 integrons among MDR *A. baumannii* clinical isolates is responsible for several nosocomial outbreaks globally (Liu et al., 2014b). The resistance rates to all the tested antibiotics, except colistin, tigecycline, minocycline, and tetracycline, of the collected CNSAb isolates were >65%. However, the presence of integron cassette arrays cannot be responsible for all these resistance phenotypes. In Iran, class 1 integrons have been reported previously in >11% of *A. baumannii* isolates (Peymani et al., 2012a; Japoni-Nejad et al., 2013). In the current study, 84% of isolates contain class 1 integrons, but no class 2 and class 3 integrons was detected. This result shows that class 2 and class 3 integrons are not the major resistant determinants in CNSAb isolates in this study, which is consistent with previous studies (Peymani et al., 2012a; Japoni-Nejad et al., 2013).

Only six categories of gene cassettes, including aminoglycoside-modifying enzymes, chloramphenicol-resistant, trimethoprim/sulfamethoxazole-resistant, and rifampicin-resistant were detected in spite of previous studies reporting integrons carrying carbapenemase genes (Japoni-Nejad et al., 2013). This result indicates that acquisitions of carbapenem-hydrolyzing β-lactamase genes plays more important role than the class 1 integrons in carbapenem resistance in CNSAb. The class 1 integrons rate (84%) here defined for a referral burn center in central Iran is higher than the rates found in other geographical regions, including Western Iran (11%) (Salimizand et al., 2014), China (74%) (Wu et al., 2012) and European countries in general (43%) (Castanheira et al., 2014). This result is similar to that of previous studies from the Northwest and center of Iran (Peymani et al., 2012a; Japoni-Nejad et al., 2013).

From literature survey, this is the first study to report carriage of class 1 integrons and associated arrays in *A. baumannii* isolates from burn patients in Iran.

Analysis of the integron cassette arrays shows that most class 1 integrons include the globally distributed *aacA4-catB8-aadA1* array (Liu et al., 2014a), and the cassette array *aacC1-orfX-orfX'-aadA1* has been documented in previous studies in Iran (Japoni-Nejad et al., 2013). Moreover, gene cassettes encoding aminoglycoside-resistance are present in the majority of CNSAb isolates in this study, suggesting a close relationship between high-level resistance rate to aminoglycoside compounds such as gentamicin and amikacin and the *A. baumannii* isolates. The different types of gene cassette array in this study and previous studies in Iran (Japoni-Nejad et al., 2013; Salimizand et al., 2014), suggests that a geographical feature plays a major role in MDR isolates formation, and it could be due to different volumes and patterns of antibiotic consumption in distinct areas. In addition, the results of this study indicate that strains indistinguishable from each other by MLST and MLVA typing can have different cassette arrays, indicating that some of these may be transmitted very efficiently. In this study, amikacin resistance recorded in 10% of the CNSAb isolates was not cassette-encoded. Analysis of the integron cassette arrays show that 50% of these isolates harbor an integron without aminoglycoside resistance genes. In the cases where the class 1 integrons could not be amplified, there were no integrons and alteration of the primer binding site or a large size of the Integron are the most likely explanations for the negative result of PCRs.

According to the findings of this research, MLVA could identify closely epidemiologically related isolates clustered by MLST in some cases (**Figure 1**). In this study, isolates with the same ST, such as ST118, have closely related MLVA type (MTs) (**Figure 1**). This result suggests that the evaluation of MLVA in CNSAb isolates shows its great ability for discrimination of genetically closely related isolates.

In agreement with previous studies (Hojabri et al., 2014), the present results substantiated that CC92 represented the recent widely distributed clone in Iran, accounting for 52.2% of CNSAb isolates examined in this study. This most likely indicates that the isolates belonging to CC92 might benefit with respect to acquiring resistance determinants and surviving in the health care environment, when encountering selection by antibiotics such as carbapenems. The international spread of the CC92 isolates has been reported from all around the world (Karah et al., 2012). This study revealed that the 28 isolates belonging to this lineage were shown to be heterogeneous by MLVA (22 MTs), followed by MLST (13 STs). The 69 CNSAb isolates were shown to be heterogeneous by MLVA (56 MTs) and MLST (40 STs). Taken together, genetic diversity and wide dissemination clones

among CNSAb isolates is consistent with recent studies from Iran that show high diversity of STs in *A. baumannii* isolates from burned patients (Hojabri et al., 2014), which are the evidence of dynamic population structure and evolution in progress. The referral of patients from other medical centers of Iran to the hospital in Tehran could be among the possible reasons for this diversity and failure to colonize a specific type. Spread of specific clonally related isolates could have been prevented by the success of infection control programs in the teaching hospital in Iran.

CONCLUSION

Evidence that XDR-CNSAb from burned patients in Iran is rapidly changing toward growing resistance to various antimicrobials, including tigecycline was presented. Despite the increasing resistance to several first-line antimicrobials, all resistant CNSAb isolates remain sensitive to the antimicrobial colistin, a viable agent in controlling XDR-CNSAb outbreaks, especially in developing countries. This is the first study to perform IC analysis and gene cassette mapping of class integrons of *A. baumannii*, obtained from burned patients in Iran. It can be confirmed that the dominant local IC is an IC variant, with the likelihood that G9 is the predominant member. In the current study, the *aacA4-catB8-aadA1* gene cassette array which confers resistance to aminoglycoside and chloramphenicol was identified as the predominant cassette array of class 1 integrons in CNSAb. This study described the widest distribution of *bla_{OXA-23}*-like-horbering IC ST118 (CC92) and ST405 (CC405) were the principal reason for the rapid increase in the carbapenem resistance rate in burned patients in Iran. The findings highlight the importance of an international drug resistance and molecular epidemiology monitoring network of *A. baumannii* isolates to effect global control measures against XDR-CNSAb.

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Dissimilar Fitness Associated with Resistance to Fluoroquinolones Influences Clonal Dynamics of Various Multiresistant Bacteria

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Fitness cost associated with resistance to fluoroquinolones was recently shown to vary across clones of methicillin-resistant *Staphylococcus aureus* and extended-spectrum β-lactamase-producing *Klebsiella pneumoniae*. The resulting dissimilar fitness should have influenced the clonal dynamics and thereby the rates of resistance for these pathogens. Moreover, a similar mechanism was recently proposed for the emergence of the H30 and H30R lineages of ESBL-producing *E. coli* and the major international clone (ribotype 027) of *Clostridium difficile*. Furthermore, several additional international clones of various multiresistant bacteria are suspect to have been selected by an analogous process. An ability to develop favorable mutations in the gyrase and topoisomerase IV genes seems to be a prerequisite for pathogens to retain fitness while showing high-level resistance to fluoroquinolones. Since, the consumption of other “non-fluoroquinolone” groups of antibiotics have also contributed to the rise in resistance rates a more judicious use of antibiotics in general and of fluoroquinolones in particular could ameliorate the international resistance situation.

Keywords: fluoroquinolones, multiresistant, fitness, clone, incidence

INTRODUCTION

Though, clonal spread has always been a hallmark of many serious pathogens it is striking and remains enigmatic why the clonal spectra of several multiresistant bacteria have undergone a reduction at some point during the last three decades. We have witnessed a worldwide clonal shrink among others in methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* and *Clostridium difficile*. The question arises what sort of driving force(s) could have reshaped the clonal landscape?

It is well-established that some clones of MRSA have for some time been on the advance replacing others and disseminating in novel geographic areas. Among the foraging MRSA clones ST22 (EMRSA-15) and CC5 proved particularly adept. In Europe where solid, up-to-date information on the clonal distribution of MRSA is available they are well-established to have become the dominant clones at the expense of multiple others (Grundmann et al., 2014). In addition, the ST22 clone has lately proved the most common sequence type in the healthcare setting in Australia (Coombs et al., 2014), and Singapore (Hsu et al., 2015). CC5 was reported the most widespread MRSA clone in the healthcare setting in Africa (Abdulgader et al., 2015) and it is dominant or on the rise in several Asian countries (Chen and Huang, 2014). CC5 has also remained the second most common clone in invasive infections in the United States though a novel

antibiotic resistant variant of the CC8 clone (USA300) has recently emerged as the most frequent type of MRSA in blood samples (Tenover et al., 2011). In contrast various long prevalent MRSA clones, especially ST30 and ST239, have been losing ground to or have been replaced by CC5 and ST22 strains in hospitals worldwide (Velazquez-Meza et al., 2004; Ma et al., 2006; Amorim et al., 2007; Conceição et al., 2007; Aires-de-Sousa et al., 2008; Knight et al., 2012; Espadinha et al., 2013; Lim et al., 2013; Coombs et al., 2014; Hsu et al., 2015; Lawes et al., 2015). Furthermore, the ST228 (South-German) clone is also on the retreat. It has been reported replaced by ST22 isolates in both German and Italian facilities (Albrecht et al., 2011; Baldan et al., 2012). The surveillance conducted by the European Staphylococcal Reference Laboratory Working Group observed the overall decline of these latter clones between 2006 and 2011 (Grundmann et al., 2014). In addition, it reported a decrease in the incidence of the ST8 clone on the continent (Grundmann et al., 2014).

A reduction has also been observed in the clonal spectrum of ESBL-producing *K. pneumoniae*. A few STs of the pathogen mostly genetically related to each other have become internationally dominant during the last decade (Damjanova et al., 2008; Lee et al., 2011; Woodford et al., 2011; Rodrigues et al., 2014; Park et al., 2015). Interestingly, a marked reduction in the abundance of ESBL-producing *K. pneumoniae* STs was associated with a shift in the type of ESBL produced: while prior to the clonal reduction ESBLs of the SHV group prevailed strains of the novel major international STs produce primarily CTX-M-15 enzymes (Damjanova et al., 2008; Woodford et al., 2011). In addition a sole sequence type of *K. pneumoniae*, ST258, related to the major international clones, contributed significantly to the dissemination of carbapenem resistance worldwide (Woodford et al., 2011; Chen et al., 2014).

Moreover, a few major clones of *C. difficile* also attained international prominence (Smits, 2013; Tickler et al., 2014; Valiente et al., 2014; Freeman et al., 2015).

Strikingly all of these events took place at some point during the last three decades.

EXPERIMENTAL PROCEDURES

Methicillin-Resistant *Staphylococcus aureus*

Two clonal replacements have been observed in HA-MRSA in Hungary during the last 15 years. A dramatic shift took place about 15 years ago when strains of the New York–Japan (ST5) and South-German (ST228) clones almost completely supplanted isolates of the then resident Hungarian/Brazilian clone (ST239; Conceição et al., 2007). Moreover, we have been witnessing another MRSA clone replacement in Hungary during the last couple of years: the Western European clone ST22 (EMRSA-15) has been gradually expanding at the expense of mainly the South-German clone (ST228; Grundmann et al., 2014). Interestingly, both clonal shifts were associated with a transient increase in the

rate for MRSA¹. The incidence of MRSA from invasive infections in Hungary between 2001 and 2014 are shown in **Figure 1**.

Since, the observed clonal shifts in MRSA were supposed to have been promoted by varying fitness cost associated with resistance to some antibiotic the impact of fluoroquinolone resistance on the vitality of MRSA has been tested by us in a clonal affiliation (Horváth et al., 2012).

Propagation assays from various clones of MRSA showing similar MIC values for fluoroquinolones established that isolates from the New York–Japan (ST5) and South-German clones (ST228) retained significantly more fitness than strains from the Hungarian/Brazilian clone (ST239) which they replaced in Hungary (Horváth et al., 2012). In addition isolates from the EMRSA-15 clone (ST22) maintained more vitality than the subsequently supplanted South-German strains. Moreover, a fluoroquinolone resistant isolate from the ST30 CA-MRSA clone suffered much greater fitness cost than strains from any other clone tested. Furthermore, fluoroquinolone resistant strains from two additional CA-MRSA clones (ST8 and ST80) also displayed fitness inferior to that of the EMRSA-15 (ST22), New York–Japan (ST5) and South-German (ST228) strains, though, this difference was smaller than that observed with the ST30 and ST239 isolates (Horváth et al., 2012). These results should account not just for the clonal shifts observed in Hungary but also for the failure of the ST30, ST80, and ST8 CA-MRSA clones to disseminate in Hungarian hospitals.

The superior fitness shown by the newly invading MRSA clones should well-explain the increase in the rate for MRSA detected transiently subsequent to both clonal replacements (**Figure 1**).

However, it must have been a rise in the use of fluoroquinolones that could have allowed the widespread dissemination of the highly fluoroquinolone resistant major clones of MRSA in Hungary. Trends in the rate for MRSA indeed proved significantly associated with trends in the consumption of fluoroquinolone type antibiotics in the country (Pearson correlation, significance two-tailed: 0.03; **Figures 1** and **2**). The MRSA rates also displayed a significant association with the consumption of second generation cephalosporins though the relationship proved somewhat weaker (Pearson correlation, significance two-tailed: 0.05). The consumption of third generation cephalosporins was unrelated to the rate for MRSA (Pearson correlation, significance two-tailed: 0.140)^{1,2}.

Subsequently, Knight et al. (2012) investigating the clonal dynamics of MRSA in a British hospital published findings consistent with our results. In addition, Holden et al. (2013) showed that the development of resistance to fluoroquinolones played a pivotal role in the widespread dissemination of the ST22 clone. Moreover, Lawes et al. (2015) considered the use of fluoroquinolones significant in the clonal shifts of MRSA in Scotland and Hsu et al. (2015) also suggested that the use of fluoroquinolones could have contributed to a clonal rearrangement in MRSA in Singapore. Moreover, the results are propped up by substantial literature on the replacement of the

¹http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database

²<http://ecdc.europa.eu/en/activities/surveillance/ESAC-Net/Pages/index.aspx>

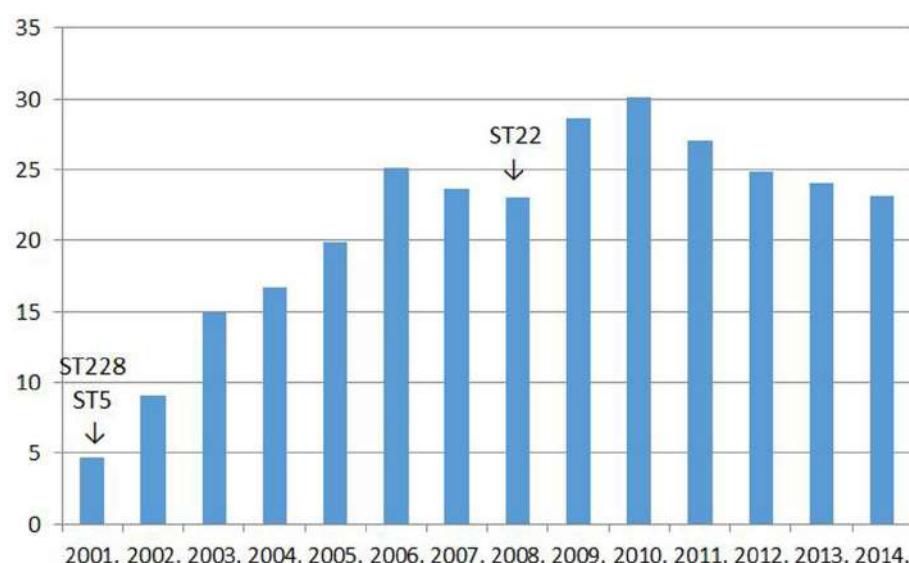


FIGURE 1 | The rate for MRSA among *Staphylococcus aureus* strains isolated from invasive infections in Hungary between 2001 and 2014% (http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database; see explanation in text).

ST30, ST239, ST228, and ST8 clones by ST22 and CC5 strains in the healthcare setting in various parts of the world referred to above.

Apart from demonstrating differences in the fluoroquinolone resistance-associated fitness cost between individual clones of MRSA our work also showed that some differences between isolates affiliated with the same STs also exist (Horváth et al., 2012). Thus, STs are having “sublineages” showing to some extent diverse fitness costs associated with resistance to fluoroquinolones. The recent dissemination of the originally CA-MRSA USA300 (CC8) clone in American hospitals should be related to the emergence of such a novel sublineage commanding better fitness relative to previous lineages when showing resistance to fluoroquinolones. It is probably no accident that Tenover et al. (2011) and Alam et al. (2015) both emphasized that in contrast to previous strains many of the recently tested USA300 isolates proved resistant to fluoroquinolones. Moreover, the emergence of a new sublineage within the USA300 clone should not come as a surprise since the clone ab ovo consisted of a genetically related but diverse group of strains (Tenover and Goering, 2009).

Though, a variety of pathogenicity factors and resistance markers to “non-fluoroquinolone” antibiotics should also affect the dissemination of MRSA the findings with fluoroquinolone resistance-associated fitness cost strongly suggest that it is the primary determinant of the epidemiology of MRSA in every area where fluoroquinolones remain in extensive use. There has to date been no report convincingly demonstrating that any other factor – with the obvious exception of the abandonment of the use of β -lactam antibiotics – could have exerted a more profound effect on the clonal dynamics of the pathogen in the healthcare setting.

Whole-genome sequencing to compare the genetic composition of various clones of MRSA and, thus, identify determinants influencing clonal dynamics have been performed by two groups. Hsu et al. (2015) reported on the displacement of the ST239 MRSA clone by the ST22 clone in Singapore. A thorough review of the genetic makeup of both clones interestingly showed that in contrast to the ST22 isolates many of the ST239 strains harbored genes of the “arginine catabolic mobile element” (ACME), nevertheless, they have been readily replaced by the ST22 isolates. No additional suspect pathogenicity factor could be demonstrated in either clones. However, authors hint that it could have been fitness cost associated with resistance to fluoroquinolones that was responsible for the success of the ST22 clone.

Moreover, Alam et al. (2015) genetically investigated the novel lineage (clade) of the USA300 clone that has recently been emerging in the USA. Though, whole-genome sequencing on many isolates have been performed they failed to identify any suspect pathogenicity factor that could have accounted for the novel clade’s dissemination. Nevertheless, they observed that, in contrast to strains of the old lineage, isolates of the emerging clade were all resistant to fluoroquinolones and harbored “classical” gyrase and topoisomerase mutations.

Though, the superior production of biofilm was reported to promote the dissemination of the ST22 (EMRSA-15) clone its general biofilm-producing capacity is in fact inferior compared with those of the ST228 and ST8 strains it readily replaces in the healthcare setting (Baldan et al., 2012).

Although, resistance markers to “non-fluoroquinolone” antibiotics should also impact the fitness of MRSA the preeminence of fluoroquinolone resistance-associated fitness cost is reflected in the observation that our CA-MRSA strains were usually more susceptible to various additional groups of

antibiotics than the major clone HA-MRSA isolates, nevertheless, they suffered more fitness cost upon acquiring resistance to fluoroquinolones than them. Interestingly, the CA-MRSA isolate suffering the most fitness cost subsequent to the induction of resistance to ciprofloxacin was the ST30 strain showing resistance exclusively to β -lactam antibiotics apart from fluoroquinolones (Horváth et al., 2012).

ESBL-Producing *K. pneumoniae* and ESBL-Producing *E. coli*

A couple of years subsequent to the “epidemiological earthquake” observed with MRSA a major clonal shift took place with ESBL-producing *K. pneumoniae* in Hungary (Damjanova et al., 2006, 2008). Prior to 2003 ESBL-producing *K. pneumoniae* had been polyclonal in the country and the isolates produced SVH type enzymes (Damjanova et al., 2007). However, after 2004 we witnessed the emergence of three major STs of ESBL-producing *K. pneumoniae* two of which (ST11, ST15) were originally detected in France³. Interestingly, in contrast to previous isolates, all of the novel strains carried the CTX-M-15 ESBL gene (Damjanova et al., 2006, 2008). The new major clones disseminated exclusively in adult hospital ward where fluoroquinolones were in extensive use and not in the perinatal intensive care units where fluoroquinolones are not considered a drug of choice (Szilágyi et al., 2010). In the perinatal intensive care units the previous epidemiological situation prevailed: the isolates remained polyclonal and continued to produce SHV type enzymes (Szilágyi et al., 2010).

Similarly, to the MRSA situation the observed clonal change was associated with a rise in the rate for ESBL-producing *K. pneumoniae* in Hungary⁴ (Data not shown). This rise, however, in contrast to MRSA, was not expected to be significantly associated with the consumption of fluoroquinolones for two reasons.

- (1) The new ESBL-producing *K. pneumoniae* clones “invaded Hungary” a couple of years subsequent to the increase in the consumption of fluoroquinolones and, thus, commenced to expand in 2004 after the advent of the widespread use of fluoroquinolones in the country (Figure 2; Damjanova et al., 2006, 2008⁵).
- (2) The dissemination of the major ESBL-producing *K. pneumoniae* clones did not prove as exclusive as that of the major HA-MRSA clones in Hungary (Szilágyi et al., 2010) and rates for clones not selected by fluoroquinolones are not supposed to be governed by changes in the consumption of these agents.

Though, varying fitness cost associated with resistance to fluoroquinolones was obviously influencing the clonal dynamics of MRSA in the hospital setting we have demonstrated an even more pronounced difference between minor clone and major clone strains in ESBL-producing *K. pneumoniae*. Some

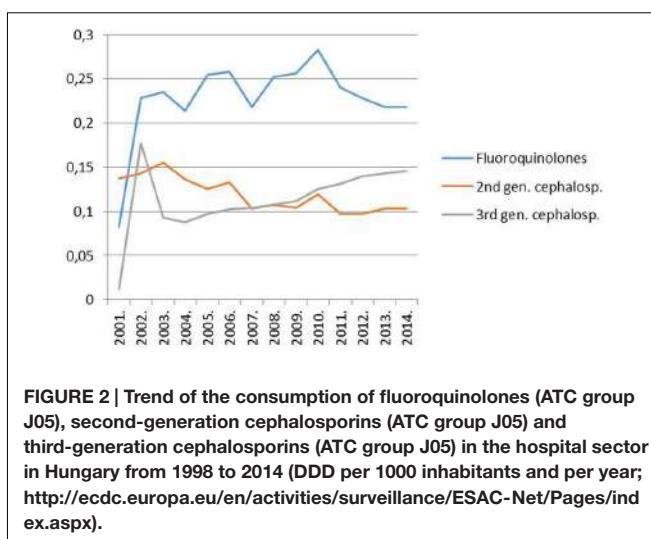


FIGURE 2 | Trend of the consumption of fluoroquinolones (ATC group J05), second-generation cephalosporins (ATC group J05) and third-generation cephalosporins (ATC group J05) in the hospital sector in Hungary from 1998 to 2014 (DDD per 1000 inhabitants and per year; <http://ecdc.europa.eu/en/activities/surveillance/ESAC-Net/Pages/index.aspx>).

of our minor clone *Klebsiella* strains, originally susceptible to fluoroquinolones, suffered a dramatic drop in fitness when resistance to ciprofloxacin was induced in them while others proved unable to assume high-level resistance to ciprofloxacin (Tóth et al., 2014). Interestingly, in contrast to major clone isolates which all carried three mutations in the *gyrA* and *parC* genes, the minor clone ESBL-producing *K. pneumoniae* strains either failed to develop any of the well-known *gyrA* and *parC* mutations or had fewer of them (Tóth et al., 2014).

Additional determinants of fluoroquinolone resistance were also investigated (Tóth et al., 2014). The *qnrA*, *B*, *C*, *D*, and *S*; *qepA* and *oqxAB* were not detected in any of our isolates. The *aac(6')-Ib-cr* was demonstrated in all of the four major clone strains and in one of the minor clones isolates (the single strain carrying a CTX-M-15 plasmid). Moreover, an active efflux system was observed in three of the four minor clone ciprofloxacin resistant isolates but in none of the major clone strains (Tóth et al., 2014).

These results should account for the clonal dynamics of ESBL-producing *K. pneumoniae* in Hungary and could explain the widespread international dissemination of the CTX-M-15 enzyme. Nevertheless it remains to be established why major clone strains proved more adept in developing favorable mutations in the *gyrA* and *parC* genes than minor clone isolates. In addition the background of clonal affiliation of fitness cost elicited by the CTX-M-15 plasmid also needs to be elucidated.

Antibiotics other than fluoroquinolones have not been observed to appreciably impact the clonal dynamics of *K. pneumoniae*. Most of our minor clone *K. pneumoniae* isolates showed similar MIC values to aminoglycosides to those of major clone strains (Tóth et al., 2014). In addition, though three of the four minor clone isolates eliminated the SHV plasmids during the induction of resistance to ciprofloxacin which was associated with a dramatic drop in β -lactam resistance they suffered much greater fitness costs than the major clone strains carrying the CTX-M-15 plasmids and showing high level resistance to β -lactams (Tóth et al., 2014).

³<http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html>

⁴http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database

⁵<http://ecdc.europa.eu/en/activities/surveillance/ESAC-Net/Pages/index.aspx>

Apart from fitness cost associated with resistance to antibiotics virulence factors should also influence the clonal dynamics of ESBL-producing *K. pneumoniae*. Nevertheless data available in the literature strongly suggest that the role of virulence factors remains inferior relative to that of fitness cost associated with resistance to fluoroquinolones.

Lascols et al. (2013) compared the virulence arsenal of major and minor clone strains of ESBL-, and carbapenemase-producing strains of *K. pneumoniae* and observed that – in contrast to expectation – the minor clone isolates harbored somewhat more virulence factors. Andrade et al. (2014) hinted that the production of biofilm could have contributed to the success of the ST11 major clone of *K. pneumoniae* and Melegh et al. (2015) found that the major clone strains of *K. pneumoniae* were more likely to produce biofilm than minor clone isolates. However, Diago-Navarro et al. (2014) observed “heterogeneity” in the formation of biofilm in ST258 strains of *K. pneumoniae* – a close relative of the ST11 clone. Moreover, Kong et al. (2012) questioned the role of biofilm formation in the development of systemic infection with *K. pneumoniae*. Furthermore, Melegh et al. (2015) also observed that the major clone isolates of *K. pneumoniae* displayed significantly higher MIC values to ciprofloxacin than the minor clone strains!

A similar mechanism was reported by an American group for *E. coli* last year. The multiresistant ST131 clone of *E. coli* emerged as an international pathogen in 2008 (Nicolas-Chanoine et al., 2008) and contributed to the worldwide spread of the CTX-M-15 ESBL gene (Nicolas-Chanoine et al., 2014; Mathers et al., 2015). Johnson et al. (2015) recently demonstrated that the main multiresistant international lineages of the clone (H30, H30R) command a “fitness advantage” relative to isolates from other clones when showing high level of resistance to fluoroquinolones. This “fitness advantage” – similarly to major clone isolates of ESBL-producing *K. pneumoniae* – was associated with the quantity of favorable mutations in the *gyrA*, *parC*, and *parE* genes and a significantly weaker efflux activity relative to isolates from other lineages (Johnson et al., 2015). Furthermore a very recent revision of the topic based on the analysis of whole-genome sequences concluded that “strong selection pressure exerted by the widespread use of fluoroquinolones and extended-spectrum cephalosporins” “most likely” played a crucial role in the emergence of the H30 and H30R lineages (Stoesser et al., 2016).

***Clostridium difficile* and Additional Pathogens**

It is well-established that the acquisition of fluoroquinolone resistance is a novel characteristic in the major international ribotype 027 of *C. difficile* compared with earlier strains of the pathogen (He et al., 2013; Spigaglia, 2016). Moreover, Wasels et al. (2015) recently showed that resistance to fluoroquinolones in ribotype 027 strains is associated with a very modest fitness cost; a trait linked to the presence of a favorable mutation (Thre82Ile) in the *gyrA* gene. This result strongly argues for a mechanism similar to that observed with HA-MRSA and ESBL-producing *K. pneumoniae* since the same mutation has also

been demonstrated in isolates of some additional international ribotypes genetically related or unrelated to ribotype 027 (Carman et al., 2009; Saxton et al., 2009; Spigaglia et al., 2010; Walkty et al., 2010; Lin et al., 2011; Solomon et al., 2011; Lee et al., 2014; Baldan et al., 2015; Kuwata et al., 2015).

The presence of the energetically favorable Thre82Ile *gyrA* mutation in many strains of the major international ribotypes of *C. difficile* should have promoted their dissemination in a fluoroquinolone affected area that may partly account for the relative diversity of the clonal spectrum of the pathogen (Bauer et al., 2011; Tickler et al., 2014; Freeman et al., 2015). This contrasts with the clonal landscape of ESBL-producing *K. pneumoniae* where the capacity of developing favorable *gyrA* and *parC* mutations seems to be the hallmark of just a few genetically related international STs (Tóth et al., 2014).

A well-documented clonal shift of *C. difficile* occurred in a Korean hospital reflecting the findings of Wasels et al. (2015). The earlier prevalent *C. difficile* ribotype 001 strains were replaced by isolates from the 014, 017, and 018 ribotypes within a couple of years (Lee et al., 2014). Interestingly, all of the novel ribotype strains carried the energetically favorable Thre82Ile *gyrA* mutation while the Korean ribotype 001 isolates harbored the Thre82Va *gyrA* mutation that has been shown by Wasels et al. (2015) to be associated with a significant fitness cost.

Moreover, the proposed “fluoroquinolone mechanism” is supported by the observation that the proportion of the ribotype 027 clone is significantly greater in adult ward than in pediatric units (McFarland et al., 2016).

Apart from favorable fitness various virulence factors produced by ribotype 027 strains have certainly contributed to the clone’s dissemination (Stabler et al., 2009; Valiente et al., 2014), although, differences between individual strains do exist (Carlson et al., 2013). Furthermore, strains of additional major ribotypes 001 and 106 have also been shown to command superior virulence relative to many other clones (Vohra and Poxton, 2011). Nevertheless Sarma et al. (2015) recently reported that a significant decrease in the consumption of fluoroquinolone type antibiotics resulted in the partial replacement of the ribotype 027, 001, and 106 strains – all reported to have been carrying the Thre82Ile *gyrA* mutation (Carman et al., 2009; Saxton et al., 2009; Cartman et al., 2010; Solomon et al., 2011) – by a variety of minor clones. Consequently, superior virulence could not prevent the demise of strains from any of these ribotypes once the selecting pressure of fluoroquinolone exposure ceased. Moreover, these findings are also in agreement with the national *C. difficile* statistics of the UK (Wilcox et al., 2012).

Furthermore, Wasels et al. (2015) demonstrated that the *gyrB* Asp426Asn and Asp426Val mutations confer an extra fitness on *C. difficile* irrespective of exposure to fluoroquinolones. These genetic alterations should also influence the clonal dynamics of the pathogen. However, to properly investigate their impact their prevalence should be investigated in a clonal affiliation.

In addition to the pathogens mentioned above the prospect of a “fluoroquinolone resistance-associated fitness mechanism” in the dissemination of a variety of multiresistant pathogens would be worth investigated. Among others the ST198 clone of *Salmonella* Kentucky (Le Hello et al., 2013) and the

fluoroquinolone resistant clone of *Streptococcus pneumoniae* (Ben-David et al., 2014).

To our understanding there may be a single species of bacteria that seems to have efficiently adapted to resistance against fluoroquinolones and varying fitness cost has to date certainly failed to select a major international clone in it: *Campylobacter jejuni* (Luo et al., 2004; Zeitouni and Kempf, 2011). The excellent adaptation of *C. jejuni* is probably due to the hyperplasticity of its genome (Stahl and Stintzi, 2011).

In summary our results and those of others indicate that diverse fitness cost associated with resistance to fluoroquinolones influenced the evolution and extensive dissemination of the major international clones of a variety of important multiresistant pathogens.

PRACTICAL CONSEQUENCES AND DISCUSSION

Since the fitness of the major clones of multiresistant pathogens mentioned above proved superior to those of the previously prevalent minor clone isolates they may have disseminated more quickly and, thus, could have influenced the rates for the multiresistant pathogens in facilities/ward where fluoroquinolones remained in extensive use. This process, as indicated above, seemed obvious in Hungary with both MRSA and ESBL-producing *K. pneumoniae*.

However, if the extensive use of fluoroquinolones – and that of additional groups of antibiotics – contributed to a rise in the incidence of various multiresistant bacteria, then a more judicious consumption of antibiotics in general and of fluoroquinolones in particular should lower the rate of resistance for these pathogens. The available quantity and quality of information in this respect varies across species. The data on MRSA is the most abundant and seem to be the most appropriate for drawing inferences from. This is no accident since the selection of all major international clones of HA-MRSA have been influenced by fluoroquinolones. Though, most studies investigating a possible link between the consumption of fluoroquinolones and the rate for MRSA did not establish the clonal affiliation of the isolates at the facilities observed, basically all surveys necessarily investigated the prevalence of “fluoroquinolone-associated clone” isolates.

Most of the literature published on the influence of fluoroquinolone consumption on the rate for MRSA show a clear relationship: the more fluoroquinolones are used the higher the rate for MRSA will rise and vice versa. The association seemed so close that both the Society of Healthcare Epidemiology of America (SHEA) in its 2003 guideline (LeDell et al., 2003) and the British Department of Health in its 2011 guideline (Byrne and Wilcox, 2011) recommended a restriction in the use of fluoroquinolones as a control measure to curb the spread of MRSA. Moreover, an abundance of papers demonstrated a direct link between fluoroquinolone use and the incidence of MRSA between 1998 and 2015 (Hill et al., 1998; Crowcroft et al., 1999; Gruson et al., 2000; Harbarth et al., 2000; Graffunder and Venezia, 2002; Weber et al., 2003; Aubert et al., 2005; Nseir

et al., 2005; Charbonneau et al., 2006; Cook et al., 2006, 2011; LeBlanc et al., 2006; Madaras-Kelly et al., 2006; Muller et al., 2006; Rogues et al., 2007; Aldeyab et al., 2008; Liebowitz and Blunt, 2008; Vernaz et al., 2008; Kaier et al., 2009; Jacoby et al., 2010; Thabet et al., 2010; Huang et al., 2011; Parienti et al., 2011; Bertrand et al., 2012; Lafaurie et al., 2012; Dancer et al., 2013; Couderc et al., 2014; Lawes et al., 2015). Nevertheless, a few researchers after controlling for multifold confounding factors failed to observe a significant association (Wibbenmeyer et al., 2010; Datta et al., 2014). In addition three papers reported that the use of distinct antibiotics from the group of fluoroquinolones had diverse effects on the rate for MRSA, contradicting partly to each other (MacDougall et al., 2005; Bosso and Mauldin, 2006; Salangsang et al., 2010). Moreover, as mentioned above, trends in the rate for MRSA and in the consumption of fluoroquinolone type antibiotics in Hungary during the past 15 years are also suggestive of a relationship (Figures 1 and 2). Despite this strong circumstantial evidence the absence of an established mechanism for how fluoroquinolones could influence the rate of β -lactam resistance in *Staphylococcus aureus* might have precluded the acceptance of a causal relationship (Fuzi, 2014).

An additional factor may complicate the situation somewhat further. One of the groups investigating a possible link between fluoroquinolone use and the rate for MRSA (Parienti et al., 2011) observed a decline in the incidence of MRSA subsequent both to a decrease and an increase in the consumption of the incriminated antibiotics. The mechanism described above posits that the development of resistance to fluoroquinolones is associated with a significant fitness cost in minor clone strains of MRSA (Horváth et al., 2012), however, the findings also imply that sooner or later all MRSA strains – even the most able isolates – will suffer some fitness cost when exposed to the pressure of fluoroquinolones in the long term, that may account for the observation of Parienti et al. (2011). Nevertheless, not surprisingly, Parienti et al. (2011) reported a much greater decline in the rate for MRSA if the use of fluoroquinolones was restricted relative to that associated with an increase in consumption. This “long-term fluoroquinolone pressure” on prevailing resident clones should also influence the rate for MRSA in the healthcare setting.

Since the recognition of novel lineages/sublineages of MRSA seem to be of utmost importance efficient techniques for the identification of newly emerging variants of the pathogen are warranted. Spa typing proved very useful in detecting variations within STs (Grundmann et al., 2014) and the continuous combined surveillance of spa types and multiple-locus variable number tandem repeat fingerprint (MLVF) types were reported to be an excellent means to monitor the dynamics of MRSA lineages and sublineages (Glasner et al., 2013). Nevertheless, it is the sequence-based approach that will provide a comprehensive and highly reliable account of lineage/sublineage distribution of the pathogen. Our results should help in establishing the disseminating potential of newly emerged lineages or sublineages. By determining the fluoroquinolone resistance-associated fitness cost of strains from newly emerged groups of MRSA (and various other pathogens) will allow the prediction of their “disseminating capacity” in ward where fluoroquinolones remain in extensive use.

The available literature on *C. difficile* is also relevant: the proportion of the “fluoroquinolone-related” clones is substantial (Tickler et al., 2014; Freeman et al., 2015); in addition many studies disclosed the clonal affiliation of the investigated isolates allowing for a specific monitoring of the “fluoroquinolone-related” ribotypes. Similarly, to MRSA an abundance of papers clearly shows that the incidence of *C. difficile* infections decreases subsequent to a reduction in the consumption of fluoroquinolone type antibiotics (Muto et al., 2007; Valiquette et al., 2007; Debast et al., 2009; Price et al., 2010; Wilcox et al., 2012; Dancer et al., 2013; Kanerva et al., 2015; Sarma et al., 2015; Gordon et al., 2016).

An additional strong argument for the influence of fluoroquinolone consumption on the clonal dynamics of *C. difficile* is the well-established fact that the incidence of the ribotype 027 strains remains significantly lower in pediatric units compared with adult ward (McFarland et al., 2016) which is in agreement with our finding with the major clones of ESBL-producing *K. pneumoniae* in Hungary (Szilágyi et al., 2010) and should reflect the differing use of fluoroquinolones in the two departments.

In contrast to MRSA and *C. difficile* the reliable impact of fluoroquinolone use on the incidence of ESBL-producing *K. pneumoniae* and ESBL-producing *E. coli* will be elucidated by future studies investigating the clonal distribution of the isolates.

The question arises how can the findings reviewed in this paper be reconciled with the abundant literature reporting only a slight fitness cost associated with antibiotic resistance and the observations that fitness cost suffered upon acquiring resistance can be reversed by developing compensatory mutations?

First, these studies cannot be compared with those reviewed here since none of them investigated a crucial aspect of the mechanism: the clonal affiliation of the isolates.

Second, fitness cost – or the absence of it – associated with resistance to antimicrobial agents is often a function of the drug of choice. For instance resistance to streptomycin and rifampicin will often not result in any fitness cost or the suffered loss in vitality can readily be reversed by compensatory mutations (Björkman et al., 1998; Maisnier-Patin et al., 2002; Sander et al., 2002; Trindade et al., 2009; Brandis et al., 2015; Durão et al., 2015). In contrast resistance in fungi to amphotericin B (Vincent et al., 2013) and resistance in plasmodium to antimalarial agents (Rosenthal, 2013) are always associated with fitness cost that cannot be wholly recovered. As we have seen resistance to fluoroquinolones is usually associated with fitness cost, though it is a function of clonal affiliation and cannot be wholly recovered at higher MIC values (Horváth et al., 2012; Knight et al., 2012; Tóth et al., 2014).

Third, the fitness cost associated with resistance to antibiotics is often related to the level of resistance of the strain tested. Fluoroquinolone type antibiotics undoubtedly belong to this group. Several authors reported small fitness cost and sometimes slight fitness gain in strains with low level resistance to quinolones (Giraud et al., 2003; O'Regan et al., 2010; Baker et al., 2013; Fàbrega et al., 2014). However, fitness costs associated with higher MIC values were usually greater and could not be reversed (Giraud et al., 2003; Komp Lindgren et al., 2005; Marcusson et al., 2005; O'Regan et al., 2010; Pope et al., 2010; Horváth et al., 2012;

Baker et al., 2013; Fàbrega et al., 2014; Tóth et al., 2014). Melnyk et al. (2015) recently reviewed much of the related literature and demonstrated that higher MIC values for many antibiotics are generally associated with higher fitness costs. They also observed that this trend unfortunately remains poorly explored since many of the investigators failed to disclose the MIC values of their isolates.

Moreover, the non-reversibility of fitness cost associated with resistance to fluoroquinolones in minor clone strains (Tóth et al., 2014) is strongly supported by international epidemiological data. The clonal landscape for the multiresistant pathogens mentioned above remains largely stable: usually a few big clones or STs are competing with each other for “territory.” If minor clone strains could easily reverse the fitness cost associated with resistance to fluoroquinolones novel international clones of various multidrugresistant pathogens should regularly emerge and replace the “resident major clones,” something we have not been witnessing.

Though, the findings discussed above show an important role for fluoroquinolones in the selection and dissemination of multiresistant clones of various bacteria additional antibiotics should also have contributed to this process, thus, a more judicious use of antibiotics in general and of fluoroquinolones in particular could improve the antibiotic resistance situation.

The genetic base of the “fluoroquinolone selection mechanism” has in part already been elucidated. The data obtained to date clearly show that favorable mutations in the gyrase and topoisomerase IV genes play a crucial role in the development of high-level resistance to fluoroquinolones with a concurrent preservation of fitness. This has been demonstrated for *K. pneumoniae* (Tóth et al., 2014), *C. difficile* (Wasels et al., 2015) and a novel emerging lineage of the ST8 MRSA clone in America (Alam et al., 2015).

However, not all changes detected in the gyrase and topoisomerase genes proved favorable. We have observed a cluster of synonymous and non-synonymous mutations in the *grlB* gene of our ST30 MRSA strain that could have contributed to its compromised fitness relative to that of isolates from other clones of MRSA (Horváth et al., 2012).

Additional mechanisms associated with resistance to fluoroquinolones seem to command an inferior role compared with mutations in the *gyrA* and *parC* genes. In our *Klebsiella* study (Tóth et al., 2014) we failed to demonstrate the presence of the plasmid-mediated quinolone resistance determinants (PMQRDs): *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, and *oqxAB* in any of our isolates. In contrast all of the major clone strains and one of the minor clone isolates possessed the *aac(6')*-*lb-cr* determinant that was related to the carriage of plasmids harboring CTX-M-15 ESBL genes. Moreover, none of the major clone strains but three of the four minor clone isolates showed efflux activity.

The data presented by us suggest that the ability of the reviewed bacteria to transmit between individuals is strongly influenced by the “speed of multiplication”; a trait we call fitness. This “speed of multiplication” can be reliably measured *in vitro* and seems to impact heavily on the disseminating capacity of the isolate. Data published on MRSA, ESBL-producing

K. pneumoniae and *C. difficile* indicate that virulence factors play an inferior role in the dissemination of these species relative to fitness. A possible reason could be the competition of bacteria during the initial colonization stage. At this stage the “speed of multiplication” could often decide the “winner” and pathogenicity factors – certainly with the exception of those involved in attachment to host or killing other bacteria – remain less essential. If a strain is getting outgrown by another in the area of colonization it will have limited value of most of its pathogenicity factors which are directed against the host. This is, of course, not to deny that virulence factors of the reviewed pathogens could strongly impact dissemination when the fitness of the competing isolates remain equal. Nevertheless, fitness studies conducted with individual strains in animals *in vivo* remain less relevant for transmissibility than propagation assays performed *in vitro*, similarly, to competitive *in vivo* studies where the infection of the animal did not resemble the usual way of natural transmission.

However, in other species of bacteria virulence factors could assume greater significance in dissemination most certainly in a clone-affiliated fashion.

In conclusion we can say that:

- (1) Major international clones of several multiresistant pathogens have emerged during the past three decades (see literature above).
- (2) Experimental findings show that diverse fitness cost associated with resistance to fluoroquinolones could have influenced the clonal dynamics in MRSA, ESBL-producing *K. pneumoniae*, ESBL-producing *E. coli*, and multiresistant *C. difficile*. These findings are based on two lines of evidence.
 - (a) The major international clones/lineages of MRSA, ESBL-producing *K. pneumoniae*, ESBL-producing *E. coli*, and *C. difficile* were shown to command favorable fitness when displaying high-level resistance to fluoroquinolones (Horváth et al., 2012; Knight et al., 2012; Tóth et al., 2014; Johnson et al., 2015; Wasels et al., 2015). For MRSA identical findings have been reported from two independent laboratories (Horváth et al., 2012; Knight et al., 2012).
 - (b) Genetic investigations demonstrated that the ability to develop favorable mutations in the gyrase and topoisomerase IV genes constitutes a prerequisite for retaining fitness in MRSA, ESBL-producing *K. pneumoniae*, multiresistant *C. difficile*, and ESBL-producing *E. coli* when showing high-level resistance to fluoroquinolones (see literature above). Some additional mechanisms of resistance to fluoroquinolones in *K. pneumoniae* seem to be either expendable (qnr type resistance) or may not provide a viable alternative (efflux; Tóth et al., 2014). The same was reported on efflux in ESBL-producing *E. coli* (Marcusson et al., 2009; Johnson et al., 2015).
- (3) *In vitro* observations concerning the fitness of various clones of MRSA (Horváth et al., 2012; Knight et al.,

2012) are supported by a plethora of papers describing clonal shifts of the pathogen worldwide. All of the clonal replacements reported in the literature (see references above) are in agreement with the results of the fitness assays obtained by us (Horváth et al., 2012) and by Knight et al. (2012). Clonal shifts and clonal distributions observed with *C. difficile* also support the “fluoroquinolone mechanism” (Lee et al., 2014; McFarland et al., 2016).

- (4) The observations that plasmids harboring the CTX-M-15 gene – in contrast to those with SHV type ESBL genes – proved impossible to eliminate from *K. pneumoniae* showing resistance to fluoroquinolones may account for the worldwide dissemination of this enzyme at the expense of SHV type ESBLs in this species (Tóth et al., 2014). The issue should be further investigated in both ESBL-producing *K. pneumoniae* and ESBL-producing *E. coli*.

The impact of fluoroquinolone consumption on the prevalence of MRSA and the major international clones of *C. difficile* is well-established (see literature above).

The influence of fluoroquinolone use on the incidence of ESBL-producing *K. pneumoniae* and ESBL-producing *E. coli* could be properly investigated by determining the clonal affiliation of the isolates and appreciating the change exclusively in the rates of those major clone strains which are known to have been selected by fluoroquinolones.

AUTHOR CONTRIBUTIONS

MF was head of the research team establishing the impact of fluoroquinolone resistance-associated fitness cost on the clonal dynamics of methicillin-resistant *Staphylococcus aureus* and ESBL-producing *Klebsiella pneumoniae*. He perused the literature related to clonality and fluoroquinolone resistance-associated fitness cost in various pathogens and compiled the manuscript himself. All the inferences presented in the paper have been drawn by him.

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Global Dissemination of Carbapenemase-Producing *Klebsiella pneumoniae*: Epidemiology, Genetic Context, Treatment Options, and Detection Methods

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The emergence of carbapenem-resistant Gram-negative pathogens poses a serious threat to public health worldwide. In particular, the increasing prevalence of carbapenem-resistant *Klebsiella pneumoniae* is a major source of concern. *K. pneumoniae* carbapenemases (KPCs) and carbapenemases of the oxacillinase-48 (OXA-48) type have been reported worldwide. New Delhi metallo-β-lactamase (NDM) carbapenemases were originally identified in Sweden in 2008 and have spread worldwide rapidly. In this review, we summarize the epidemiology of *K. pneumoniae* producing three carbapenemases (KPCs, NDMs, and OXA-48-like). Although the prevalence of each resistant strain varies geographically, *K. pneumoniae* producing KPCs, NDMs, and OXA-48-like carbapenemases have become rapidly disseminated. In addition, we used recently published molecular and genetic studies to analyze the mechanisms by which these three carbapenemases, and major *K. pneumoniae* clones, such as ST258 and ST11, have become globally prevalent. Because carbapenemase-producing *K. pneumoniae* are often resistant to most β-lactam antibiotics and many other non-β-lactam molecules, the therapeutic options available to treat infection with these strains are limited to colistin, polymyxin B, fosfomycin, tigecycline, and selected aminoglycosides. Although, combination therapy has been recommended for the treatment of severe carbapenemase-producing *K. pneumoniae* infections, the clinical evidence for this strategy is currently limited, and more accurate randomized controlled trials will be required to establish the most effective treatment regimen. Moreover, because rapid and accurate identification of the carbapenemase type found in *K. pneumoniae* may be difficult to achieve through phenotypic antibiotic susceptibility tests, novel molecular detection techniques are currently being developed.

Keywords: carbapenemase, *Klebsiella pneumoniae*, epidemiology, KPC, NDM, OXA-48-like

INTRODUCTION

The increasing prevalence of antibiotic resistance and the lack of new antibiotic drug development has gradually reduced the treatment options for bacterial infections (Lee et al., 2013a; Nathan and Cars, 2014). In 2013, the Centers for Disease Control and Prevention (CDC) named three microorganisms that pose an urgent threat to public health: carbapenem-resistant (CR) *Enterobacteriaceae* (CRE), drug-resistant *Neisseria gonorrhoeae*, and *Clostridium difficile* (Zowawi et al., 2015). Carbapenems (imipenem, meropenem, biapenem, ertapenem, and doripenem) are antibiotics used for the treatment of severe infections caused by multi-resistant *Enterobacteriaceae*, such as *Klebsiella pneumoniae* and *Escherichia coli* (Nordmann et al., 2009). However, over the past 10 years, CRE have increasingly been reported worldwide (Nordmann et al., 2011a). In particular, *K. pneumoniae* have acquired carbapenemases, which are enzymes capable of breaking down most β -lactams including carbapenems, and thus conferring resistance to these drugs (Jeon et al., 2015). High mortality rates have been reported in patients with bloodstream infections caused by CR *K. pneumoniae* (Munoz-Price et al., 2013). Carbapenemases can be divided according to their dependency on divalent cations for enzyme activation into metallo-carbapenemases (zinc-dependent class B) and non-metallo-carbapenemases (zinc-independent classes A, C, and D; Jeon et al., 2015). The class A carbapenemases, such as the *K. pneumoniae* carbapenemase (KPC) enzymes, have been identified worldwide in *K. pneumoniae* (Tangden and Giske, 2015). Various class B and D carbapenemases have also been detected in hospital-acquired multi-resistant *K. pneumoniae* (Nordmann et al., 2011a), whereas class C carbapenemases have rarely been reported. In this review, we summarize the epidemiology of the major four classes of carbapenemases and discuss their molecular genetics, methods used for their detection, and the therapeutic options available for their treatment.

THE EPIDEMIOLOGY, GENETIC CONTEXT, TREATMENT OPTIONS, AND DETECTION METHODS OF CARBAPENEM-RESISTANT *K. pneumoniae*

Class A Carbapenemases

Epidemiology

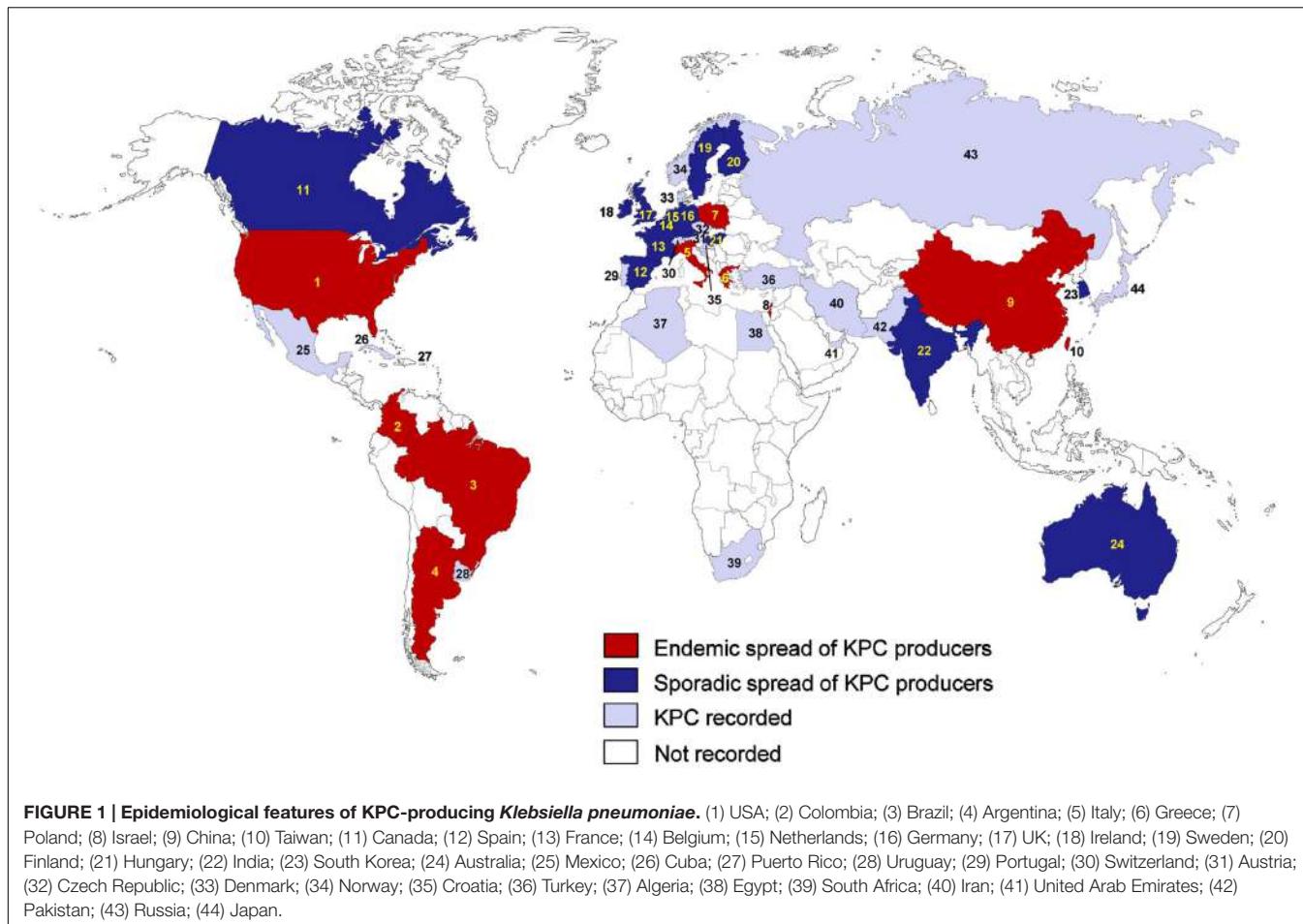
Various class A carbapenemases forming six distantly related branches have been identified (Jeon et al., 2015). While some carbapenemases are chromosome-encoded (IMI-1, NMC-A, SME enzymes, SHV-38, and SFC-1), others are plasmid-encoded (KPC enzymes, GES enzymes, and IMI-2). KPCs have been the most frequently observed class A carbapenemases since their first description in the eastern USA in 1996 (Yigit et al., 2001). Of the many different KPC family variants (KPC-1 to KPC-22), the most well-characterized variants are KPC-2 and KPC-3. KPCs are mostly plasmid-encoded enzymes and bacteria producing these

enzymes are susceptible to only a few antibiotics such as colistin, aminoglycosides, and tigecycline. Therefore, the mortality of the patient's bloodstream infections caused by these bacteria is very high (Munoz-Price et al., 2013).

The epidemiology of *K. pneumoniae* producing KPCs varies geographically. The endemic spread of these bacteria has been reported in the USA, China, Italy, Poland, Greece, Israel, Brazil, Argentina, Colombia, and Taiwan (Munoz-Price et al., 2013; Figure 1). Sporadic spread of KPC-producing *K. pneumoniae* has also been observed in many European countries including Spain, France, Germany, the Netherlands, the UK, Ireland, Belgium, Sweden, and Finland, and in several countries in the Asia-Pacific region, including India, South Korea, and Australia (Munoz-Price et al., 2013; Nordmann and Poirel, 2014). In the USA, the transmission of CR *K. pneumoniae* is primarily driven by the spread of organisms carrying KPC enzymes (Kaiser et al., 2013), but other carbapenemase enzymes, such as the New-Delhi metallo- β -lactamase (NDM), have also emerged (Lascols et al., 2013). Within the USA, the prevalence of KPC-positive isolates was relatively stable between 2007 and 2009 (5.9% in 2007, 4.9% in 2008, and 5.7% in 2009; Kaiser et al., 2013), and KPC-2 and KPC-3 were the most frequently identified carbapenemases in *K. pneumoniae* (Deshpande et al., 2006).

The outbreaks caused by KPC-producing *K. pneumoniae* have been reported in the USA (Woodford et al., 2004) and Israel (Leavitt et al., 2007), but recently, similar outbreaks associated with patients traveling to endemic areas have also been reported in many European countries. Since KPC-producing *K. pneumoniae* was identified in France, Italy, and Greece (Naas et al., 2005; Tsakris et al., 2008; Giani et al., 2009), the sporadic spread of KPC-producing *K. pneumoniae* has been observed in many European countries including Spain (Robustillo Rodela et al., 2012), France (Carbonne et al., 2010), Germany (Wendt et al., 2010; Steinmann et al., 2011), the Netherlands (Weterings et al., 2015), the UK (Woodford et al., 2008; Virgincar et al., 2011), Ireland (Roche et al., 2009; Morris et al., 2012), Belgium (Bogaerts et al., 2010), Sweden (Samuelson et al., 2009), and Finland (Osterblad et al., 2012; Kanerva et al., 2015). KPC-producing *K. pneumoniae* were also recently detected in eastern European countries including the Czech Republic (Hrabak et al., 2013b), Hungary (Toth et al., 2010), and Croatia (Bedenic et al., 2012).

In Greece, KPC-producing *K. pneumoniae* was first isolated in August 2007 (Tsakris et al., 2008), and the prevalence of KPC-producers among carbapenemase-producing *K. pneumoniae* isolates collected at a tertiary Greek hospital increased from 0% in 2003 to 38.3% in 2010 (Zagorianou et al., 2012). Most of the genotyped KPC-producing *K. pneumoniae* in Greece harbored KPC-2 (Zagorianou et al., 2012). While many carbapenemase-producing *K. pneumoniae* in the USA and Greece had KPC enzymes (Nordmann et al., 2009; Zagorianou et al., 2012), several studies in Spain showed that most carbapenemase-producing *K. pneumoniae* harbored OXA-48-like or class B carbapenemases, and the rate of KPC-producing *K. pneumoniae* was very low (2–3%; Oteo et al., 2013b; Palacios-Baena et al., 2016). These results indicate that the prevalent genotype of carbapenemase-producing *K. pneumoniae* varies geographically. For example, in



Italy which is a representative southern European country where KPC is becoming endemic, 89.5% of carbapenemase producers have been reported to have KPC-type enzymes, followed by VIM-1 (9.2%) and OXA-48 (1.3%; Giani et al., 2013).

In America, the endemic spread of KPCs has been reported in Colombia (Villegas et al., 2006; Rojas et al., 2013), Brazil (Peirano et al., 2009; Fehlberg et al., 2012), and Argentina (Pasteran et al., 2008; Gomez et al., 2011). In Canada, KPC-producing *K. pneumoniae* has sporadically been reported (Goldfarb et al., 2009; Lefebvre et al., 2015), and since plasmid-mediated KPC-producing *K. pneumoniae* was first detected in Ottawa in Goldfarb et al. (2009), a laboratory surveillance program found a high frequency (89.3%) of KPC-type enzymes among carbapenemase producers between 2010 and 2012 (Lefebvre et al., 2015). The emergence of KPCs in Argentina was characterized by two patterns of dispersion: the first was the irregular occurrence of diverse *Enterobacteriaceae* harboring *bla*_{KPC-2} in the IncL/M transferable plasmid in distant regions and the second was the sudden clonal spread of *K. pneumoniae* ST258 harboring *bla*_{KPC-2} in Tn4401a (Gomez et al., 2011). KPC-producing *K. pneumoniae* was recently also detected in Cuba (Quinones et al., 2014), Mexico (Garza-Ramos et al., 2014), Uruguay (Marquez et al., 2014), and Puerto Rico (Gregory et al., 2010).

In the Asia-Pacific region, the endemic dissemination of KPC-producing *K. pneumoniae* has been reported in China (Li et al., 2014) and Taiwan (Tseng et al., 2015), and the sporadic spread has been reported in India (Shanmugam et al., 2013), South Korea (Yoo et al., 2013), and Australia (Partridge et al., 2015). A novel KPC-15 variant which is closely homologous with KPC-4 was discovered in China (Wang et al., 2014b) and its enzymatic activity and phenotype was characterized (Wang et al., 2014a). In China, the frequency of KPC-type enzymes among carbapenemase producers was high (63%; Li et al., 2014). While ST258 is the predominant clone observed in European countries and the USA (Giani et al., 2013; Chen et al., 2014e; Bowers et al., 2015), ST11, which is closely related to ST258, is the prevalent clone associated with the spread of KPC-producing *K. pneumoniae* in Asia (particularly in China and Taiwan; Qi et al., 2011; Yang et al., 2013; Tseng et al., 2015). KPC-producing ST11 strain has also been reported in Latin America (Munoz-Price et al., 2013). Although it is unknown why ST11 is prevalent, a recent report showed that the KPC-producing *K. pneumoniae* ST11 clone was resistant to serum killing (Chiang et al., 2016). In a Chinese hospital, another nosocomial outbreak of KPC-2-producing *K. pneumoniae* was caused by multiple *K. pneumoniae* strains including ST37, ST392, ST395, and ST11, implying the horizontal transfer of *bla*_{KPC-2} gene between

different *K. pneumoniae* clones in China (Yang et al., 2013). In Taiwan, two novel KPC variants were identified; KPC-16 and KPC-17 differed from KPC-2 by two (P202S and F207L) and a single (F207L) amino acid substitutions, respectively (Yu et al., 2015). A nationwide survey in Taiwan between 2011 and 2013 reported the national spread of KPC-2 and KPC-17 (Tseng et al., 2015). KPC-producing *K. pneumoniae* was recently also detected in Japan (Saito et al., 2014), Pakistan (Pesesky et al., 2015), Iran (Nobari et al., 2014), and United Arab Emirates (Sonnevend et al., 2015a). In the Arabian Peninsula, the prevalence of KPC-producing *K. pneumoniae* was very low in comparison to NDM-1 and OXA-48-like carbapenemases (Sonnevend et al., 2015b). Sonnevend et al. (2015a), two *K. pneumoniae* ST14 strains producing KPC-2 were first identified in the United Arab Emirates of the Arabian Peninsula. In Africa, several countries such as South Africa (Brink et al., 2012), Algeria (Bakour et al., 2015b), and Egypt (Metwally et al., 2013), have also isolated KPC-producing *K. pneumoniae*.

The coexistence of KPCs and other carbapenemases in *K. pneumoniae* was frequently reported worldwide, including in Italy (KPC-3/VIM-2 and KPC-2/VIM-1; Richter et al., 2012; Perilli et al., 2013), Colombia (KPC-2/VIM-24; Rojas et al., 2013), Brazil (KPC-2/NDM-1; Pereira et al., 2015), China (KPC-2/NDM-1, KPC-2/CMY-2, and KPC-2/IMP-4; Hu et al., 2014; Dong et al., 2015; Liu et al., 2015), Canada (KPC-3/CMY-2; Leung et al., 2012), and Greece (KPC-2/VIM-1; Giakkoupi et al.,

2009), indicating the worldwide prevalence of *K. pneumoniae* co-harboring two carbapenemases.

Aside from KPC-type carbapenemases, other class A carbapenemases, such as GES-2, GES-4, GES-5, GES-6, GES-11, GES-14, GES-18, SFC-1, SHV-38, NMC-A, SME-1, and IMI-type enzymes, were rarely found in *K. pneumoniae* (Table 1).

Molecular and Genetic Context

The *bla*_{KPC} in *K. pneumoniae* has been reported on numerous plasmid types, such as IncF, IncI2, IncX, IncA/C, IncR, and ColE1 (Garcia-Fernandez et al., 2012; Chen et al., 2014e; Pitout et al., 2015), but the predominant plasmid type is IncF with FII_K replicons (Pitout et al., 2015). IncF often contains several additional genes responsible for resistance to other antibiotics, including aminoglycosides, tetracyclines, quinolones, trimethoprim, and sulfonamides (Pitout et al., 2015). Many *bla*_{KPC} genes are associated with a promiscuous transposon-related structure Tn4401, which is approximately 10 kb in size and consists of a transposase gene, a resolvase gene, the *bla*_{KPC} gene, and two insertion sequences, ISKpn6 and ISKpn7 (Figure 2A; Naas et al., 2008). This transposon has jumped to numerous plasmids that are commonly conjugative (Chen et al., 2014e). In China, a novel genetic environment was detected (Shen et al., 2009). It contains an integration structure consisting of a Tn3-based transposon and partial Tn4401 segment, with the gene order Tn3-transposase, Tn3-resolvase, ISKpn8, the *bla*_{KPC-2}

TABLE 1 | The epidemiology of various carbapenemases in *Klebsiella pneumoniae*.

Molecular class	Carbapenemase	Geographical distribution
A	SME types	Not found
	IMI types	Not found
	GES types	Greece (Vourli et al., 2004), Finland (Osterblad et al., 2012), Brazil (Picao et al., 2010), and South Korea (Jeong et al., 2005; Bae et al., 2007)
B	SFC-1, SHV-38, and NMC-A	France (Poirel et al., 2003) and Brazil (Tollentino et al., 2011)
	OXA-23, OXA-24/40, OXA-51, OXA-58, OXA-134, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235	Not found
C	DHA-1	Taiwan (Lee et al., 2012b), South Korea (Park et al., 2013), and China (Hu et al., 2014)
D	CMY-2 and CMY-10	China (Hu et al., 2014), Canada (Leung et al., 2012), and Greece (Pournaras et al., 2010b)
	ADC-68	Not found
	IMP types	Malaysia (Hamzan et al., 2015), Taiwan (Tseng et al., 2015), China (Chen et al., 2015), Thailand (Rimrang et al., 2012), Ireland (Morris et al., 2016), Greece (Lascols et al., 2013), Spain (Lascols et al., 2013), Italy (Lascols et al., 2013), Turkey (Lascols et al., 2013), Austria (Zarfel et al., 2011), the USA (Limbago et al., 2011; Rojas et al., 2013), and Mexico (Gales et al., 2012)
E	VIM types	Greece (Pournaras et al., 2010b), Ireland (Morris et al., 2016), Spain (Pena et al., 2014), Australia (Lascols et al., 2013), Croatia (Zujic Atalic et al., 2014), the Czech Republic (Hrabak et al., 2013b), Hungary (Melegh et al., 2014), Italy (Giani et al., 2013), Norway (Naseer et al., 2012), Austria (Zarfel et al., 2011), Finland (Osterblad et al., 2012), Germany (Steinmann et al., 2011), France (Birgy et al., 2011), China (Liu et al., 2015), India (Castanheira et al., 2011), Philippines (Lascols et al., 2013), Iran (Rajabnia et al., 2015), Taiwan (Tseng et al., 2015), Colombia (Rojas et al., 2013), Mexico (Gales et al., 2012), and Algeria (Rodriguez-Martinez et al., 2010)
	GIM-1, KHM-1, and SPM-1	Not found

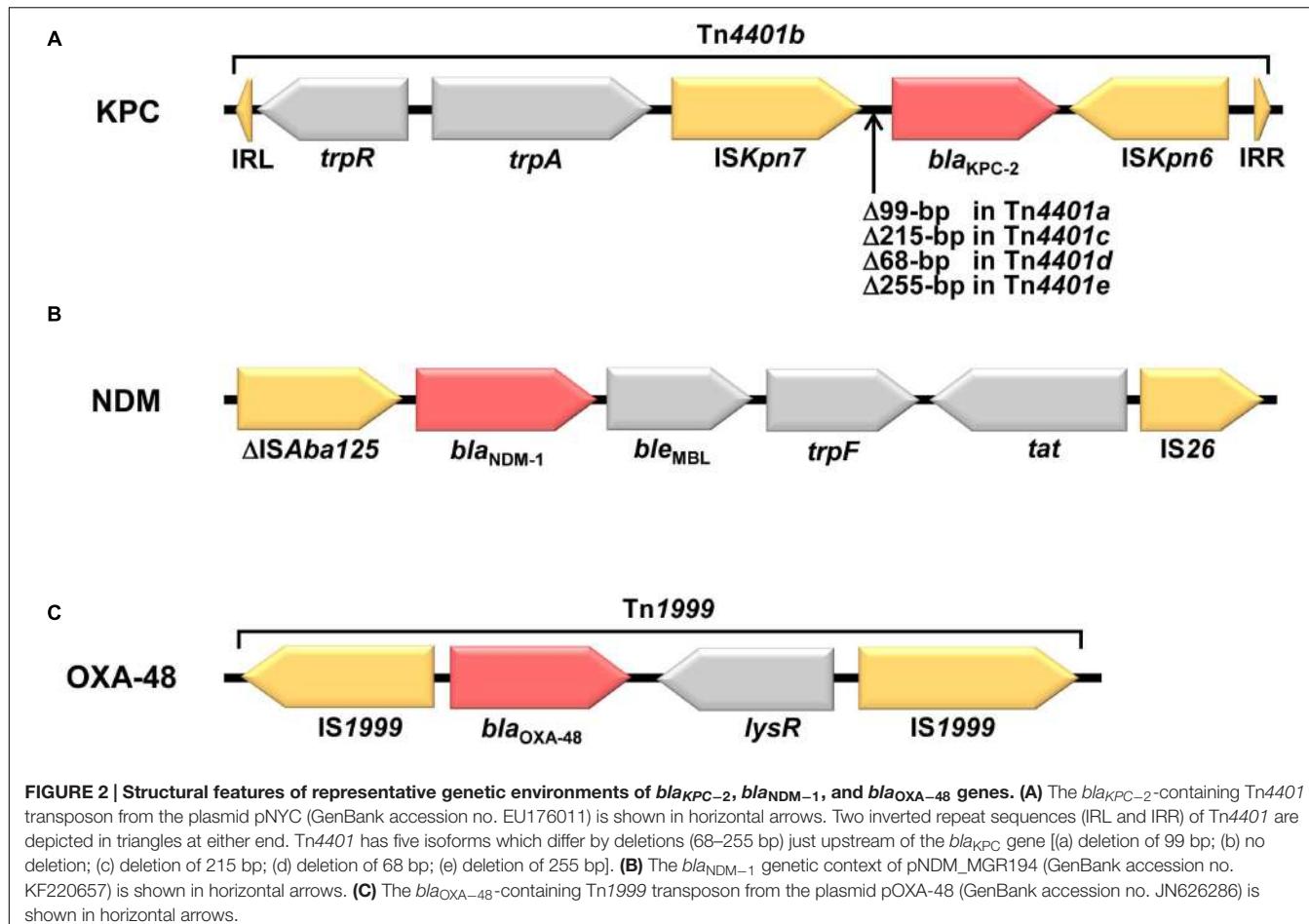
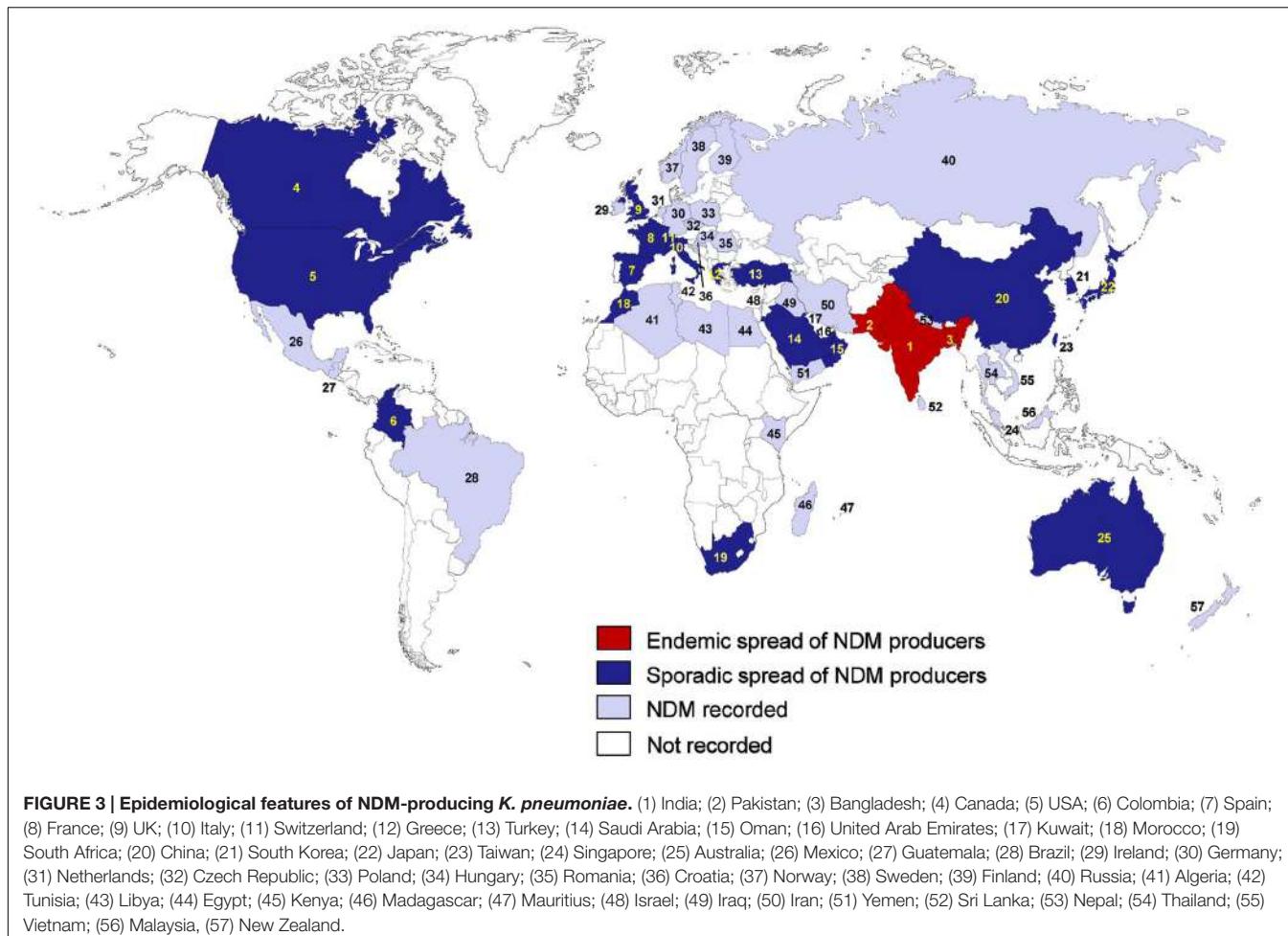


FIGURE 2 | Structural features of representative genetic environments of *bla_{KPC-2}*, *bla_{NDM-1}*, and *bla_{OXA-48}* genes. (A) The *bla_{KPC-2}*-containing Tn4401 transposon from the plasmid pNYC (GenBank accession no. EU176011) is shown in horizontal arrows. Two inverted repeat sequences (IRL and IRR) of Tn4401 are depicted in triangles at either end. Tn4401 has five isoforms which differ by deletions (68–255 bp) just upstream of the *bla_{KPC}* gene [(a) deletion of 99 bp; (b) no deletion; (c) deletion of 215 bp; (d) deletion of 68 bp; (e) deletion of 255 bp]. **(B)** The *bla_{NDM-1}* genetic context of pNDM_MGR194 (GenBank accession no. KF220657) is shown in horizontal arrows. **(C)** The *bla_{OXA-48}*-containing Tn1999 transposon from the plasmid pOXA-48 (GenBank accession no. JN626286) is shown in horizontal arrows.

gene, and the *ISKpn6*-like element (Shen et al., 2009). This genetic structure is the chimera form of several transposon-associated elements. This transposon was also identified in many other countries (Chen et al., 2014e), and several variants with various fragment insertions between the *ISKpn8* and *bla_{KPC}* gene have been found among *Enterobacteriaceae* in China (Shen et al., 2009; Li et al., 2011; Qi et al., 2011). Tn4401 has five isoforms which differ by deletions (68–255 bp) just upstream of the *bla_{KPC}* gene [(a) deletion of 99 bp; (b) no deletion; (c) deletion of 215 bp; (d) deletion of 68 bp; (e) deletion of 255 bp; Chen et al., 2014e]. Notably, in many cases, different Tn4401 isoforms were associated with different *bla_{KPC}*-harboring plasmids. Tn4401a was frequently found in the *bla_{KPC-3}*-harboring IncFII_{K2} plasmids (Leavitt et al., 2010; Garcia-Fernandez et al., 2012; Chen et al., 2014d), and Tn4401b and Tn4401d were often associated with the IncN and IncFIA plasmids, respectively (Chen et al., 2013a, 2014c,e). Up to now, more than 30 *bla_{KPC}*-harboring plasmids obtained from *K. pneumoniae* have been sequenced (Gootz et al., 2009; Shen et al., 2009; Jiang et al., 2010; Leavitt et al., 2010; Almeida et al., 2012; Chen et al., 2013a,b, 2014b,c,e). One of common features shared by these sequenced plasmids is the presence of the *tra* operon, which encodes the plasmid conjugation machinery proteins that induce the spread of plasmids (Chen et al., 2014e).

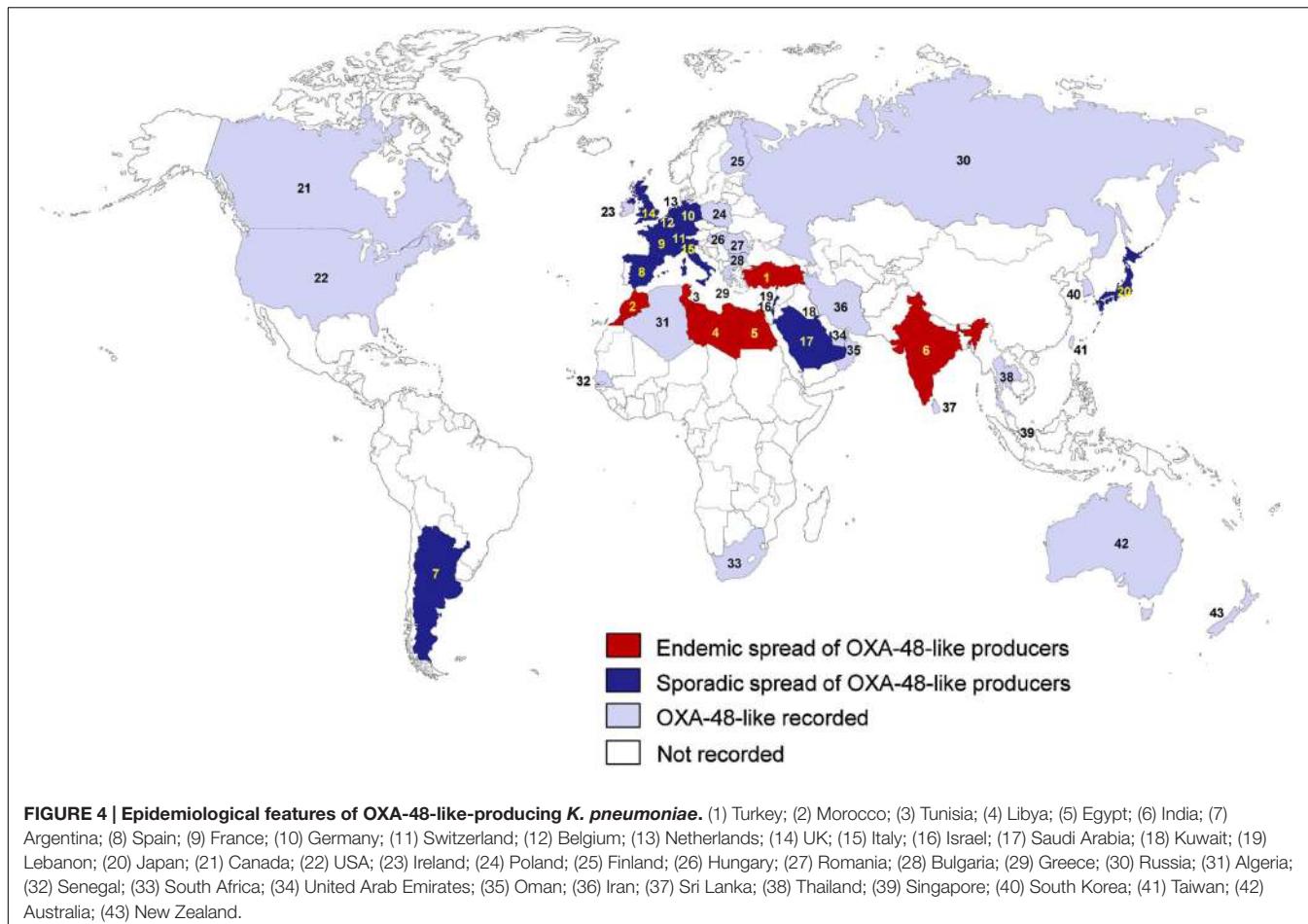
These genes may be important for the successful dissemination of *bla_{KPC}*-harboring plasmids. The IncFII plasmids are one of predominant *bla_{KPC}*-harboring plasmids. pKpQIL, which is an IncFII_{K2} plasmid harboring Tn4401a, was initially identified in Israel in 2006 (Leavitt et al., 2010), and then this plasmid and its variants are believed to have spread to Italy, Poland, the UK, Colombia, the Czech Republic, the USA, and other countries (Baraniak et al., 2011; Garcia-Fernandez et al., 2012; Hidalgo-Grass et al., 2012; Warburg et al., 2012; Hrabak et al., 2013b; Chen et al., 2014d,e), suggesting the wide dissemination of this plasmid. The *bla_{KPC}* gene has also been identified in other non-Tn4401 mobile elements that mostly have partial *ISKpn6* genes (Shen et al., 2009; Gomez et al., 2011). Based on the insertion sequence upstream of the *bla_{KPC}* gene, they can be divided into three groups: group I, no insertion (Shen et al., 2009; Liu et al., 2012; Chen et al., 2014g); group II, insertion of truncated *bla_{TEM}* (Gomez et al., 2011); group III, insertion of Tn5563/IS6100 (Wolter et al., 2009). These non-Tn4401 genetic elements harboring *bla_{KPC}* sometimes have an IS26 transposon (Liu et al., 2012; Chen et al., 2014g).

Carbapenemase genes often spread worldwide through clonal expansion in several successful pathogenic strains (Chen et al., 2014e). For example, the dissemination of KPC-producing *K. pneumoniae* in most countries including the USA and



European countries is largely caused by expansion of a single dominant strain, ST258 (Giani et al., 2013; Chen et al., 2014e; Bowers et al., 2015). This strain is a prototype of a high-risk clone of *K. pneumoniae*. Recent data from Israel showed that the KPC-producing *K. pneumoniae* ST258 clone remains the predominant clone, representing 80% of the KPC-producing *K. pneumoniae* population (Adler et al., 2015). ST258 may be a hybrid clone that was created by a large recombination event between ST11 and ST442 (Pitout et al., 2015). It is unknown why the ST258 lineage is the most prevalent clone of KPC-producing *Klebsiella* species. The ST258 clone is highly susceptible to serum killing in animal models and lacks well-known *K. pneumoniae* virulence factors, such as aerobactin genes, K1, K2, and K5 capsular antigen genes, and the regulator of the mucoid phenotype gene *rmpA* (Tzouvelekis et al., 2013; Pitout et al., 2015). Two recent reports revealed that the ST258 strains consist of two distinct genetic clades and genetic differentiation between the two clades (-1 and *cps*-2) results from an approximately 215-kb region of divergence that includes *cps* genes involved in capsule polysaccharide synthesis (Chen et al., 2014e; Deleo et al., 2014). Multiplex PCR for the identification of two capsular types in *K. pneumoniae* ST258 strains revealed a significant association between the *cps* type and KPC variant: the *cps*-1 clade is largely associated with

KPC-2, while the *cps*-2 clade is primarily associated with KPC-3 (Chen et al., 2014a). Because the capsule polysaccharide can help *K. pneumoniae* to evade phagocytosis, the global success of this strain may involve the capsule polysaccharide biosynthesis regions *cps*-1 and *cps*-2. A recent report revealed a relationship between the integrative conjugative element ICEKp258.2 and the global success of the ST258 clone (Adler et al., 2014). ICEKp258.2 contains two specific gene clusters, a type IV pilus gene cluster (i.e., *pilV*) associated with the uptake and exchange of plasmids and adherence to living and non-living surfaces, and a gene cluster of a type III restriction-modification system determining host specificity in the exchange of certain compatible plasmids or mobile elements (Adler et al., 2014). Because these genes associated with the restriction of plasmids and specific mobile elements were present only in ST258 and genetically related sequence types, this difference may explain the divergence of ST258 predominantly harboring KPC and ST11, another high-risk clone that lacks ICEKp258.2, harboring a broad range of plasmids and carbapenemases, including KPC, NDM, OXA-48, VIM, and IMP (Chen et al., 2014f; Pitout et al., 2015). Although the ICEKp258.2 of ST258 strains may contribute to global success, the precise reason for the predominance of the ST258 strain in KPC-producing *K. pneumoniae* is still not entirely



understood. Recently, an outbreak of non-ST258 KPC-producing *K. pneumoniae* clones has been reported in the USA and Europe (Ruiz-Garbajosa et al., 2013; Bonura et al., 2015; Garbari et al., 2015).

The habitat of *K. pneumoniae* is not limited to humans but extends to the ecological environment, such as soil, water, and sewage, and *K. pneumoniae* can survive in extreme environments for long periods of time (Pitout et al., 2015). Therefore, *K. pneumoniae* producing KPCs were detected in various nosocomial environments, such as gowns and gloves (Rock et al., 2014) and wastewater (Chagas et al., 2011; Galler et al., 2014). The frequency of KPC-producing *K. pneumoniae* contamination of gowns and gloves of healthcare workers is similar to that of contamination with methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* (Rock et al., 2014), indicating fast transmission of KPC-producing *Klebsiella* species in a nosocomial environment. A long-term observation in a hospital with low-frequency outbreaks of KPC-producing *K. pneumoniae* suggested the possible role of a persisting environmental reservoir of resistant strains in the maintenance of this long-term outbreak (Tofteland et al., 2013). After discharge from the hospital, long-term (>3 years) carriage of KPC-producing *K. pneumoniae* is also possible (Lübbert et al., 2014), and lateral gene transfer of KPC among *Enterobacteriaceae*

colonizing the human intestine appears frequent, for example from *K. pneumoniae* to *E. coli* (Richter et al., 2011; Gona et al., 2014). Therefore, reservoirs in healthcare workers, patients, or the hospital environment may be a principle mode of spread in nosocomial outbreaks.

Treatment Options

Carbapenemase-producing *K. pneumoniae* strains are currently one of the most important nosocomial pathogens. Hospital outbreaks of KPC-producing *K. pneumoniae* mainly affect severely ill patients and are associated with an increased risk of death (Ducombe et al., 2015; Tumbarello et al., 2015). KPC-producing *K. pneumoniae* bloodstream infections in intensive care unit (ICU) have also been associated with increased mortality (Chang et al., 2015).

Because carbapenemase-producing *K. pneumoniae* are mostly resistant to several important antibiotic classes (β -lactam drugs, fluoroquinolones, and aminoglycosides), antibiotics, such as polymyxin B, colistin (polymyxin E), fosfomycin, tigecycline, and sometimes selected aminoglycosides, are the last-resort agents. KPC-producing *K. pneumoniae* are usually resistant to all β -lactam antibiotics, but temocillin can be active against some KPC-producing *K. pneumoniae*, particularly in the case of lower urinary tract infections (Adams-Haduch et al., 2009).

To maximize bacterial killing and minimize bacterial resistance, combined therapy is sometimes recommended. Combination therapy including a carbapenem, such as a combination of tigecycline, colistin, and meropenem, was strongly effective in the treatment of KPC-producing *K. pneumoniae*, including colistin-resistant isolates (Tumbarello et al., 2012, 2015; Giamarellou et al., 2013; Hong et al., 2013; Daikos et al., 2014). The synergistic combination of colistin and rifampin was also effective in the treatment of colistin-resistant KPC-producing *K. pneumoniae* by slowing the selection of heteroresistant subpopulations during colistin therapy (Tascini et al., 2013). However, several reports have shown that combination therapy was not superior to monotherapy (de Oliveira et al., 2015; Toledo et al., 2015). Thus, extensive studies will be required to assess the effectiveness of combination therapy. A triple combination of colistin-doripenem-ertapenem was effective only in isolates with high levels of OmpK35 and OmpK36 porin expression (Hong et al., 2013). The expression level of OmpK36 was also involved in the rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing *K. pneumoniae* populations (Adams-Sapper et al., 2015). Therefore, molecular characterization of the KPC-producing *K. pneumoniae* strain, such as the determination of the expression level of OmpK35 and OmpK36, can be used to identify effective combination regimens. However, as a minor effect of OmpK35 and OmpK36 on carbapenem resistance of *K. pneumoniae* was also reported (Zhang et al., 2014), more extensive studies on the role of these proteins on *K. pneumoniae* carbapenem resistance are also required.

Colistin (polymyxin E), an agent discovered more than 60 years ago, is a key component of the combination of antimicrobial regimens used for the treatment of severe KPC-producing *K. pneumoniae* infections (Cannatelli et al., 2014b). Since the global spread of KPC-producing *K. pneumoniae*, the emergence of colistin resistance in KPC-producing *K. pneumoniae* have been reported in many countries, including Italy (Cannatelli et al., 2014b; Giani et al., 2015), the USA (Bogdanovich et al., 2011), Greece (Kontopoulou et al., 2010), Hungary (Toth et al., 2010), and Turkey (Labarca et al., 2014). The increasing prevalence of colistin-resistant *K. pneumoniae* producing KPC poses a threat to public health because colistin resistance increases the mortality due to KPC-producing *K. pneumoniae* bloodstream infections and reduces therapeutic options. A multicenter case-control-control study in Italy showed that the rate of colistin resistance among KPC-producing *K. pneumoniae* blood isolates increased more than threefold during the 4.5-years study period, and the 30-days mortality due to colistin-resistant KPC-producing *K. pneumoniae* bloodstream infections was approximately 51% (Giacobbe et al., 2015). Data collected from 21 hospital laboratories in Italy between 2013 and 2014 also showed that 43% of carbapenemase-producing *K. pneumoniae* were resistant to colistin, 6% were resistant to tigecycline, 16% were resistant to gentamicin, 82% were resistant to trimethoprim-sulfamethoxazole, and 1% were resistant to all four antibiotics, and colistin-resistant isolates were detected in all participating hospital laboratories (Monaco et al., 2014). The progressive increase of colistin resistance

was also reported elsewhere (Pena et al., 2014; Bonura et al., 2015; Parisi et al., 2015). These results indicate that the strict rules for colistin use are required to diminish the spread of colistin resistance in the endemic regions of KPC-producing *K. pneumoniae*.

Molecular and biochemical studies have shown that insertional inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the PhoQ-PhoP signaling system, can be responsible for colistin resistance in KPC-producing *K. pneumoniae*, due to the resulting up-regulation of the *Pmr* lipopolysaccharide modification system (Cannatelli et al., 2013, 2014b). A recent study analyzing a series of colistin-resistant *K. pneumoniae* isolates of worldwide origin identified a single amino acid change (T157P) in the *PmrB* protein as being responsible for the overexpression of *pmrCAB* and *pmrHFIIJKL* operons involved in lipopolysaccharide modification, leading to colistin resistance (Jayol et al., 2014). The relationship between colistin resistance and inactivation of the *mgrB* gene was further supported by analysis of clinical colistin-resistant *K. pneumoniae* isolates producing KPC (Bonura et al., 2015; Giani et al., 2015). The emergence of colistin resistance was also associated with low-dosage colistin treatment (Cannatelli et al., 2014a). A recent report showed that the plasmid carrying the *mcr-1* gene, which encodes a phosphoethanolamine transferase enzyme catalyzing the addition of phosphoethanolamine to lipid A, is a major contributor to colistin resistance in Gram-negative bacteria and is spread through horizontal gene transfer (Liu et al., 2016). This *mcr-1*-harboring plasmid was also detected in *E. coli* isolates collected from 78 (15%) of 523 samples of raw meat, 166 (21%) of 804 animals, and 16 (1%) of 1322 samples from inpatients with infection, indicating the emergence of this plasmid-mediated colistin resistance mechanism (Liu et al., 2016).

Fosfomycin is a broad-spectrum antibiotic that inhibits bacterial cell wall biogenesis by inactivating the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA (Brown et al., 1995). Fosfomycin has been used to treat KPC-producing *K. pneumoniae*, but recently, a high fosfomycin resistance rate was observed in countries with higher usage (Giske, 2015). Only 43.4% of KPC-producing *K. pneumoniae* strains retained susceptibility to fosfomycin in a Chinese university hospital (Li et al., 2012) and a similar fosfomycin susceptibility rate (39.2%) was observed in KPC-producing *K. pneumoniae* collected from 12 hospitals in China (Jiang et al., 2015). Like colistin, fosfomycin resistance seems to be associated with the plasmid containing the *fosA3* gene which encodes glutathione S-transferase to modify fosfomycin and was characterized first in CTX-M-producing *E. coli* in Japan (Wachino et al., 2010). In China, the *fosA3*-harboring plasmid was attributed to 55.6% of fosfomycin-resistant KPC-producing *K. pneumoniae* strains (Jiang et al., 2015). Although the *fosA3* gene is mainly associated with the *blaCTX-M* gene, the *fosA3* gene has also been characterized in atypical *blaKPC*-harboring plasmids (Jiang et al., 2015; Li et al., 2015). In pFOS18 (Jiang et al., 2015) and pKP1034 (Xiang et al., 2015), the *fosA3* and *blaKPC-2* genes were located on different transposon systems, whereas in pHS102707 belonging to the IncP1 group (Li et al.,

2015), two genes were co-located in the same Tn1721-Tn3-like transposon.

Tigecycline, a derivative of minocycline, is the first member of the glycylcycline class that acts as a protein synthesis inhibitor by blocking the interaction of aminoacyl-tRNA with the A site of the ribosome (Rose and Rybak, 2006). Due to the increased clinical use of tigecycline for treatment of KPC-producing *K. pneumoniae*, increased tigecycline resistance was reported (Zagorianou et al., 2012; Papadimitriou-Olivgeris et al., 2014; Weterings et al., 2015). In the Netherlands, all KPC-producing *K. pneumoniae* isolates exhibited reduced susceptibility to tigecycline (Weterings et al., 2015). Another report showed that during ICU stay, 17.9% (39/257) of patients became colonized by tigecycline-resistant KPC-producing *K. pneumoniae* during their stay (Papadimitriou-Olivgeris et al., 2014). In a Greek tertiary hospital during 2004 to 2010, 11.3% (34/301) of KPC-producing isolates were resistant to tigecycline (Zagorianou et al., 2012). Overproduction of efflux pumps such as AcrAB and overexpression of RamA, a positive regulator of the AcrAB efflux system, seem to be major factors for decreased sensitivity of *K. pneumoniae* strains to tigecycline (Ruzin et al., 2005; Rosenblum et al., 2011; Sun et al., 2013). A recent report in China showed that the OqxAB efflux pump was also contributed to tigecycline resistance in *K. pneumoniae* isolates (Zhong et al., 2014).

Because KPC-producing *K. pneumoniae* sometimes remains susceptible to several aminoglycosides such as gentamicin (Tzouvelekis et al., 2014), aminoglycosides can be used alone or in combination therapy to treat KPC-producing *K. pneumoniae* infections. Actually, gentamicin monotherapy or in combination with tigecycline was recently reported to reduce the mortality from sepsis caused by *K. pneumoniae* ST512 clone producing KPC-3, SHV-11, or TEM-1 (Gonzalez-Padilla et al., 2015). New weapons are always indispensable for combating KPC-producing *K. pneumoniae* infections (Lee et al., 2007, 2015a). The effectiveness of some antibiotics in development was also estimated against KPC-producing *K. pneumoniae*. Potent inhibitors of serine β -lactamases, such as avibactam and MK7655, were effective against KPC-producing *K. pneumoniae* infections (Temkin et al., 2014). Combination therapy with avibactam and ceftazidime exhibited significant synergistic effects against organisms with combinations of extended-spectrum β -lactamases (ESBLs), AmpCs, and KPC-2 (Wang et al., 2014e). Plazomicin (a novel aminoglycoside) also exhibited significant activity against KPC-producing *K. pneumoniae* (Temkin et al., 2014). The novel polymyxin derivatives with lower nephrotoxicity are under development (Vaara, 2010). A recent report suggested that synthetic peptides with antimicrobial and antibiofilm activities are a promising strategy in the treatment of infections caused by KPC-producing *K. pneumoniae* (Ribeiro et al., 2015). The *in vitro* activity of the next-generation aminoglycoside plazomicin alone and in combination with colistin, meropenem, fosfomycin or tigecycline was tested against carbapenemase-producing *Enterobacteriaceae* (CPE) strains. When plazomicin was combined with meropenem, colistin or fosfomycin, synergy was observed against CPE isolates (Rodriguez-Avial et al., 2015).

Detection Methods

Because a delay in the appropriate antibiotic therapy for severe infections of KPC-producing *K. pneumoniae* is strongly associated with unfavorable prognosis and increased mortality rates (Karaiskos and Giamarellou, 2014), rapid detection of CR strains is essential for the effective management of these infections (Lee et al., 2005b, 2006b, 2016). Various methods for the identification of KPCs have been developed, including multiplex PCR assay (Spanu et al., 2012; Adler et al., 2014), real-time PCR assay (Wang et al., 2012; Lee et al., 2015c), DNA microarray (Peter et al., 2012; Braun et al., 2014), Raman spectroscopic analysis (Willemse-Erix et al., 2012), single-colony whole-genome sequencing (Koser et al., 2014), matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS; Carvalhaes et al., 2014), loop-mediated isothermal amplification (LAMP) method (Nakano et al., 2015), chromogenic medium (Vrioni et al., 2012), and a new phenotypic test, called the carbapenem inactivation method (CIM; van der Zwaluw et al., 2015). False positive results can occur when the modified Hodge test is used to detect carbapenemases in carbapenemase-negative *K. pneumoniae* clinical isolates (Wang et al., 2011). Therefore, to improve the efficiency in the phenotypic detection of KPC-producing *K. pneumoniae* isolates, the modified Hodge test can be combined with an EDTA disk test (Yan et al., 2014) or a disk test using boronic acid compounds (Pournaras et al., 2010a). These methods enhanced the sensitivity and specificity of KPC detection in *K. pneumoniae* isolates. In a new developed phenotypic test, called the CIM, a susceptibility-testing disk containing carbapenem was immersed in the suspension made by suspending an inoculation loop of bacterial culture (van der Zwaluw et al., 2015). After incubation, the disk was placed on an agar plate inoculated with a susceptible *E. coli* indicator strain. If the bacterial isolate produces carbapenemase, the susceptibility-testing disk will allow the growth of the susceptible indicator strain. This method showed high concordance with results obtained by PCR (van der Zwaluw et al., 2015). Nevertheless, these culture-based phenotypic tests are time-consuming and cannot easily detect ESBLs and carbapenemases produced by *Enterobacteriaceae*, owing to varied levels of enzyme expression and the poor specificity of some antibiotic combinations (Okeke et al., 2011; Swayne et al., 2013). To overcome these limitations of phenotypic methods, various molecular-based diagnostic methods have been developed. Especially, the direct detection of the carbapenemase gene using multiplex PCR, real-time PCR, and DNA microarray method can increase the speed and accuracy of detecting CR strains (Okeke et al., 2011; Solanki et al., 2014). Because of the high genetic diversity of genes coding for carbapenemase, the precise design of primers or probes is necessary for correctly amplifying or detecting only expected carbapenemase genes. Therefore, already developed methods for *bla* gene detection are restricted to the detection of only limited types of *bla* genes (Lee et al., 2015b). However, the large-scale *bla*Finder was recently developed on the basis of multiplex PCR, and this large-scale detection method can detect almost all *bla* genes, including KPCs, NDMs, OXA-48-likes, present in bacterial pathogens (Lee et al., 2015b).

Recently, mass spectrometry-based methods, such as MALDI-TOF MS and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), have been shown to be capable of characterizing carbapenemase-producing bacteria (Carricajo et al., 2014; Carvalhaes et al., 2014). These methods are fast and accurate to routinely identify bacterial isolates with great specificity and sensitivity (Hrabak et al., 2011; Patel, 2013; Carvalhaes et al., 2014; Lasserre et al., 2015), but these systems do not accurately provide the carbapenem minimum inhibitory concentrations (MICs) for carbapenemase-producing *K. pneumoniae* (Patel, 2013). Several experiments showed that this method is more rapid and accurate for detection of carbapenemase activity in Gram-negative bacteria than some methods including the modified Hodge test (Lee et al., 2013b; Chong et al., 2015). The LAMP method has emerged as a powerful gene amplification assay for the rapid identification of microbial infections (Notomi et al., 2000). This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. The assay amplifies the DNA under isothermal conditions (63–65°C) with high degrees of specificity, efficiency, and speed (Reddy et al., 2010). The cycling reaction continues with accumulation of 10⁹ copies of target in less than an hour. The assay can be conducted in a water bath or heating block instead of the thermal cycling using a PCR machine (Notomi et al., 2000). The LAMP assay can be applied for detection of KPC producers in the clinical laboratory (Nakano et al., 2015) and has greater sensitivity, specificity, and rapidity compared to the phenotypic methods and PCR for the detection of KPC-producing *K. pneumoniae* (Solanki et al., 2013).

Colistin has often been used as a therapeutic option for the treatment of CR *K. pneumoniae* infections. However, the imprudent use of colistin has caused rapid spread of colistin resistance in *K. pneumoniae* producing carbapenemases, particularly the KPC-type carbapenemases (Monaco et al., 2014; Giacobbe et al., 2015). This situation demonstrates the need for the development of accurate and reliable methods for detecting colistin resistance. Recently, several methods for the identification of colistin resistance were reported, including various routine colistin MIC testing methods, such as BMD, BMD-P80, AD, Etest, MTS, and Vitek2 (Dafopoulos et al., 2015; Humphries, 2015); capillary electrophoresis method according to characteristic surface properties of bacteria (Sautrey et al., 2015); and the micromax assay based on evaluation of the efficacy of antibiotics that affect cell wall integrity (Tamayo et al., 2013). Because a recent report showed that the *mcr-1* gene, involved in the modification of lipid A, is a major contributor to colistin resistance in Gram-negative bacteria (Liu et al., 2016), detection of this gene may be important in the detection of colistin resistance.

Class B Carbapenemases

Epidemiology

Class B β-lactamases are metallo-β-lactamases that require zinc or another heavy metal for catalysis. Class B β-lactamases have a broad substrate spectrum and can catalyze the hydrolysis of

virtually all β-lactam antibiotics including carbapenems except for monobactams (Jeon et al., 2015). Class B carbapenemases were mostly identified in *Enterobacteriaceae* and include VIMs, IMPs, and the emerging NDM group (Jeon et al., 2015). Among them, NDM (New Delhi metallo-β-lactamase) is one of the most clinically significant carbapenemases. NDM-1 was first detected in 2008 in *K. pneumoniae* and *E. coli* in a patient returning to Sweden from India and has since spread worldwide (Yong et al., 2009; Jeon et al., 2015). Thus far, 15 NDM variants have been assigned (Jeon et al., 2015), and most of them originated from Asia (Nordmann and Poirel, 2014). NDMs shares very little identity with other metallo-β-lactamases (Nordmann and Poirel, 2014).

Since 2008, *K. pneumoniae* producing NDMs rapidly spread in many countries (Berrazeg et al., 2014; **Figure 3**). NDM-producing *K. pneumoniae* are considered to be endemic in the Indian subcontinent, including India, Pakistan, and Bangladesh (Nordmann et al., 2011b; Nordmann and Poirel, 2014). The sporadic spread has been reported in the USA (Rasheed et al., 2013; Centers for Disease Control and Prevention, 2014; Doi et al., 2014), Canada (Mulvey et al., 2011; Borgia et al., 2012; Lowe et al., 2013), Colombia (Escobar Perez et al., 2013; Ocampo et al., 2015), Spain (Oteo et al., 2013b; Seara et al., 2015), France (Arpin et al., 2012; Robert et al., 2014), Switzerland (Poirel et al., 2011g; Spyropoulou et al., 2016), Italy (Gaibani et al., 2011), the UK (Kumarasamy et al., 2010; Giske et al., 2012), Greece (Voulgari et al., 2014; Spyropoulou et al., 2016), Turkey (Poirel et al., 2014; Kilic and Baysallar, 2015), Morocco (Poirel et al., 2011b; Barguigua et al., 2013), South Africa (Brink et al., 2012; de Jager et al., 2015), Singapore (Chen et al., 2012; Balm et al., 2013; Ling et al., 2015), Saudi Arabia (Shibl et al., 2013; Zowawi et al., 2014), Oman (Poirel et al., 2011a; Zowawi et al., 2014), United Arab Emirates (Sonnevend et al., 2013; Dash et al., 2014), Kuwait (Jamal et al., 2012, 2013; Sonnevend et al., 2015b), China (Qin et al., 2014; Jin et al., 2015; Liu et al., 2015), Japan (Yamamoto et al., 2013; Nakano et al., 2014), Taiwan (Chiu et al., 2013; Tseng et al., 2015), South Korea (Kim et al., 2012; Cho et al., 2015), and Australia (Shoma et al., 2014; Wailan et al., 2015). In India, NDM-1 was the most common carbapenemase type detected and accounted for 75.22% of the carbapenemase-producing isolates (Kazi et al., 2015). In Singapore and the United Arab Emirates, NDM-1 also was the most common carbapenemase type observed (44.4 and 100%, respectively; Dash et al., 2014; Ling et al., 2015). The endemic spread of NDM-producing *K. pneumoniae* has also been reported in the UK, which has close relationships with India and Pakistan (Nordmann and Poirel, 2014). In China, NDM-1 has been found mostly in *Acinetobacter* spp., but data obtained from patients between June 2011 and July 2012 showed that 33.3% of the CRE isolates, including *K. pneumoniae*, had NDM-1, suggesting the possible transmission of *bla*_{NDM-1}-containing sequences from *Acinetobacter* spp. to *Enterobacteriaceae* (Qin et al., 2014). These findings reveal the emergence and active transmission of NDM-1-producing *K. pneumoniae* in China. Comparative analyses of the conserved NDM-1-encoding region among different plasmids from *K. pneumoniae* and *E. coli* suggested that

the transposable elements and two unknown inverted repeat-associated elements flanking the NDM-1-encoding region aided the spreading of this resistance determinant (Chen et al., 2012). Recently, in China, eight *K. pneumoniae* isolates producing NDM-1 were identified in the neonatal ward of a teaching hospital (Zhang et al., 2015), and four diverse types (NDM-1, KPC-2, VIM-2, and IMP-4) of carbapenemase of *K. pneumoniae* clones were identified in a single hospital in China (Liu et al., 2015).

The Balkan states (Livermore et al., 2011; Voulgari et al., 2014), the Arabian Peninsula (Nordmann and Poirel, 2014), and North African countries (Dortet et al., 2014c), have also been recently considered as an additional reservoir of NDM producers. In the Arabian Peninsula, NDM-1 was the most frequently encountered carbapenemase (46.5%) followed by OXA-48-like carbapenemases (32.5%; Sonnevend et al., 2015b). In Greece, among 132 non-repetitive CRE isolates between 2010 and 2013, 78 *K. pneumoniae* isolates with the *bla*_{NDM-1} gene were identified (Voulgari et al., 2014). In the USA, KPC-producing *K. pneumoniae* have been responsible for much of the increase in carbapenemase-producing bacteria detection, but recent increases in NDM-producing *K. pneumoniae* have the potential to add to this burden (Rasheed et al., 2013; Centers for Disease Control and Prevention, 2014).

The movement of patients between countries may be a trigger for the international spread of carbapenemase-producing *K. pneumoniae* (Berrazeg et al., 2014). Carbapenemase-producing Gram-negative bacteria including *K. pneumoniae*, obtained from patients that had recently traveled outside Canada between 2010 and 2013, were found to be NDM-producing *K. pneumoniae*, belonging to various sequence types (ST15, ST16, ST147, ST258, ST340, ST512, and ST972) with different plasmids (IncF, IncA/C, and IncL/M), and were imported from India to Canada (Peirano et al., 2014). Therefore, more careful attention is required when treating patients with a recent history of foreign hospitalization in countries where carbapenemase-producing bacteria are prevalent. The international transportations of patients between countries recently resulted in the detection of NDM-producing *K. pneumoniae* in Mexico (Barrios et al., 2014), Guatemala (Pasteran et al., 2012), Brazil (Quiles et al., 2015), the Netherlands (Bathoorn et al., 2015), Ireland (McDermott et al., 2012), Poland (Baraniak et al., 2016), Czech Republic (Studentova et al., 2015), Croatia (Kocsis et al., 2016), Russia (Ageevets et al., 2014), Tunisia (Ben Nasr et al., 2013), Romania (Lixandru et al., 2015), Egypt (Bathoorn et al., 2013), Kenya (Poirel et al., 2011f), Madagascar (Chereau et al., 2015), Iraq (Poirel et al., 2011e), Yemen (Gharout-Sait et al., 2014), Iran (Shahcheraghi et al., 2013), Mauritius (Poirel et al., 2012b), Sri Lanka (Dortet et al., 2013), Thailand (Rimrang et al., 2012), Nepal (Tada et al., 2013), Vietnam (Hoang et al., 2013), Malaysia (Al-Marzooq et al., 2015), and New Zealand (Williamson et al., 2012).

The global dissemination of NDM-producing *K. pneumoniae* also has a serious impact on neonatal mortality rates, particularly in low-income countries where the burden of neonatal sepsis is high (Zaidi et al., 2005). Colonization of NDM-producing *K. pneumoniae* isolates in pregnant women in the community

in Madagascar highlighted the potential for mother-to-child transmission (Chereau et al., 2015). In India, analysis of *Enterobacteriaceae*, including *K. pneumoniae*, isolated from the blood of septicaemic neonates, indicated that 14% of the isolates possessed *bla*_{NDM-1}, and there was a significantly higher incidence of sepsis caused by NDM-1-harboring isolates (Datta et al., 2014). In China, an outbreak of *bla*_{NDM-1}-producing *K. pneumoniae* ST20 and ST17 isolates was identified in a neonatal unit (Jin et al., 2015). In Turkey, the spread of NDM-1-producing *K. pneumoniae* in a neonatal ICU was reported (Poirel et al., 2014). In Colombia, NDM-1-producing *K. pneumoniae* strains were identified from an outbreak that affected six neonatal patients (Escobar Perez et al., 2013).

As with KPC, coexistence of NDMs and other carbapenemases in *K. pneumoniae* has also been reported worldwide, in Brazil (NDM-1/KPC-2; Pereira et al., 2015), Malaysia (NDM-1/OXA-232; Al-Marzooq et al., 2015), South Korea (NDM-5/OXA-181; Cho et al., 2015), China (NDM-1/IMP-4; Chen et al., 2015), India (NDM-1/OXA-232; Al-Marzooq et al., 2015), Turkey (NDM-1/OXA-48; Kilic and Baysallar, 2015), Pakistan (NDM-1/KPC-2; Sattar et al., 2014), Switzerland (NDM-1/OXA-48; Seiffert et al., 2014), United Arab Emirates (NDM-1/OXA-48-like; Dash et al., 2014), Australia (NDM-1/OXA-48; Sidjabat et al., 2015), Morocco (NDM-1/OXA-48; Barguigua et al., 2013), Singapore (NDM-1/OXA-181 and NDM-5/OXA-181; Balm et al., 2013), and the USA (NDM-1/OXA-232; Doi et al., 2014).

Besides NDM-type carbapenemases, the IMP and VIM groups have also been detected worldwide in *K. pneumoniae*, but other carbapenemases, such as GIM-1, KHM-1, and SPM-1, have been not found in *K. pneumoniae* (Table 1). Since IMP and VIP were first detected in *Serratia marcescens* in 1991 and in *Pseudomonas aeruginosa* in 1996, respectively (Osano et al., 1994; Lauretti et al., 1999), IMP- and VIM-producing *K. pneumoniae* have spread in Europe and Asia, but were rarely reported in other regions, such as America and Africa (Table 1). Although in this review we have focused only on carbapenemase-producing *K. pneumoniae*, the VIM group is one of the most commonly reported carbapenemases worldwide if we consider all bacteria species (the VIM groups have been mainly identified in *P. aeruginosa*; Poirel et al., 2007).

Molecular and Genetic Context

The *bla*_{NDM} gene is frequently observed in the transposon Tn125 (with two flanking ISAb125 elements) within NDM-producing species of the genus *Acinetobacter* (Partridge and Iredell, 2012; Wailan et al., 2015). The *bla*_{NDM} gene was hypothesized to originate in the genus *Acinetobacter* (Toleman et al., 2012). In *Enterobacteriaceae*, the ISAb125 elements of the Tn125 structure carrying *bla*_{NDM} are frequently truncated (Tn125) at various lengths and the Tn125 structure frequently has different IS elements (Figure 2B; Partridge and Iredell, 2012; Wailan et al., 2015). The *bla*_{NDM} genes in *K. pneumoniae* have been reported on numerous broad-host-range plasmid types, including IncA/C (Hudson et al., 2014), IncF (Hishinuma et al., 2013), IncR (Studentova et al., 2015), IncH (Villa et al., 2012), IncN (Chen et al., 2014a), IncL/M (Peirano et al., 2014), and IncX types (Wang et al., 2014d). The

predominant plasmid type responsible for spreading *bla*_{NDM-1} is the IncA/C type plasmids (Poirel et al., 2011d; Pitout et al., 2015). Many IncA/C plasmids with *bla*_{NDM-1} also carry various antibiotic resistance genes including 16S rRNA methylases (RmtA and RmtC), associated with aminoglycoside resistance; CMY-type β -lactamases, associated with broad-spectrum cephalosporin resistance; and QnrA, associated with quinolone resistance (Pitout et al., 2015). Consequently, many NDM-producing *K. pneumoniae* were susceptible only to colistin, fosfomycin, and tigecycline (Nordmann and Poirel, 2014). A novel NDM-1 variant (NDM-9) located on a novel IncH variant plasmid was recently identified in a clinical *K. pneumoniae* isolate in China (Wang et al., 2014c). Because the *bla*_{NDM} genes are located in numerous broad-host-range plasmids, the spread of NDM-1 is facilitated by horizontal gene transfer between bacteria. In China, CRE 21 strains harboring the *bla*_{NDM-1} gene were found to consist of multiple *Enterobacteriaceae* species including nine *Enterobacter cloacae*, three *E. coli*, three *Citrobacter freundii*, two *K. pneumoniae*, two *K. oxytoca*, and two *Morganella morganii* strains (Wang et al., 2015).

Unlike KPC, the *bla*_{NDM} genes were detected in various *K. pneumoniae* clones. ST11, a major high-risk sequence type of KPC-producing *K. pneumoniae* in Asia, has also been associated with *bla*_{NDM-1} in *K. pneumoniae* identified in many countries, such as the USA (Rasheed et al., 2013), Greece (Voulgari et al., 2014), Australia (Shoma et al., 2014), Switzerland (Seiffert et al., 2014), the Czech Republic (Studentova et al., 2015), Spain (Oteo et al., 2013b), and Thailand (Netikul et al., 2014). The recent outbreak of NDM-producing *K. pneumoniae* ST11 in Poland was caused by a clone similar to an isolate identified in the Czech Republic in 2013 (Baraniak et al., 2016), indicating the local spread of this clone. ST11 has also been associated with OXA-48-like enzymes from isolates found in Argentina, Turkey, and Spain (Lascols et al., 2013; Oteo et al., 2013b). ST14, ST147, and ST340 have sometimes been associated with *bla*_{NDM} in *K. pneumoniae* in many countries (Peirano et al., 2011, 2014; Poirel et al., 2011d; Giske et al., 2012; Osterblad et al., 2012; Lascols et al., 2013; Gharout-Sait et al., 2014; Lee et al., 2014; Shoma et al., 2014; Wang et al., 2014d; Izdebski et al., 2015; Sonnevend et al., 2015b). Over 50% of NDM-producing *K. pneumoniae* isolates from India belonged to either ST11 or ST147 (Lascols et al., 2013). The analysis of clinical isolates of NDM-1-producing *K. pneumoniae* from India, the UK, and Sweden, showed that the most frequently detected sequence types were ST14, ST11, ST149, ST231, and ST147 (Giske et al., 2012). Although ST258 is a high-risk KPC-producing *K. pneumoniae* clone, ST258 harboring *bla*_{NDM} has never been reported, to the best of our knowledge. This phenomenon may result from the integrative conjugative element ICEKp258.2, present only in ST258 and genetically related sequence types (Adler et al., 2014). This genetic locus contains a type IV pilus gene cluster and a type III restriction-modification system. Because these genes are associated with the restriction of plasmids and specific mobile elements, most ST258 may predominantly harbor plasmids with the *bla*_{KPC} gene. Therefore, PCR for this unique region (ICEKp258.2) can

provide a reliable tool for the rapid detection of the ST258 clone.

High-resolution genomic analysis of multidrug-resistant hospital outbreaks of *K. pneumoniae* through whole-genome sequencing revealed the emergence of a capsule switching NDM-1 bearing *K. pneumoniae* ST15 strain, suggesting that further studies should concentrate on the diversity and spread of this specific clone (Chung The et al., 2015). ST15 harboring *bla*_{NDM-1} has often been reported in many countries, including Spain (Ruiz-Garajosa et al., 2013), Croatia (Kocsis et al., 2016), Thailand (Netikul et al., 2014), Canada (Peirano et al., 2014), China (Hu et al., 2013), France (Arpin et al., 2012), and Morocco (Poirel et al., 2011b). In Bulgaria, this clone was responsible for the clonal dissemination of KPC-2-producing *K. pneumoniae* (Markovska et al., 2015). Another whole-genome sequencing analysis of CR *K. pneumoniae* strains, which were isolated from 26 individuals involved in infections in a Nepali neonatal unit, showed that three temporally separated cases were caused by a single NDM-producing *K. pneumoniae* strain with four conserved plasmids including a plasmid carrying *bla*_{NDM-1} (Stoesser et al., 2014). The plasmids contained a large number of antimicrobial resistance and plasmid maintenance genes, which may explain their persistence (Stoesser et al., 2014). These reports suggest that whole-genome sequencing analysis play an important role in the elucidation of the factors that allow emergence and persistence of resistance.

Treatment Options

New-Delhi metallo- β -lactamase-producing *K. pneumoniae* are usually resistant to most β -lactam antibiotics but remain susceptible to aztreonam (Nordmann et al., 2012a). As with the case of KPC-producing *K. pneumoniae*, the effect of combination therapy was tested in the treatment of NDM-producing *K. pneumoniae* infections. When double- and triple-antibiotic combinations of aztreonam, ciprofloxacin, colistin, daptomycin, fosfomycin, meropenem, rifampin, telavancin, tigecycline, and vancomycin were used in patients infected with two NDM-producing *K. pneumoniae* strains susceptible to colistin, the combination of rifampin-meropenem-colistin was the most effective regimen against these strains (Tangden et al., 2014). The *in vitro* synergistic effect of the combination therapy of colistin and fosfomycin against NDM-producing *K. pneumoniae* has also been reported (Bercot et al., 2011). The combination of polymyxin B and chloramphenicol used against NDM-producing *K. pneumoniae* substantially enhanced bacterial killing and suppressed the emergence of polymyxin resistance (Abdul Rahim et al., 2015). Combination therapy including aztreonam and avibactam (a novel inhibitor of serine β -lactamases under development) was effective in the treatment of metallo- β -lactamase-producing bacterial infections (Wang et al., 2014e).

In the case of NDM-1-producing *Enterobacteriaceae* infections, carbapenems have been suggested to still represent a viable treatment option (Wiskirchen et al., 2014b). Despite unfavorable *in vitro* MICs of NDM-producing *K. pneumoniae*, recent *in vivo* studies have demonstrated the efficacy of carbapenems against NDM-1-producing isolates

in immunocompetent-mouse and neutropenic-mouse thigh infection models (Wiskirchen et al., 2013, 2014b; MacVane et al., 2014). Although high-dose, prolonged infusions of ertapenem or doripenem induced reduction in bacterial density, bacterial density was also reduced in standard infusions of ertapenem at 1 g every 24 h or of doripenem at 500 mg every 8 h (Wiskirchen et al., 2013, 2014b). Notably, these efficacies were observed only against NDM-1-producing *K. pneumoniae* (Wiskirchen et al., 2013, 2014b). In addition to carbapenems, this discordance between *in vitro* and *in vivo* activities against NDM-1-producing *K. pneumoniae* was also observed in human simulated regimens of ceftazidime at 2 g every 8 h or ceftazidime/avibactam at 2,000/500 mg every 8 h (MacVane et al., 2014). Despite experiments in immunocompetent-mouse and neutropenic-mouse thigh infection models, these results show that standard infusions of ertapenem and doripenem could reduce bacterial density. Therefore, further experiments in human are required to determine whether carbapenems are sometimes a viable treatment option for NDM-1-producing *K. pneumoniae* infections.

The copy number of *bla*_{NDM-1} was assessed using Southern blotting and quantitative PCR under different conditions. The *bla*_{NDM-1} sequence was maintained under antibiotic selection; however, removal of the antibiotic selection led to the emergence of susceptible bacterial populations with a reduced copy number or even the complete loss of the *bla*_{NDM-1} gene (Huang et al., 2013). The dynamic nature of the copy number of *bla*_{NDM-1} provides a strong argument for the prudent use of clinically important antibiotics to reduce the development and dissemination of antibiotic resistance among pathogenic bacteria (Huang et al., 2013).

Detection Methods

Because NDM-producing *K. pneumoniae* infections are also associated with significant in-hospital mortality (de Jager et al., 2015), the rapid and accurate detection of NDM-producing *K. pneumoniae* is becoming a major issue in limiting the spread of CR bacteria. Several methods recently developed to detect NDM-producing *K. pneumoniae* include Xpert[®] Carba-R based on real time PCR (Anandan et al., 2015), the Carba NP test based on the color change of a pH indicator (Nordmann et al., 2012b), and its derivatives (Pires et al., 2013; Doret et al., 2014b), and a method based on MALDI-TOF (Hrabak et al., 2013a). Xpert[®] Carba-R based on real time PCR effectively identified various carbapenemases of KPC, NDM, IMP, and VIM, with 100% sensitivity and 77% specificity (Anandan et al., 2015). However, this method failed to detect OXA-48-like carbapenemases, in contrast to multiplex PCR (Anandan et al., 2015).

The Carba NP test using chromogenic medium is based on the color change of a pH indicator (Nordmann et al., 2012b). The enzymatic hydrolysis of the β -lactam ring of a carbapenem (usually imipenem) causes the acidification of an indicator solution (phenol red for the Carba NP test) that changes its color due to pH modification (Garcia-Fernandez et al., 2016). This method could rapidly detect KPC, IMP, VIM, NDM, and OXA-48-like producers with sensitivity and specificity of 97.9

and 100%, respectively, directly from spiked blood cultures (Doret et al., 2014a). Comparative evaluation of the Carba NP test with other detection methods was tested in many reports (Vasoo et al., 2013; Huang et al., 2014; Yusuf et al., 2014; Gallagher et al., 2015; Lifshitz et al., 2016). When the Carba NP test and the modified Hodge test were compared, the Carba NP test was more specific (100% versus 80%) and faster (Vasoo et al., 2013; Huang et al., 2014; Yusuf et al., 2014). When it was compared the performance of the Carba NP test and the commercially available imipenem hydrolysis-based rapid test (the Rosco Rapid CARB screen kit) for detecting CPE and *P. aeruginosa*, the Carba NP test showed superior specificity and sensitivity (Huang et al., 2014; Yusuf et al., 2014; Gallagher et al., 2015). A novel simplified protocol of the Carba NP test designed for carbapenemase detection direct from bacterial cultures (instead of bacterial extracts) showed enhanced detection of carbapenemase producers (Pasteran et al., 2015).

However, several reports showed that false-negative results in the Carba NP test were associated with mucoid strains or linked to enzymes with low carbapenemase activity, particularly OXA-48-like (Tijet et al., 2013; Osterblad et al., 2014). To overcome these problems, several derivatives of the Carba NP test were developed, such as the Rapidec Carba NP test (bioMérieux; Poirel and Nordmann, 2015), the CarbAcineto NP test for rapid detection of carbapenemase-producing *Acinetobacter* spp. (Doret et al., 2014b), the Rapid CARB Screen (Rosco Diagnostica; Doret et al., 2015), the Blue-Carba test using bromothymol blue as a pH indicator solution (Garcia-Fernandez et al., 2016), a modified Carba NP test (Bakour et al., 2015a), and the BYG Carba test based on an electro-active polymer biosensing technology (Bogaerts et al., 2016). Many studies evaluated the performance of the Rapidec Carba NP test (bioMérieux), which was introduced into the market for the detection of carbapenemase production (Doret et al., 2015; Hombach et al., 2015; Kabir et al., 2016; Lifshitz et al., 2016). These report showed that this method was user-friendly and had a high overall performance, making it an attractive option for clinical laboratories (Kabir et al., 2016). Recently, performance evaluation of two biochemical rapid tests commercialized (the Rapidec Carba NP test and the Rapid CARB Screen) was reported and compared with the home-made Carba NP test (Doret et al., 2015). The Rapidec CARBA NP test possesses the best performance for rapid and efficient detection of CPE (Doret et al., 2015). The BYG Carba test based on a new and original electrochemical method detects the variations of conductivity of a polyaniline (an electro-sensing polymer)-coated electrode which is highly sensitive to the modifications of pH and of redox activity occurring during the imipenem enzymatic hydrolysis reaction (Bogaerts et al., 2016). In comparison with PCR results, the BYG Carba test displayed sensitivity of 95% and specificity of 100% versus 89% and 100%, respectively, for the Carba NP test (Bogaerts et al., 2016). The development of these detection methods based on inexpensive and affordable techniques can limit the spread of CR bacteria.

Class D Carbapenemases

Epidemiology

Class D β -lactamases were referred to as oxacillinas (OXAs) because they commonly hydrolyze isoxazolylpenicillins (oxacillin, cloxacillin, and dicloxacillin) much faster than benzylpenicillin (Jeon et al., 2015). Of over 400 Class D β -lactamases, only some variants actually possess carbapenemase activity. Based on their amino acid sequence, class D carbapenemases were recently reclassified into 12 subgroups: OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235 (Jeon et al., 2015). Among them, only several subgroups such as OXA-23, OXA-48, OXA-51, and OXA-58 are reported in *K. pneumoniae* (Evans and Amyes, 2014). OXA-48 is the most efficient class D carbapenemase for imipenem and is one of the most prevalent class D carbapenemases (Jeon et al., 2015). The OXA-48 was first identified in *K. pneumoniae* in Turkey in 2003 (Poirel et al., 2004), and thus far, 10 variants of the *bla*_{OXA-48} gene have been identified (Jeon et al., 2015). Turkey may be one of the main reservoirs of OXA-48-producing *K. pneumoniae* (Nordmann and Poirel, 2014). Since 2003, the endemic spread of these bacteria has been reported in countries such as Turkey, Morocco, Libya, Egypt, Tunisia, and India (Nordmann and Poirel, 2014; **Figure 4**). The sporadic spread has been reported in France (Liapis et al., 2014; Semin-Pelletier et al., 2015), Spain (Oteo et al., 2013b; Pena et al., 2014), Italy (Giani et al., 2013, 2014), Belgium (Cuzon et al., 2008; Huang et al., 2013), the Netherlands (Kalpoe et al., 2011; Dautzenberg et al., 2014), the UK (Dimou et al., 2012; Thomas et al., 2013), Germany (Pfeifer et al., 2012; Kola et al., 2015), Switzerland (Potron et al., 2012; Seiffert et al., 2014), Argentina (Poirel et al., 2011c; Lascols et al., 2013), Lebanon (Beyrouty et al., 2014), Israel (Adler et al., 2013, 2015), Kuwait (Poirel et al., 2012a; Zowawi et al., 2014), Saudi Arabia (Shibl et al., 2013; Liu et al., 2015), and Japan (Nagano et al., 2013; Hashimoto et al., 2014). The prevalence of OXA-48 carbapenemases among carbapenemase-producing *K. pneumoniae* in Spain and France was particularly high (74 and 78%, respectively; Robert et al., 2014; Palacios-Baena et al., 2016). In Africa, OXA-48-producing *K. pneumoniae* have been mainly reported in the northern countries, such as Morocco, Libya, Egypt, Tunisia, and Algeria (**Figure 4**). In the Arabian Peninsula, the prevalence of OXA-48-like carbapenemases among carbapenemase-producing *K. pneumoniae* was also high (32.5–56%; Zowawi et al., 2014; Sonnevend et al., 2015b). Among CRE isolates in Lebanon, 88% produced OXA-48 carbapenemase (Beyrouty et al., 2014). In Saudi Arabia, 78% of carbapenemase-producing *K. pneumoniae* isolates harbored *bla*_{OXA-48}, and three strains of 47 *bla*_{OXA-48}-positive *K. pneumoniae* isolates were resistant to colistin, suggesting that colistin resistance is emerging in Saudi Arabia (Shibl et al., 2013).

In France, examination of the epidemiologic features of an outbreak of OXA-48-producing *K. pneumoniae* in an ICU revealed that the outbreak was caused by environmental persistence of OXA-48-producing *K. pneumoniae* over 20 months (Pantel et al., 2016). This report emphasizes the importance of early environmental screening to interrupt the transmission of carbapenemase-producing *K. pneumoniae* (Pantel et al., 2016).

Similarly, a large outbreak of OXA-48 carbapenemase-producing *K. pneumoniae* in a French university hospital was recently attributed to the late implementation of successive cohort units and a high level of staff movement between the infectious diseases and internal medicine ward (Semin-Pelletier et al., 2015). These results suggest that practical guidelines are required to help hospitals confronting uncontrolled outbreaks. Because the gut of colonized patients is the main source of CPE, accurate and stringent hygiene of endoscopic instruments is also important. A recent report partially attributed an outbreak of OXA-48-producing *K. pneumoniae* in a German University hospital to complex instruments such as the duodenoscope (Kola et al., 2015). Strict hygiene regulations for various nosocomial environments, including endoscopic instruments as well as gowns and gloves, are required.

OXA-48-producing *K. pneumoniae* was recently also detected in Canada (Ellis et al., 2013), the USA (Mathers et al., 2013), Ireland (Wrenn et al., 2014), Poland (Izdebski et al., 2015), Hungary (Janvari et al., 2014), Greece (Voulgari et al., 2013), Romania (Lixandru et al., 2015), Bulgaria (Markovska et al., 2015), Finland (Osterblad et al., 2012), Russia (Fursova et al., 2015), Algeria (Cuzon et al., 2015), United Arab Emirates (Ahn et al., 2015), Iran (Azimi et al., 2014), South Africa (Brink et al., 2013), Senegal (Moquet et al., 2011), Taiwan (Ma et al., 2015), Singapore (Ling et al., 2015), South Korea (Jeong et al., 2015), and Australia (Espedido et al., 2013; **Figure 4**). In North America, the frequency of OXA-48-like enzymes among carbapenemase-producing *K. pneumoniae* isolates was very low (11%; Lascols et al., 2013). A recent report in Romania showed that among 65 carbapenemase-producing *K. pneumoniae*, the most frequently identified gene was the *bla*_{OXA-48} gene (78%), 12% were positive for *bla*_{NDM-1} gene, 6% had the *bla*_{KPC-2} gene (Lixandru et al., 2015). The recent spread of OXA-48 and OXA-244 carbapenemase genes in Russia was reported among *Proteus mirabilis*, *E. aerogenes*, and *E. cloacae* as well as *K. pneumoniae* (Fursova et al., 2015). In South Africa, the emergence of a colistin-resistant OXA-181-producing *K. pneumoniae* isolate was also reported (Brink et al., 2013), and in the Netherlands, an OXA-48-producing *K. pneumoniae* was reported to infect two patients (Kalpoe et al., 2011), and a hospital-wide outbreak was successfully controlled (Dautzenberg et al., 2014).

OXA-181, a derivative of OXA48 with the substitution of a single amino acid, was first identified in India in Potron et al. (2011), and then has been spread to many different countries, such as the UK (Dimou et al., 2012), Romania (Székely et al., 2013), Canada (Peirano et al., 2014), Oman (Potron et al., 2011), Singapore (Balm et al., 2013), Sri Lanka (Hall et al., 2014), South Korea (Cho et al., 2015), Australia (Sidjabat et al., 2015), Japan (Kayama et al., 2015), and New Zealand (Williamson et al., 2011). However, in many cases, the infections have been associated with India (Potron et al., 2011; Williamson et al., 2011; Dimou et al., 2012; Hall et al., 2014; Peirano et al., 2014). Other OXA-48-derivatives, such as OXA-204 (Potron et al., 2013), OXA-232 (Potron et al., 2013), and OXA-163 (Poirel et al., 2011b), were recently identified in Tunisia, France, and Argentina, respectively, and OXA-244 and

OXA-245 were first reported in Spain (Oteo et al., 2013a). All of these countries are regions with a high prevalence of OXA-48-producing *K. pneumoniae* (**Figure 4**). In addition, OXA-232-producing *K. pneumoniae* has been reported in various countries, such as the USA (Doi et al., 2014), Singapore (Teo et al., 2013), India (Al-Marzooq et al., 2015), and South Korea (Jeong et al., 2015). These results indicate that besides OXA-48, its many derivatives also spread worldwide. OXA-163, which differs from OXA-48 by a four amino acid deletion and a single amino acid substitution, has lower carbapenemase activity than OXA-48, but this enzyme exhibits extended activity against expanded-spectrum cephalosporins, and its activity is partially inhibited by clavulanic acid, a β -lactamase inhibitor (Poirel et al., 2011c). OXA-247, a derivative of OXA-163 with a single amino acid substitution, was recently reported in Argentina (Gomez et al., 2013), where OXA-163-producing *K. pneumoniae* were often reported (Lascols et al., 2013).

The coexistence of OXA-48-like and other carbapenemases in *K. pneumoniae* was also frequently reported worldwide, such as in Turkey (OXA-48/NDM-1; Kilic and Baysallar, 2015), Switzerland (OXA-48/NDM-1; Seiffert et al., 2014), United Arab Emirates (OXA-48-like/NDM-1; Dash et al., 2014), Australia (OXA-48/ NDM-1; Sidjabat et al., 2015), Morocco (OXA-48/NDM-1; Barguigua et al., 2013), India (OXA-181/VIM-5; Castanheira et al., 2011), Singapore (OXA-181/NDM-1 and OXA-181/NDM-5; Balm et al., 2013), the USA (OXA-232/NDM-1; Doi et al., 2014), and India (OXA-232/NDM-1; Al-Marzooq et al., 2015).

Other class D carbapenemases, such as OXA-23, OXA-24/40, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235, were mainly identified in *Acinetobacter* species such as *A. baumannii* but not in *K. pneumoniae* (**Table 1**; Evans and Amyes, 2014).

Molecular and Genetic Context

Unlike KPCs and NDMs, one highly transferable IncL group plasmid (pOXA-48a) was mainly responsible for the spread of the *bla*_{OXA-48} gene in *K. pneumoniae* (**Figure 2C**; Pitout et al., 2015). The molecular epidemiology of OXA-48 in European and North African countries showed that in 92.5% of the isolates, the *bla*_{OXA-48} gene was located on this self-conjugative IncL/M type plasmid (Potron et al., 2013). The *bla*_{OXA-48} gene was found only in the IncL group of IncL/M type plasmids (Carattoli et al., 2015). In contrast to the IncA/C plasmids of NDM-1, the pOXA-48a plasmid contains *bla*_{OXA-48}, a unique antibiotic resistance gene (Pitout et al., 2015). The conjugation rate of the pOXA-48a plasmid was very high (1×10^{-1}); therefore, this self-conjugative plasmid can conjugate at a very high frequency to *Enterobacteriaceae* (Potron et al., 2014). Inactivation of the *tir* gene, which is known to encode a transfer inhibition protein, was recently reported to be responsible for a 50- to 100-fold increase in the efficiency of transfer of the pOXA-48a plasmid (Potron et al., 2014), explaining the spread of the pOXA-48a plasmid with *bla*_{OXA-48}. Recently, the *bla*_{OXA-48-like} gene has also been reported on other plasmids and genetic elements, such as IncA/C types (Ma et al., 2015), IncH types (Wang et al., 2014c), and Tn1999 (Poirel et al., 2012a). In comparison to the pOXA-48a plasmid, the transmission frequency of the Tn1999

composite transposon was very low ($<1.0 \times 10^{-7}$; Aubert et al., 2006).

OXA-48 has most often been found in *K. pneumoniae*, but OXA-48 was also found in various *Enterobacteriaceae*, because of the high conjugation rate of the pOXA-48a (Potron et al., 2014). Molecular and epidemiological analyses in a German hospital showed the horizontal gene transfer of the OXA-48-containing plasmid from *K. pneumoniae* to *E. coli* (Gottig et al., 2015). Besides *E. coli*, OXA-48 has also been identified in *K. oxytoca*, *Enterobacter* spp., *Providencia rettgeri*, *C. freundii*, and *S. marcescens* (Poirel et al., 2012c; Berger et al., 2013). The *bla*_{OXA-48} gene was identified in all *Enterobacteriaceae* isolates from the index case in Spain, indicating the capacity of OXA-48 carbapenemase to spread among *Enterobacteriaceae* by the horizontal gene transfer (Arana et al., 2015).

Similar to NDM, the *bla*_{OXA-48-like} genes were detected in various *K. pneumoniae* clones. ST11 has often been associated with *bla*_{OXA-48-like} in *K. pneumoniae* isolated in many countries, such as Spain (Oteo et al., 2013a,b, 2015; Ruiz-Garbajosa et al., 2013; Branas et al., 2015), Taiwan (Ma et al., 2015), Libya (Lafeuille et al., 2013), Turkey (Lascols et al., 2013), Argentina (Lascols et al., 2013), and Greece (Voulgari et al., 2013). In Spain, a large outbreak was initiated in 2013 by a OXA-48-producing *K. pneumoniae* ST11 clone, and this strain was detected in 44 patients (Branas et al., 2015). The ST11 isolates carried various carbapenemases, including NDM-1, VIM-1, OXA-48, KPC-2, and OXA-245 (Lascols et al., 2013; Oteo et al., 2013b, 2015). In addition, ST14, ST15, ST101, ST147, and ST405 harboring *bla*_{OXA-48-like} have often been reported in many countries, such as the USA (Lascols et al., 2013), Spain (Oteo et al., 2013b, 2015; Ruiz-Garbajosa et al., 2013; Arana et al., 2015; Cubero et al., 2015), the Czech Republic (Hrabak et al., 2015), Libya (Lafeuille et al., 2013), India (Lascols et al., 2013), Germany (Gottig et al., 2015), Finland (Osterblad et al., 2012), France (Liapis et al., 2014), and Japan (Hashimoto et al., 2014). Recent results from 83 hospitals in Spain showed that OXA-48 (71.5%) and VIM-1 (25.3%) were the most frequently detected carbapenemases, and the most prevalent sequence types were ST11 and ST405 for *K. pneumoniae* (Oteo et al., 2015). However, the molecular epidemiology of OXA-48-producing enterobacterial isolates collected from European and north-African countries between 2001 and 2011 indicated that ST101 was the most commonly observed sequence type in *K. pneumoniae* isolates, accounting for 17 out of 67 isolates (25.4%), followed by ST395 and ST15 (each seven isolates, 10.5%; Potron et al., 2013). Two outbreaks of OXA-48-producing *K. pneumoniae* ST101 clones were reported in Spain (Pitart et al., 2011; Cubero et al., 2015). OXA-48-producing *K. pneumoniae* appear to vary geographically. As with NDM, to the best of our knowledge, ST258 harboring *bla*_{OXA-48-like} has never been reported.

Treatment Options

OXA-48-like-producing *K. pneumoniae* are usually resistant to most β -lactam antibiotics, but OXA-48-producing *K. pneumoniae* without ESBLs remain susceptible to the expanded-spectrum cephalosporins (Nordmann et al., 2012a; Munoz-Price et al., 2013). In addition, OXA-48-like-producing

K. pneumoniae sometimes remains susceptible to several aminoglycosides such as gentamicin (Tzouvelekis et al., 2014). Unlike for NDMs (Wiskirchen et al., 2013, 2014b; MacVane et al., 2014), carbapenems may not be a reliable treatment option for OXA-48 producer infection (Wiskirchen et al., 2014a). Combination therapy with sulbactam, meropenem, and colistin was more effective in isolates producing NDM carbapenemase than those producing OXA-48-like carbapenemases, suggesting that the identification of the carbapenemase type helps determine the combination most likely to clear the infection (Laishram et al., 2015). The combination of fosfomycin with imipenem, meropenem, and tigecycline was also synergistic against OXA 48-positive *K. pneumoniae* strains *in vitro* with the ratios of 42, 33, and 33%, respectively (Evren et al., 2013). Similarly, the *in vitro* assays indicate that imipenem-containing combinations were effective against serine- β -lactamase producers (KPC, OXA-48), while no synergy was observed for all NDM-1 producers (Poirel et al., 2016). However, because carbapenems were effective against NDM-1-producing isolates *in vivo* (Wiskirchen et al., 2013, 2014a; MacVane et al., 2014), the effect of carbapenem combination therapy on carbapenemase-producing isolates should be determined *in vivo*, particularly in the case of NDM-1-producing bacteria.

Despite the effectiveness of combination therapies, the prognosis for bloodstream infections caused by OXA-48-producing *Enterobacteriaceae* remains poor, and the 30-days mortality reached 50% (Navarro-San Francisco et al., 2013). A similar result was reported in OXA-48-producing *K. pneumoniae* infections in a tertiary hospital in Spain (Pano-Pardo et al., 2013). Although OXA-48-producing *K. pneumoniae* were susceptible to several antibiotics, including amikacin (97.2% susceptible), colistin (90.1%), tigecycline (73%), and fosfomycin (66.2%), in-hospital mortality among patients with OXA-48-producing *K. pneumoniae* infections was 43.5% (Pano-Pardo et al., 2013). Therefore, to prevent delay in diagnosis and initiation of optimal antimicrobial therapy, rapid identification of OXA-48-producing isolates is required.

Detection Methods

As with KPCs and NDMs, various detection methods were developed to identify OXA-48-like carbapenemases (Tsakris et al., 2010; Giske et al., 2011; Girlich et al., 2013; Naas et al., 2013; Lee et al., 2015c). Accurate differentiation of the various carbapenemase types, such as KPC-type, NDM-type, and OXA-48-type enzymes, is crucial for controlling the spread of carbapenem resistance among *Enterobacteriaceae* (Nordmann et al., 2012a). Many phenotypic detection methods to allow differentiation between class A and class B carbapenemases were developed using boronic acid derivatives and EDTA or dipicolinic acid (Tsakris et al., 2010; Giske et al., 2011). Recently, a specific phenotypic method to differentiate a single OXA-48 producer from those producing other carbapenemase types (e.g., KPC-types, NDM-types) was also developed (Tsakris et al., 2015). This method was based on an imipenem disk and two blank disks adjacent to the imipenem disk, loaded with the tested strain and impregnated with EDTA and EDTA plus phenyl boronic acid, respectively (Tsakris et al., 2015). This novel method exhibited

96.3% sensitivity and 97.7% specificity (Tsakris et al., 2015). ChromID OXA-48, based on chromogenic media, have been commercialized for the direct isolation of CPE from clinical samples (Girlich et al., 2013), with an estimated 91% sensitivity and 100% specificity (Girlich et al., 2013).

Emerging Class C Carbapenemases

Class C β -lactamases confer resistance to penicillins, cephalosporins, and cephemycins (cefoxitin and cefotetan) and are not significantly inhibited by clinically applied β -lactamase inhibitors such as clavulanic acid (Jeon et al., 2015). Although four class C carbapenemases (ACT-1, CMY-2, CMY-10, and ADC-68) have been reported, ACT-1 and CMY-2 exhibit reduced susceptibility to carbapenems, particularly ertapenem, only when combined with permeability defects, due to their low catalytic efficiencies (K_{cat}/K_m) for imipenem (0.007 and 0.04 M⁻¹.S⁻¹, respectively; Mammeri et al., 2010). CMY-10 with the catalytic efficiency of 0.14 M⁻¹.S⁻¹ for imipenem was the first reported carbapenemase among plasmidic class C β -lactamases (Kim et al., 2006), and this enzyme was also a class C extended-spectrum β -lactamase with extended substrate specificity for extended-spectrum cephalosporins (Lee et al., 2009, 2012a; Jeon et al., 2015). Among the chromosomal class C β -lactamases, ADC-68 identified in *A. baumannii* was the first reported enzyme possessing both class C extended-spectrum β -lactamase and carbapenemase activities (Jeon et al., 2014, 2015), and its catalytic efficiency for imipenem is 0.17 M⁻¹.S⁻¹. CMY-10-producing *K. pneumoniae* was identified only in South Korea (Lee et al., 2005a, 2006a) and ADC-68-producing *K. pneumoniae* was never reported.

Many reports showed that carbapenem resistance can be triggered by the loss of two major porins, OmpK35 and OmpK36, in combination with ESBLs or Ambler class C AmpC cephalosporinases, and the production of carbapenemase (Wang et al., 2009; Shin et al., 2012; Tsai et al., 2013). A recently genetically engineered mutant of *K. pneumoniae* showed that several carbapenems (imipenem, meropenem, and doripenem) remain effective against these carbapenemase-independent CR strains (Tsai et al., 2013). Therefore, laboratory testing for susceptibility to imipenem, meropenem, and doripenem can improve the accuracy of identification of these isolates (Tsai et al., 2013).

CONCLUSION

We analyzed the epidemiology of *K. pneumoniae* producing true carbapenemases (Ambler molecular class A, B, D, and several carbapenemases of class C) responsible for non-susceptibility to carbapenems without additional permeability defects. Many types of CR *K. pneumoniae* have been identified worldwide (Figures 1, 3, and 4). During the past 3 years, many countries have reported the arrival of carbapenemases previously unreported in those countries. For example, although *K. pneumoniae* producing KPC- and NDM-type carbapenemases have been extensively reported in the USA, a *K. pneumoniae* producing OXA-48-type carbapenemases was recently detected

in the USA (Mathers et al., 2013). Since a first report of NDM-1 in 2008, this carbapenemase has rapidly spread worldwide, and NDM-producing *K. pneumoniae* still continues to be found in new countries, implying that NDM-producing *K. pneumoniae* is still spreading quickly. Despite the global dissemination of KPC, NDM, and OXA-48, the prevalence of carbapenemases varies geographically. The frequency of KPC- and NDM-producing strains was significantly higher in the USA, Canada, Greece, Taiwan, Colombia, and China, whereas OXA-48-producing strains were rarely found in those countries (**Figures 1, 3, and 4**). In Argentina, despite the extensive spread of KPC- and OXA-48-producing strains, no NDM-producing *K. pneumoniae* has been reported. Although NDM- and OXA-48-producing *K. pneumoniae* significantly spread in Turkey, KPC-producing strains have rarely been reported there. In Brazil, KPC-producing *K. pneumoniae* has been mainly reported. In the Arabian Peninsula, OXA-48 and NDM producers are common, whereas KPC-type, VIM-type, or IMP-type producers are rare. In India, Spain, France, Italy, and the UK, all three types of carbapenemases have been frequently reported (**Figures 1, 3, and 4**).

ST258 is an important strain responsible for the extensive global spread of KPC-producing *K. pneumoniae*. Although the precise reason for the predominance of the ST258 strain in KPC-producing *K. pneumoniae* is not fully understood, recent molecular studies unveiled the genetic characteristics of this strain. The ST258 strains consists of two distinct genetic clades (*cps-1* and *cps-2*) derived from genetic differentiation in genes involved in capsule polysaccharide biosynthesis. In addition, the integrative conjugative element ICEKp258.2, present only in ST258 and genetically related sequence types, may be linked to the global success of the ST258 clone. ICEKp258.2 contains a type IV pilus gene cluster and a type III restriction-modification system. The type IV pilus gene cluster ICEKp258.2, particularly *pilV*, may contribute to the global success of ST258 clone. The type III restriction-modification system associated with the restriction of plasmids and specific mobile elements may explain the differences observed between ST258 predominantly harboring KPC and ST11, another high-risk clone that lacks ICEKp258.2, harboring various carbapenemases, such as NDM-1, OXA-48, KPC-2, VIM-1, and OXA-245. IncF with FII_K replicons, a plasmid most commonly identified in ST258 with *bla*_{KPC}, often contains several genes associated with resistance to other antibiotics, such as aminoglycosides, tetracyclines, quinolones, trimethoprim, and sulfonamides. The features of this plasmid may also play an important role in the current global success of ST258.

The rapid global spread of NDM-type carbapenemases may be partly attributed to the dissemination of various epidemic broad-host-range plasmids bearing the *bla*_{NDM} genes. NDM-type carbapenemases were found in various plasmids such as IncA/C, IncF, IncR, IncH, IncN, IncL/M, and IncX types. The IncA/C type plasmids, most common plasmids associated with spread of the *bla*_{NDM} genes, often have various antibiotic resistance genes, such as 16S rRNA methylases associated with aminoglycoside resistance, CMY-type β -lactamases associated with broad-spectrum cephalosporin resistance, and QnrA

associated with quinolone resistance. These features may be linked to the current global success of NDM-producing *K. pneumoniae*.

The current spread of OXA-48-producing bacteria is attributed to the pOXA-48a plasmid, which belongs to the IncL group of IncL/M type plasmids. Although the pOXA-48a plasmid contains *bla*_{OXA-48}, a unique antibiotic resistance gene, the conjugation rate of the pOXA-48a plasmid was very high, which may be responsible for its global spread in *K. pneumoniae*. The high pOXA-48a conjugation rate was recently attributed to mutations in the *tir* gene known to encode a transfer inhibition protein, which may lead to a 50- to 100-fold increase in the efficiency of transfer of the pOXA-48a plasmid. Furthermore, the pOXA-48a plasmid is self-conjugative. Therefore, these specific features of the pOXA-48a plasmid may explain the global dissemination of OXA-48-type carbapenemases.

ST11, ST14, ST101, ST147, and ST258 are major carbapenemase-producing *K. pneumoniae* clones. ST258 was mainly found in KPC-producing *K. pneumoniae*, whereas other clones were found in various carbapenemase-producing *K. pneumoniae* regardless of carbapenemase types. Well-designed epidemiological and molecular studies will be required to understand the dynamics of transmission, risk factors, and reservoirs of these *K. pneumoniae* clones. This will provide information essential for preventing infections and the spread of these risky sequence types.

Most currently available antibiotics may be not sufficiently effective for the treatment of all types of carbapenemase producers in monotherapy. Combination therapy of carbapenems with polymyxin B, colistin, rifampin, fosfomycin, or tigecycline has been reported to effectively treat carbapenemase-producing *K. pneumoniae*. Despite these data supporting the use of combination therapy for treatment of severe carbapenemase-producing *K. pneumoniae* infections, current clinical evidence for treatment guidelines are limited and more accurate randomized controlled *in vivo* studies are required. Moreover, considerable caution is required when applying these therapies. For example, temocillin can actively treat against some KPC-producing *K. pneumoniae*, particularly lower urinary tract infections, and NDM-producing *K. pneumoniae* is often susceptible to aztreonam. OXA-48-producing *K. pneumoniae* remain susceptible to the expanded-spectrum cephalosporins in approximately 20% of cases without ESBLs. In the case of NDM-1-producing *K. pneumoniae*, carbapenems were recently reported to represent a viable treatment option for infections caused by these bacteria, despite unfavorable *in vitro* MICs. Because these results imply that carbapenems can sometimes be a viable treatment option for infection with carbapenemase producers, more extensive studies on the effect of carbapenem monotherapy will be required in the case of NDM-producing *K. pneumoniae* infections.

The accurate and rapid detection of the genotype of carbapenemases can minimize the delay to appropriate prescription of antibiotics. Many detection kits based on various phenotypic or molecular techniques, such as multiplex PCR assay, real-time PCR assay, DNA microarray, Raman spectroscopic analysis, single-colony whole-genome sequencing,

MALDI-TOF MS, loop-mediated isothermal amplification method, chromogenic medium, and new phenotypic test methods, have been developed. Through the imprudent use of colistin which is a key component used for the treatment of severe carbapenemase-producing *K. pneumoniae* infections, the rapid spread of colistin resistance was recently reported in *K. pneumoniae* producing carbapenemases, particularly KPC-type carbapenemases. This situation strongly demonstrates the need for the development of novel accurate and reliable methods for detecting resistance to clinically important antimicrobial agents, such as colistin. Hospital interventions can effectively reduce the spread of carbapenemase-producing *K. pneumoniae*. Standard infection control guidelines should be implemented upon the detection of carbapenemase-producing *K. pneumoniae*, and carbapenemase-producing *K. pneumoniae* positive patients should be individually isolated and treated according to strict standard guidelines.

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C-RL, JL, and SL contributed to the conception and the design of the review and C-RL, JL, KP, YK, BJ, and SL researched and wrote the review.

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Increased prevalence of carbapenem resistant *Enterobacteriaceae* in hospital setting due to cross-species transmission of the *bla*_{NDM-1} element and clonal spread of progenitor resistant strains

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This study investigated the transmission characteristics of carbapenem-resistant *Enterobacteriaceae* (CRE) strains collected from a hospital setting in China, in which consistent emergence of CRE strains were observable during the period of May 2013 to February 2014. Among the 45 CRE isolates tested, 21 (47%) strains were found to harbor the *bla*_{NDM-1} element, and the rest of 24 CRE strains were all positive for *bla*_{KPC-2}. The 21 *bla*_{NDM-1}-borne strains were found to comprise multiple *Enterobacteriaceae* species including nine *Enterobacter cloacae*, three *Escherichia coli*, three *Citrobacter freundii*, two *Klebsiella pneumoniae*, two *Klebsiella oxytoca*, and two *Morganella morganii* strains, indicating that cross-species transmission of *bla*_{NDM-1} is a common event. Genetic analyses by PFGE and MLST showed that, with the exception of *E. coli* and *E. cloacae*, strains belonging to the same species were often genetically unrelated. In addition to *bla*_{NDM-1}, several CRE strains were also found to harbor the *bla*_{KPC-2}, *bla*_{VIM-1}, and *bla*_{IMP-4} elements. Conjugations experiments confirmed that the majority of carbapenem resistance determinants were transferable. Taken together, our findings suggest that transmission of mobile resistance elements among members of *Enterobacteriaceae* and clonal spread of CRE strains may contribute synergistically to a rapid increase in the population of CRE in clinical settings, prompting a need to implement more rigorous infection control measures to arrest such vicious transmission cycle in CRE-prevalent areas.

Keywords: carbapenem-resistant *Enterobacteriaceae*, NDM-1, clonal spread, mobile element

Introduction

β -Lactams have been a cornerstone in treatment of infections caused by Gram-negative bacterial pathogens due to their high efficacy and low toxicity to humans, among which carbapenems are considered agents of the last resort, especially in cases where extended-spectrum β -Lactamase (ESBL) producing organisms were involved (Dalhoff and Thomson, 2003). In the past two decades, usage of carbapenems such as imipenem and meropenem has been substantially increased due to the emergence of multidrug-resistant organisms (Goel et al., 2011; Zilberberg and Shorr, 2013). However, increased carbapenem consumption in turn initiated a vicious cycle in which carbapenem-resistant Gram-negative pathogens (CRGNP), which often cause untreatable hospital infections (Livermore, 2004, 2009; Karaikos and Giamarellou, 2014), further gained selection advantage. Species belonging to the family *Enterobacteriaceae* are common human pathogens which can cause a wide range of community-acquired and nosocomial infections (Stock, 2014). The emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) has posed a huge challenge to clinical infection control. Carbapenem resistance in CRE was mainly mediated by the production of carbapenemases, among which KPC, Metallo- β -lactamases (VIM, IMP, NDM) and OXA-48 type of enzymes were the most common (Nordmann et al., 2011). New Delhi Metallo- β -lactamase-1 (NDM-1) was one of the most important carbapenemases of CRE. Since its first discovery in 2008 in a *Klebsiella pneumoniae* isolate recovered from a patient at a hospital in New Delhi, India, it has been transmitted to many species of *Enterobacteriaceae* in various countries (Yong et al., 2009). NDM-1 is most frequently identified in the Indian subcontinent, followed by the Balkans region and the Middle East, and is mainly associated with community-acquired infections.

In China, the first clinical report of *bla*_{NDM-1} involved carbapenem-resistant *Acinetobacter baumannii* strains detectable in four patients indifferent provinces in 2011 (Chen et al., 2011). Since then it has been recoverable in most species of *Enterobacteriaceae*, including *K. pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Citrobacter freundii*. To date, NDM-1-producing isolates have been reported in various cities in China including Beijing, Changsha, Chongqing, Fuzhou, Guangzhou, Hangzhou, Hebei, Hong Kong, and Zhengzhou (Berrazeg et al., 2014; Qin et al., 2014). Although various *bla*_{NDM-1}-carrying CRE strains have been sporadically identified, few outbreaks of CRE carrying the *bla*_{NDM-1} element have been reported in China, suggesting that the transmission of NDM-1 is mainly mediated by conjugative plasmids, which is consistent with the features of NDM-1 transmission observable in other parts of the world (Hu et al., 2014). In this study, we report an increasing prevalence of *bla*_{NDM-1}-positive *Enterobacteriaceae* in a Jiaxing hospital, which is located in Zhejiang Province, China. Through time-series analysis of the molecular features and epidemiological linkage of CRE recovered within the hospital, we demonstrated that emergence of new CRE strains was due to a combination of clonal spread of existing *bla*_{NDM-1}-carrying strains and efficient

horizontal transfer of the *bla*_{NDM-1} elements from such strains to other drug sensitive organisms. This combinatorial mode of transmission of both CRE organisms and the resistance elements that they harbor can theoretically result in an exponentially increasing rate of spread of NDM positive CRE in clinical settings if proper infection control measures are not implemented to disrupt the transmission routes.

Materials and Methods

Bacterial Strains and Species Identification

From May 2013 to February 2014, a total of 6598 clinical *Enterobacteriaceae* strains were isolated from different specimens (urine, feces, and sputum) collected from patients in Second People's Hospital of Jiaxingin Zhejiang Province, China. All isolates were identified using the Vitek 2 system (bioMérieux, Marcy-l'Etoile, France), and confirmed by the MALDI-TOF MS apparatus (Bruker Microflex LT, Bruker Daltonik GmbH, Bremen, Germany). These isolates were screened for their ability to produce carbapenemases by a disc diffusion test, in which 10-mg imipenem discs were used (Oxoid, Basingstoke, UK) (Zhou et al., 2015). A total of 45 CRE isolates were recovered from these *Enterobacteriaceae* strains.

Molecular Detection of Resistance Genes

PCR and nucleotide sequencing were employed to screen for the presence of carbapenemase-encoding genes, including *bla*_{VIM}, *bla*_{IMP}, *bla*_{KPC}, *bla*_{OXA-48} and *bla*_{NDM-1}, as well as ESBL genes, including *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV}, as described previously (Dallenne et al., 2010). An imipenem-EDTA double-disc synergy test and a modified Hodge test were used to screen for the presence of Metallo- β -lactamases(MBLs) and carbapenemases, respectively, and were analyzed according to CLSI guidelines (Zhou et al., 2015).

Antimicrobial Susceptibility Testing

The MICs of 10 antibiotics, including imipenem, meropenem, ceftazidime, cefotaxime, aztreonam, piperacillin-tazobactam, fosfomycin, amoxicillin-clavulanic acid, amikacin, tigecycline, were determined using the agar dilution method, and the results were analyzed according to the CLSI criteria of 2014 (Zhou et al., 2015). The 2014 EUCAST breakpoints were used (available at http://www.eucast.org/clinical_breakpoints/) for tigecycline.

PFGE and MLST

Clonal relationships between *bla*_{NDM-1}-positive isolates were investigated by PFGE of *Xba*I-digested genomic DNA using a Rotaphor System 6.0 instrument (Whatman Biometra, Goettingen, Germany), with a running time of 24 h and pulse times of 3–40 s. *Salmonella* strain H9812 was used as the control strain. Bands were stained with ethidium bromide (0.5 μ g/mL) prior to visualization under UV light. A dendrogram depicting the genetic relatedness of the test strains was generated from the homology matrix with a 0.2% coefficient by the unweighted pair-group method, and by using arithmetic averages (UPGMA), to describe the relationships of the PFGE profiles. Isolates were

allocated to the same PFGE group if their dice similarity index was $\geq 85\%$.

MLST was performed using seven housekeeping genes in *bla*_{NDM-1}-producing *E. coli*, *K. pneumonia* and *E. cloacae* isolates, which were amplified using primers listed in the online databases (<http://pubmlst.org/ecloacae/> for *E. cloacae*, <http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html> for *K. pneumoniae* and <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli> for *E. coli*). The resultant PCR products were purified and sequenced. Sequence types (STs) were assigned using online database tools.

Conjugation Experiments

The conjugation experiment was carried out using the mixed broth method as previously described (Borgia et al., 2012). Both the donor (*bla*_{NDM-1}-positive *Enterobacteriaceae*) and the recipient strains (sodium azide-resistant *E. coli* J53) were mixed on Luria-Bertani agar at a ratio of 1:1, and the mixtures were incubated for 24 h at 35°C. Transconjugants were selected on LB agar supplemented with sodium azide (100 mg/L) and meropenem (0.3 mg/L). Colonies that grew on the selective medium were picked for identification by the Vitek MS system. Transformants that harbored *bla*_{NDM-1} and exhibited resistance to carbapenems and cephalosporins were defined as transconjugants.

Results

The study period and venue were May 2013 to February 2014 and the Second Hospital of Jiaxing, Zhejian Province, China, respectively. During this period, a total of 45 CRE isolates were recovered from 6598 clinical specimens (urine, feces and sputum), among which 21 (47%) were found to harbor the *bla*_{NDM-1} elements; the rest of 24 CRE strains were all positive for *bla*_{KPC-2}. The objective of the study was to investigate the molecular events that lead to emergence of CRE in the hospital, with a focus on understanding the molecular and epidemiological features of transmission of both *bla*_{NDM-1}-borne strains and the *bla*_{NDM-1} element itself. The 21 *bla*_{NDM-1}-borne CRE strains were found to comprise a variety of different species of *Enterobacteriaceae* including nine *E. cloacae*, three *E. coli*, three *C. freundii*, two *K. pneumoniae*, two *K. oxytoca* and two *M. morganii* strains, indicating that *bla*_{NDM-1} can be efficiently acquired by various *Enterobacteriaceae* species. Most of these *bla*_{NDM-1}-borne CRE were recovered from patients in the Neurosurgery Department (43%), who were subjected to various neuronal system surgical procedures, followed by patients from the Respiratory Department (19%) suffering mainly from pulmonary infection, patients from the Pediatric Department and Recovery department (14% each), and one patient each from the Cardio-Thoracic Surgery and Breast Department (Table 1). These *bla*_{NDM-1} borne CRE strains were isolated from urine (85%), sputum (10%), and breast secretion (5%), respectively.

Clinical records showed that most of patients from whom *bla*_{NDM-1}-borne CRE were recovered have been subjected to different types of antimicrobial treatment including the use of cephalosporins (ceftazidime, cefoperazone/sulbactam, cefuroxime, piperacillin/tazobactam), amikacin, levofloxacin and

tigecycline, and the vast majority of patients recovered and were discharged from the hospital. Our record also showed that although *bla*_{NDM-1}-borne CRE were resistant to all cephalosporins, clinical treatment with cephalosporins for non-blood infections caused by *bla*_{NDM-1}-borne CRE remained effective (Table 1). In addition, levofloxacin could be a choice for treatment of infections caused by fluoroquinolone-susceptible CREs. Likewise, amikacin was also a good choice since most of the CRE were susceptible to this antibiotic.

The first case of CRE infection occurred in the Neurosurgery Department on June 24, 2013, with the causative organism being identified as *K. oxytoca*, recurrent infection due to the same clone with identical PFGE pattern occurred on August 15, 2013, suggesting the long-term persistence of similar clones in a hospital. Interestingly, non-clonal spread was seen in specific species of CRE such as *C. freundii* and *K. pneumoniae*. For instance, the first case of NDM-producing *C. freundii* infection was recorded on June 26, 2014 in the Neurosurgery Department, followed by the second case in the Respiratory Department on August 17, 2013; the third case was recorded back in Neurosurgery Department on February 20, 2014, however, pneumonia-associated *C. freundii* strains recoverable from each of these three cases were found to be genetically non-identical (Figure 1, Table 1). Likewise, two genetically un-related strains of *K. pneumoniae* were found to cause infections in different departments at different dates (Figure 1, Table 1). On the other hand, both non-clonal and clonal spread could be seen upon analysis of infection caused by *E. coli* and *E. cloacae*. The first case of NDM-producing *E. coli* was observable in the Pediatric Department on September 26, 2013, whereas a different strain was found to cause infections in the Recovery Department in 2014. Although the NDM-1 producing *E. coli* strains recovered belonged to two different clones, three of the strains were found to belong to ST167 (Figure 1, Table 1). *E. cloacae* were the most common CRE species in this hospital. The first case of *E. cloacae* was reported on June 29, 2013 in the Pediatric Department. The same clone, which belonged to ST114, was causing an outbreak in the Neurosurgery Department, in which four infections recorded during the period of August to October, 2013. In August 2013, infections caused by a different clone also occurred in the Pediatric Department, and other clones were also found to cause infections in different departments at different times. These clones belonged to different ST types such as ST93, ST190, and ST66 (Figure 1, Table 1). Finally, NDM-1 producing *Morganella morganii* was reported for the first time in this hospital. Two very similar clones were found to cause infections in different departments and dates (Figure 1, Table 1).

Antimicrobial susceptibilities were performed on all CRE strains. All 21 *bla*_{NDM-1}-positive isolates were resistant to most β -lactam antibiotics, including expanded-spectrum cephalosporins and the carbapenems. Moreover, all isolates were resistant to fosfomycin (≥ 256 mg/L) and amoxicillin-clavulanic acid (Table 2). In addition, 10 isolates were resistant to amikacin (≥ 64 mg/L). Only two isolates were sensitive to aztreonam (≤ 4 mg/L). Sixteen isolates exhibited intermediate susceptibility to tigecycline (MICs ≤ 1 mg/L), and 11 were tigecycline-resistant (MICs > 2 mg/L). The high frequency of resistance observed to

TABLE 1 | Origin and molecular features of *Enterobacteriaceae* isolates harboring *bla*_{NDM-1}, and clinical outcome of diseases that they caused.

Isolate	Isolation date	Location of acquisition	Culture type	Diseases	Treatment	Outcome	PFGE	MLST
EC-06	26/9/2013	PD	Urine	Neonatal pneumonia	Ceftazidime	Recovered	Ec1	ST167
EC-33	18/01/2014	ECD	Urine	Myelitis	NT	Uracratia	Ec2	ST167
EC-34	12/02/2014	RCD	Urine	Myelitis, Urinary tract infection	NR	NR	Ec2	ST167
KO-03	24/06/2013	ND	Urine	Subarachnoid hemorrhage, Oculomotor paralysis, Pneumonia	NR	Recovered	Ko1	ND
KO-16	15/08/2013	ND	Urine	Cerebroma recurrence	NT	Discharged	Ko1	ND
CF-05	26/06/2013	ND	Urine	Traumatic subarachnoid hemorrhage	Levofloxacin, Amikacin	Recovered	Cf1	ND
CF-17	20/08/2013	RD	Sputum	Pulmonary infection	Tigecycline	Recovered	Cf2	ND
CF-35	20/02/2013	ND	Urine	Cerebral contusion	Levofloxacin	Recovered	Cf3	ND
ECL-07	29/06/2013	PD	Urine	Neonatal pneumonia	Ceftazidime	Recovered	Ecl1	ST114
ECL-08	05/08/2013	ND	Urine	Cerebral hemorrhage	Levofloxacin	Transferred	Ecl1	ST114
ECL-09	07/08/2013	ND	Urine	Intracranial aneurysm	Piperacillin/Tazobactam	Discharged	Ecl2a	ST93
ECL-10	09/08/2013	ND	Urine	Traumatic subdural hemorrhage	Levofloxacin	Recovered	Ecl1	ST114
ECL-18	28/08/2013	PD	Urine	Neonatal infections	Ceftazidime	Recovered	Ecl3	ST190
ECL-19	28/08/2013	RD	Urine	Pulmonary infection	Mupirocin ointment	Recovered	Ecl1	ST114
ECL-22	02/10/2013	ND	Urine	Subarachnoid hemorrhage	Levofloxacin	Recovered	Ecl1	ST114
ECL-27	11/11/2013	CTS	Sputum	Abdominal aortic aneurysm	Cefuroxime	Recovered	Ecl4	ST66
ECL-28	27/11/2013	RD	Urine	Chronic obstructive bronchitis	Cefoperazone/Sulbactam		Ecl2b	ST93
KP-04	26/06/2013	RD	Urine	Bladder neoplasmpulmonary infection	Ceftazidime, Piperacillin/Tazobactam	Discharged	Kp1	ST147
KP-14	08/08/2013	ND	Urine	Cerebral hernia	Levofloxacin	Discharged	Kp2	ST1724
MM-23	04/10/2013	BD	Secretion	Cholangitis	Amikacin, Metronidazole	Recovered	Mm1a	ND
MM-26	29/10/2013	RCD	Urine	Spinal cord injury	Amikacin	Recovered	Mm1b	ND

EC, *Escherichia coli*; KO, *Klebsiella oxytoca*; CF, *Citrobacter freundii*; ECL, *Enterobacter cloacae*; KP, *Klebsiella pneumoniae*; MM, *Morganella morganii*. JH, the Second People's Hospital of JiaXing; NT, not treated; NR, no record; ND, not determined. PD, Pediatric Department; RCD, Recovery Department; ND, Neurosurgery Department; CTS, Cardio-Thoracic Surgery; RD, Respiratory Department; BD, Breast Department.

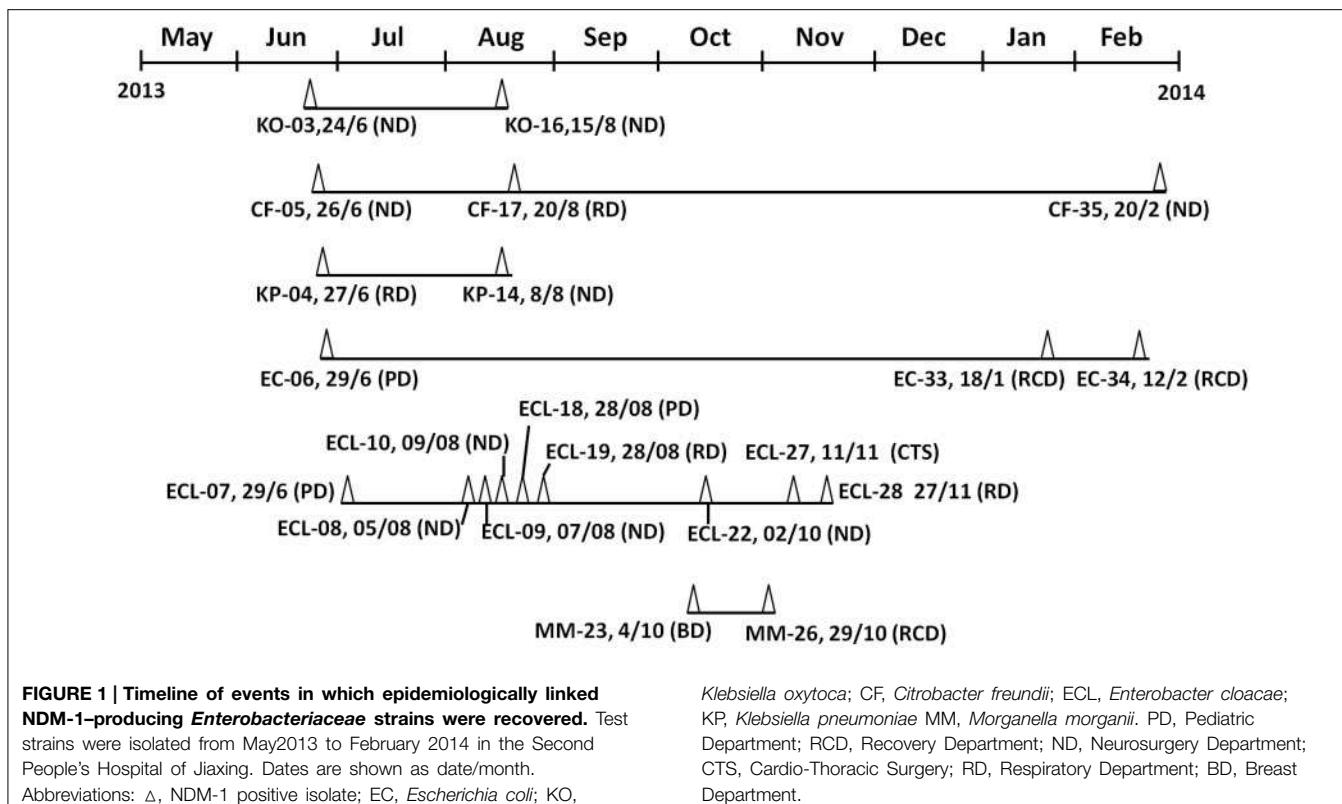
alternative therapeutic antibiotics is a great concern for clinicians in charge of treating infections caused by these CREs.

Transferability of resistance determinants in these CRE strains, in particular those producing NDM-1, was investigated. Conjugation experiments were successful for all isolates except for two (ECL-8 and CF-17) *bla*_{NDM-1}-positive isolates. The resulting 19 transconjugants all exhibited resistance to carbapenems and cephalosporins. Importantly, fosfomycin and amikacin resistance determinants could also be transferred to the recipient *E. coli* strain (Table 2). Phenotypic resistance to all β -lactams in these isolates suggested that they might express additional β -lactamases since NDM-1 would not mediate resistance to aztreonam. In view of the discrepancy between the existence of known carbapenemase genes and the drug susceptibility profiles of the test strains, all CRE strains and their corresponding transconjugants were screened for the presence of additional β -lactamase genes. Our data revealed that in addition to *bla*_{NDM-1}, strains CF35, ECL19, and KP14 also harbored *bla*_{KPC-2}, strain KP04 was found to harbor

*bla*_{VIM-1}, and *bla*_{IMP-4} was detectable in ECL18. To the best of our knowledge, this is the first case of *K. pneumoniae* containing both *bla*_{NDM-1} and *bla*_{VIM-1}, hence further works are required to elucidate the mechanisms governing the uptake of multiple carbapenemase genes in a single organism. In addition, 18 out of the 21 (86%) *bla*_{NDM-1}-positive isolates were found to harbor ESBL genes in various combinations (Table 2). It should also be noted that the additional resistance genes detectable in several isolates, including *bla*_{SHV-12}, *bla*_{CTX-M-3/14}, *bla*_{TEM-1} and *bla*_{KPC-2}, could be co-transferred to the *E. coli* recipient strain J53, along with the *bla*_{NDM-1} element (Table 2).

Discussion

Since the first report of their emergence, NDM-1-producing *Enterobacteriaceae* have become a worldwide public health concern (Patel and Bonomo, 2013). In China, currently available data tend to suggest that *bla*_{NDM-1} is only present at a relatively low frequency and spreading sporadically amongst



Enterobacteriaceae (Wang et al., 2013; Hu et al., 2014). A recent study reported a high rate [33.3% (16/48)] of NDM-1 positive CRE organisms in a hospital in Henan Province, China, most of which were due to plasmid mediated transmission of the *bla*_{NDM-1} elements among different members of *Enterobacteriaceae* (Qin et al., 2014). Although the prevalence of *bla*_{NDM-1}-positive CRE has been increasing over the past several years in China, very few epidemiological data are available to elucidate the underlying mechanisms that mediate the increased rate of transmission of NDM-1 positive CRE strains in hospitals. In this study, we identified 21 NDM-1-producing strains of *Enterobacteriaceae* in a hospital over a short period of time, from May 2013 to February 2014, with the majority of the strains (18/21) being collected during the period of June–November 2013. Our data suggest that a combination of outbreak of CRE infections and sporadic emergence of genetically unrelated resistant organisms contributed to the dramatic increase of CRE infections in this hospital during this period, prompting a need to investigate the molecular basis of these events. It should be noted that the series of CRE infections investigated in this study represents the most serious of its kind in China to date (Berrazeg et al., 2014; Zhou et al., 2014).

MLST-diverse NDM-1-producing *E. coli* (ST410, ST131, ST684, and ST101) and *K. pneumoniae* strains (ST147, ST14, ST11, and ST340) have been identified worldwide (Poirel et al., 2011). In this work, the NDM-1 positive *E. coli* strains tested were found to belong to ST167 with two distinct PFGE types. NDM-1 positive ST167 *E. coli* were previously shown to be an

animal-associated clone recoverable in both France and China (Cuzon et al., 2013; Zhang et al., 2013; Yang et al., 2014). Repeated emergence of these NDM-1 positive *E. coli* strains in China represents a significant clinical and public health concern. The two *K. pneumoniae* isolates reported in the current study belonged to ST147 and a novel type, ST1724. Clinical NDM-1-producing *K. pneumoniae* ST147 strains are frequently detected worldwide, suggesting that they play an important role in the dissemination of the *bla*_{NDM-1} elements to other *K. pneumoniae* strains (Giske et al., 2012; Peirano et al., 2014). Identification of carbapenem-resistant *K. pneumoniae* strains belonging to the novel STs (ST359 and ST1724) in this study infers that the size of the existing pool of NDM-1-producing strains has been further expanded.

In contrast to *E. coli* and *K. pneumoniae*, MLST type of NDM-1-producing *E. cloacae* strains in this study included both epidemic ST types and new ST types. A carbapenem-resistant *E. cloacae* strain belonging to ST89 and producing the OXA-48 carbapenemase was isolated in Poland in 2014 (Majewski et al., 2014). Izdebski et al. reported that the ST66, ST78, and ST114 types have spread worldwide and were commonly associated with production of ESBLs and carbapenemases in many countries (Izdebski et al., 2014). NDM positive *E. cloacae* strains are among the most frequently reported CRE species and are known to cause occasional hospital outbreaks and community-acquired infections (Rozales et al., 2014; Yanik et al., 2014; Stoesser et al., 2015). In this study, we reported a similar situation in which *E. cloacae* belonging to the ST114 type was linked to five infection

TABLE 2 | Profiles of antimicrobial drug susceptibility and detectable-lactamase determinants of NDM-1 positive CRE strains and the corresponding transconjugants.

Strains	MICs (mg/L)											Additional β lactamase determinants	
	IPM	MEM	CAZ	CTX	ATM	TZP	SCF	AMC	LEV	FOS	AK	TIG	
EC-06	16	32	>256	>256	>256	>256	>256	128	8	32	4	2	CTX-M-3, CTX-M-14, SHV-12
T-EC06	16	32	>256	>256	2	>256	>256	128	<0.125		1	1	–
EC-33	4	4	>256	>256	>256	>256	>256	64	32	32	2	1	CTX-M-15
T-EC33	4	4	>256	>256	>256	>256	>256	64	<0.125		1	1	CTX-M-15
EC-34	4	8	>256	>256	>256	>256	>256	128	32	32	2	2	CTX-M-15, TEM-1, SHV-12
T-EC34	4	4	>256	>256	>256	>256	>256	64	<0.125		1	1	TEM-1, SHV-12
KO-03	8	16	>256	>256	>256	>256	>256	128	16	16	1	4	SHV-12
T-KO03	16	32	>256	>256	2	>256	>256	128	<0.125		1	1	–
KO-16	8	16	256	>256	>256	>256	>256	128	16	16	1	4	SHV-12
T-KO16	16	32	>256	>256	>256	>256	>256	128	<0.125		1	1	SHV-12
CF-05	16	32	>256	>256	>256	>256	>256	256	4	8	1	2	SHV-12
T-CF05	16	32	>256	>256	>256	>256	>256	128	<0.125		1	1	SHV-12
CF-17	8	8	>256	>256	256	>256	>256	256	8	32	16	2	SHV-12
CF-35	8	32	>256	>256	>256	>256	>256	>256	8	16	>64	1	CTX-M-3, KPC-2, SHV-12
T-CF35	2	1	16	8	128	128	32	128	<0.125	0.25	>64	0.5	SHV-12
ECL-07	4	16	>256	>256	>256	>256	>256	256	16	64	>64	2	CTX-M-14, TEM-1, SHV-12
T-ECL07	4	2	>256	128	64	256	128	256	<0.125	0.5	1	1	TEM-1
ECL-08	4	4	>256	>256	>256	>256	>256	128	16	64	>64	4	CTX-M-14, TEM-1, SHV-12
ECL-09	4	16	>256	>256	>256	>256	>256	256	32	32	>64	4	CTX-M-3, CTX-M-14, TEM-1, SHV-12
T-ECL09	4	1	>256	256	32	256	256	256	0.25	0.5	1	1	CTX-M-14, TEM-1
ECL-10	8	16	>256	>256	>256	>256	>256	256	16	>64	>64	4	CTX-M-14, TEM-1, SHV-12
T-ECL10	8	8	>256	128	128	256	256	256	<0.125	<0.125	1	1	–
ECL-18	4	32	256	>256	256	256	>256	256	0.5	1	>64	2	IMP-4, TEM-1
T-ECL18	4	8	>256	128	16	256	256	256	<0.125	<0.125	>64	1	TEM-1
ECL-19	8	8	>256	>256	>256	>256	>256	256	16	64	>64	2	CTX-M-14, KPC-2
T-ECL19	8	4	>256	128	64	256	256	256	<0.125	<0.125	1	1	–
ECL-22	8	4	>256	>256	>256	>256	>256	256	32	32	>64	4	CTX-M-14, TEM-1, SHV-12
T-ECL22	8	8	>256	128	128	>256	256	256	0.25	0.5	1	1	TEM-1, SHV-12
ECL-27	4	4	256	>256	256	>256	>256	128	0.5	2	0.5	2	CTX-M-3, CTX-M-14, TEM-1, SHV-12
T-ECL27	8	8	>256	128	128	>256	256	256	<0.125	0.5	1	1	TEM-1
ECL-28	4	4	>256	>256	256	>256	>256	256	64	>64	4	4	TEM-1, SHV-12
T-ECL28	4	8	>256	>256	256	>256	>256	256	0.25	1	2	1	TEM-1, SHV-12
KP-04	8	16	>256	>256	>256	>256	>256	128	2	4	1	2	CTX-M-14, VIM-1, TEM-1, SHV-12
T-KP04	8	16	>256	>256	>256	>256	>256	128	<0.125	0.5	1	1	SHV-12
KP-14	16	16	256	>256	>256	>256	>256	128	0.15	0.5	1	2	CTX-M-14, KPC-2, SHV-12
T-KP14	16	16	256	>256	>256	>256	>256	128	0.15	0.5	1	2	SHV-12
MM-23	8	4	128	>256	2	256	>256	>256	1	4	4	2	–
T-MM23	4	4	>256	128	1	>256	256	256	2	4	1	2	–
MM-26	8	4	64	>256	2	64	>256	>256	16	16	4	4	–
T-MM26	4	8	>256	128	1	>256	256	128	1	2	1	2	–

T denotes transconjugant. IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; TZP, piperacillin-tazobactam; CPS, cefoperazone/sulbactam; AMC, amoxicillin-clavulanic acid; LEV, levofloxacin; FOS, fosfomycin; AK, amikacin; TIG, tigecycline.

cases, organisms belonging to the ST93 type were linked to two cases of infections, and two new ST types, ST66 and ST190 were responsible for one infection each. Since the five ST114 isolates were collected from different wards at the Second People's Hospital of Jiaxing, we speculated that NDM-1-producing *E. cloacae* strain ST114 may have already spread in the hospital.

In the United Kingdom and the Indian subcontinent, *bla*_{NDM-1} has been found to be located in plasmids of various sizes (ca. 50–300 kb), which generally belong to at least four different Inc groups, including A/C, L/M, FI/FII and an undefined type (Kumarasamy et al., 2010; Poirel et al., 2011). In our study, most of *bla*_{NDM-1} elements recoverable from CRE isolates were

harbored by self-transmissible plasmids, which also encoded multiple β -lactamases and other determinants of amikacin and fosfomycin resistance. This finding is consistent with currently available data in China and various other countries. Finally, our finding that transmission of conjugative plasmids encoding various carbapenemases and clonal spread of strains containing such plasmids were both responsible for a significant increase in the number of NDM-1 positive CRE infections in a hospital in China raised an alarming possibility that the prevalence of CRE can increase rapidly in a hospital setting within a short period. Whether the rate of infections due to such strains increases at a similar rate in the future depends on numerous factors including the effectiveness of infection control measures of the hospital concerned and the immune status of the infected patients. More works are urgently needed to investigate factors that determine the rate of transmission of CRE and the mobile resistance elements in order to help design appropriate intervention

strategies that pinpoint the core of the problem, that is, to target the pool of existing CRE and the resistance elements that they harbor.

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Attributes of carbapenemase encoding conjugative plasmid pNDM-SAL from an extensively drug-resistant *Salmonella enterica* Serovar Senftenberg

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A carbapenem resistant *Salmonella enterica* serovar Senftenberg isolate BCH 2406 was isolated from a diarrheal child attending an outpatient unit of B.C. Roy Hospital in Kolkata, India. This isolate was positive for the *bla*_{NDM-1} in the PCR assay, which was confirmed by amplicon sequencing. Except for tetracycline, this isolate was resistant to all the tested antimicrobials. The *bla*_{NDM-1} was found to be located on a 146.13-kb mega plasmid pNDM-SAL, which could be conjugally transferred into *Escherichia coli* and other enteric pathogens such as *Vibrio cholerae* O1 Ogawa and *Shigella flexneri* 2a. However, the expression of β-lactam resistance is not the same in different bacteria. The whole genome sequence of pNDM-SAL was determined and compared with other pNDM plasmids available in public domain. This plasmid is an IncA/C incompatibility type composed of 155 predicted coding sequences and shares homology with plasmids of *E. coli* pNDM-1_Dok01, *Klebsiella* pNDM-KN, and *Citrobacter* pNDM-CIT. In pNDM-SAL, gene cluster containing *bla*_{NDM-1} was located between IS26 and IS4321 elements. Between the IS26 element and the *bla*_{NDM-1}, a truncated ISAb125 insertion sequence was identified. Downstream of the *bla*_{NDM-1}, other genes, such as *ble*_{MBL}, *trpF*, *tat*, and an ISCR1 element with class 1 integron containing *aac(6')*-*lb* were detected. Another β-lactamase gene, *bla*_{CMY-4} was found to be inserted in IS1 element within the type IV conjugative transfer loci of the plasmid. This gene cluster had *blc* and *sugE* downstream of the *bla*_{CMY-4}. From our findings, it appears that the strain S. Senftenberg could have acquired the NDM plasmid from the other members of Enterobacteriaceae. Transfer of NDM plasmids poses a danger in the management of infectious diseases.

Keywords: NDM, carbapenemase, *S. Senftenberg*, enteric pathogens, pNDM-SAL

Introduction

Emergence of carbapenem resistance among Gram-negative bacteria is a major public health problem as they are associated with critical infections. This drug has been considered as one of the vital drugs against pathogens which produce extended spectrum β-lactamases (ESBLs). New Delhi Metallo-β-lactamase-1(NDM-1), which is a recent addition to the carbapenemase has become a

major concern worldwide due to its rapid spread across different members of Enterobacteriaceae and other Gram-negative bacteria. The NDM-1 encoding gene (*bla*_{NDM-1}) was first detected in *Klebsiella pneumoniae* and *Escherichia coli* recovered from a Swedish patient who had undergone treatment in New Delhi, India (Yong et al., 2009; Ghosh et al., 2014). Thereafter, this gene was identified in different Gram-negative bacteria in several countries including the USA, Canada, France, Sweden, UK, Germany, Japan, Austria, Africa, and Australia (Rolain et al., 2010). Presence of *bla*_{NDM-1} was generally associated with resistance to most of the antimicrobials, including fluoroquinolones, aminoglycosides, and β -lactams. The *bla*_{NDM} was found to be located of different large plasmids, which were readily transferable to other bacterial species (Kumarasamy et al., 2010; Rolain et al., 2010). The spread of the *bla*_{NDM-1} gene acquired by InCA/C MDR plasmids has drastically reduced the therapeutic options available to the physicians (Ghosh et al., 2014). In this study, we report the isolation of a *Salmonella enterica* serovar Senftenberg (S. Senftenberg) isolate carrying the *bla*_{NDM-1} gene on a large plasmid, which also possessed several genes responsible for extensively drug resistance (EDR).

Materials and Methods

Bacterial Strains

A carbapenem resistant *Salmonella enterica* isolate (BCH 2406) was isolated in 2012 from a five years old child who attended the outpatient department of B.C. Roy Memorial Hospital for Children, Kolkata for the treatment of diarrhea. This isolate was serotyped according to White-Kauffmann-Le Minor scheme with commercially available antisera (S&A Reagents Lab Ltd., Bangkok, Thailand). Tetracycline resistant XL1-Blue (TET^R) and sodium azide resistant *E. coli* J53 (Az^R) strains were used for conjugation experiments. In addition, ampicillin sensitive *Vibrio cholerae* O1 Ogawa (IDH 5313) and *Shigella flexneri* 2a (IDH 3077), isolated and identified from diarrheal patients were used as recipients. All the strains were preserved in Luria Bertani (LB) broth (Difco, Sparks, MD, USA) containing 15% glycerol at -80°C. Transconjugants were also maintained in nutrient agar (Difco) stab supplemented with 15 μ g/ml ceftriaxone. *E. coli* ATCC 25922 was served as control in antimicrobial susceptibility testing.

Detection of Carbapenem Resistance Encoding Gene

Presence of *bla*_{NDM} was identified by PCR with previously described primers (Chen et al., 2011) using Taq DNA polymerase (Roche, Mannheim, Germany). Amplicons were purified using a PCR product purification kit (Qiagen, Hilden, Germany) and sequenced using the ABI Big Dye terminator cycle sequencing ready reaction kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) in an automated DNA sequencer (ABI 3730, Applied Biosystems). The sequences were assembled and analyzed using DNASTAR software (DNASTAR, Inc., Madison, WI, USA).

Conjugation

To test the mobility and promiscuity of the *bla*_{NDM-1} harboring plasmid, conjugation by broth mating technique was carried out using NDM-positive *Salmonella* isolate as donor with four different recipients, namely *E. coli* XL1-Blue (TET^R), *E. coli* J53 (Az^R), *V. cholerae* O1 Ogawa, and *S. flexneri* 2a. In brief, overnight cultures of the bacteria were diluted in LB broth and allowed to grow as late-exponential phase culture. Cell density was adjusted to 1.5×10^8 cells/ml. Donor and recipient cells were mixed at 1:2 donor-to-recipient ratios in 1 ml of LB broth and allowed to grow overnight at 37°C. In all cases, the donor and recipient suspensions were also diluted in phosphate buffer saline (PBS) with a dilution of 10⁻³ and 10⁻⁵ and plated on MacConkey agar (Difco) to confirm the purity and count the colonies.

To recover *bla*_{NDM-1} positive transconjugants, several selective media were used. Transconjugants (conjugally transferred, CT) of XL1-Blue (CT-*E. coli* XL1-Blue) and *S. flexneri* 2a (CT-*S. flexneri*) were selected on xylose lysine desoxycholate (XLD, Difco) agar supplemented with tetracycline (30 μ g/ml) and ceftriaxone (5 μ g/ml). Similarly, transconjugants of *V. cholerae* (CT-*V. cholerae*) were obtained with selection based on growth on ceftriaxone (5 μ g/ml) containing thiosulphate citrate bile salts sucrose (TCBS, Eiken, Tokyo, Japan) agar and for transconjugants of *E. coli* J53 (CT-*E. coli* J53), MacConkey agar containing both ceftriaxone (5 μ g/ml) and sodium azide (100 μ g/ml) was used. Transconjugants were confirmed as *bla*_{NDM-1} positive by PCR analysis followed by PCR amplicon sequencing. The transfer frequencies were expressed as the number of transconjugants per donor cell.

Antimicrobial Susceptibility Testing

To confirm the transfer of resistance phenotype, antibiotic susceptibility patterns of the donor, recipients, and transconjugants were determined after their growth on Mueller-Hinton (MH, Difco) agar by disk diffusion method in accordance with Clinical and Laboratory Standards Institute [CLSI] (2014) using commercially available disks (Becton Dickinson Company, Sparks, MD) namely, ampicillin (AMP), cefuroxime (CXM), ceftriaxone (CRO), cefotaxime (CTX), cefotaxime/clavulanic acid (CTX-CLA), ceftazidime (CAZ), ceftazidime/clavulanic acid (CAZ-CLA), chloramphenicol (CHL), nalidixic acid (NA), ciprofloxacin (CIP), ofloxacin (OFX), norfloxacin (NOR), imipenem (IPM), streptomycin (STR), azithromycin (AZM) tetracycline (TET), trimethoprim/sulfamethoxazole (SXT). To measure the increase of resistance in the transconjugants, MICs of antibiotics (ceftriaxone, imipenem, tetracycline, and sulfamethoxazole/trimethoprim) were determined by *E* test (AB bioMérieux, Solna, Sweden) in comparison with the wild NDM-positive *Salmonella* isolate.

Plasmid analysis

Plasmid DNA was extracted from donor, recipients, and transconjugants by Kado and Liu's (1981) method and analyzed by gel electrophoresis using 0.8% agarose. Presence of *bla*_{NDM-1}

in the purified plasmid of the donor and transconjugants was determined by PCR. For size determination, plasmid analysis by S1 nuclease-pulsed-field gel electrophoresis (PFGE) (Barton et al., 1995) was performed with the donor and CT-*E. coli* J53. Total bacterial DNA prepared in agarose plugs was digested with S1 nuclease (Fermentas, Waltham, MA, USA) and separated using a CHEF-Mapper PFGE system (Bio-Rad, Hercules CA, USA), as reported previously (Kumarasamy et al., 2010). The PFGE conditions were run time 18 h gradient 6 V/cm, temperature 14°C, included angle 120° and initial and final pulses conducted for 2.16 s and 54.17 s, respectively. DNA of *S. enterica* serovar Braenderup H9812 digested with *Xba*I (Roche) was included as control size marker. Incompatibility group of plasmid was determined for the *Salmonella* isolate and *E. coli* J53 transconjugant (as *E. coli* J53 Az^R was devoid of plasmids) using the PCR-based replicon typing (PBRT) method as described by Carattoli et al. (2005).

Sequencing

Plasmid DNA was prepared from the *E. coli* J53 transconjugant using Qiagen Maxiprep kit (Qiagen). Paired-end libraries (300–500 bp fragments) were constructed by using the Illumina H TruSeqTM DNA Sample preparation kit (Illumina). Each library was deposited onto a HiSeq Flow Cell and sequenced using an Illumina HiSeq-2000 next-generation DNA sequencer. The distributions of “base quality” “base composition” and %GC of the plasmid were checked. Based on these distributions, the first 15 bases and last one base were trimmed to avoid specific sequence bias and poor quality bases. Errors were corrected in the sequence data using the HiTEC tool. Contig assembly and predicted gaps were then confirmed and filled by PCR-based gap closure, confirmed by DNA sequencing of the amplicons (Applied Biosystems). To assemble the contigs, ABySS and Edena software were used by varying the parameter ‘k’ and ‘overlap cut-off’, respectively. This step produced several contigs for each parameter setting. The contigs were then integrated using contig integrator for sequence assembly (CISA). A blastN search was made against the ‘nt’ database for each contig and two contigs were retained for further downstream analysis. The open reading frames (ORFs) from the contigs were generated by CISA using Glimmer-MG program. For all these ORFs, the nucleotide sequence and amino acid sequences were obtained and translated in the appropriate frame. The predicted ORFs were annotated using an in-house pipeline (CANoPI-Contig Annotator Pipeline) that also includes blastX search for each ORF sequence against ‘nr’ database provided by NCBI. ORF search and gene prediction was performed for the complete plasmid sequence with Lasergene software (DNASTAR, Inc., Madison, WI, USA) and pairwise alignment was analyzed by blastN and blastP homology search using the NCBI database (<http://www.ncbi.nlm.nih.gov/blast>).

Nucleotide Sequence Accession Number

The complete sequence of plasmid pNDM-SAL was submitted to GenBank under accession number KP742988.1

Results

Identification and Characterization of *bla*_{NDM-1}-Positive Isolate

The isolated *Salmonella enterica* was found to be positive for *bla*_{NDM-1}, which was confirmed by amplicon sequencing. The sequence of the *bla*_{NDM-1} showed 100% homology with those reported previously (Chen et al., 2011; Ho et al., 2011; Sekizuka et al., 2011; Bonnin et al., 2012; Carattoli et al., 2012; Fu et al., 2012; Dolejska et al., 2013). By serology, this isolate was identified as *S. Senftenberg* presenting antigenic formula as 1,3,19 : g,s,t : -. The isolate was found to be resistant to almost all antibiotics, including nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol, streptomycin, azithromycin, sulfamethoxazole/trimethoprim, ampicillin, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, and also to β-lactamase inhibitor combinations (cefotaxime/clavulanate and ceftazidime/clavulanate). However, it was susceptible to tetracycline and showed reduced susceptibility towards imipenem.

Conjugation and Transfer of Resistance

Plasmid harboring the *bla*_{NDM-1} was transferable to *E. coli* strains J53, XL1-Blue and to other enteric pathogens like *V. cholerae* O1 Ogawa and *S. flexneri* 2a isolated from the diarrheal patients. Conjugation frequencies were observed at higher rates (~10⁻⁵ transconjugants per donor cell) while using *E. coli* J53 and *S. flexneri* 2a as recipients than *E. coli* XL1-Blue (~10⁻⁶). The transfer of NDM plasmid into *V. cholerae* O1 Ogawa was even less efficient (~10⁻⁸) compared to the other strains (Table 1). In addition, the transconjugants acquired additional resistance against β-lactam antibiotics, namely ampicillin, cefuroxime, ceftriaxone, cefotaxime, ceftazidime, and also toward cephalosporin inhibitor combinations like cefotaxime/clavulanate and ceftazidime/clavulanate (Table 1). Interestingly, sulfamethoxazole resistance was noticed only in *S. flexneri* transconjugant. Though the CT-*E. coli* J53 was susceptible to sulfamethoxazole/trimethoprim, its MIC was found to increase by 2.7-fold. In CT-*S. flexneri*, the MIC of SXT rose by 31.6-fold, suggesting that sulfamethoxazole resistance was greatly expressed in CT-*S. flexneri* but not in CT-*E. coli* strains.

The CT-*E. coli* showed reduced susceptibility to carbapenems (Table 1). On the contrary, the transconjugants of clinical strains (CT-*V. cholerae* and CT-*S. flexneri*) showed high level resistance to imipenem, far greater than the donor strain *S. Senftenberg* (>5-fold). The MIC values for imipenem were found to be higher in CT-*S. flexneri* (128-fold) than those in CT-*V. cholerae* (>21-fold), CT-*E. coli* XL1-Blue (24-fold), and CT-*E. coli* J53 (12-fold). For ceftriaxone, more than 300-fold rise in MIC was observed for all CTs compared to the respective recipients.

Plasmid Analysis

Plasmid profiles of the donor and transconjugants (Figure 1) revealed the transfer of a mega plasmid from *S. Senftenberg* which conferred β-lactam resistance to the recipient strains of *E. coli*, *V. cholerae*, and *S. flexneri*. NDM-1 PCR assay confirmed

TABLE 1 | Antibiotic susceptibilities of donor, recipients, and transconjugants.

Strain	Bacteria	Resistance profile	MIC value ($\mu\text{g/ml}$)			
			TET	CRO	IPM	SXT
BCH 2406 (Donor)	<i>Salmonella</i> Senftenberg	NA, NOR, CIP, OFX, SXT, CHL, AZM, STR, AMP, CXM, CRO, CTX, CTX-CLA, CAZ, CAZ-CLA, IPM (I)	3	>256	6	>32
XL1-Blue (Recipient)	<i>Escherichia coli</i>	NA, TET	192	0.5	0.125	
CT-E. coli XL1-Blue (Transconjugant)	<i>E. coli</i>	NA, TET, AMP, CXM, CRO, CTX, CTX-CLA, CAZ, CAZ-CLA	96	>256	3	
J53 (Recipient)	<i>E. coli</i>	AZD		0.5	0.125	0.047
CT-E. coli J53 (Transconjugant)	<i>E. coli</i>	AMP, CXM, CRO, CTX, CTX-CLA, CAZ, CAZ-CLA, AZD	>256	1.5	0.125	
IDH 5313 (Recipient)	<i>Vibrio cholerae</i> O1 Ogawa	NA, SXT, STR		0.5	1.5	>32
CT-V. cholerae (Transconjugant)	<i>V. cholerae</i> O1 Ogawa	NA, SXT, STR, AMP, CXM, CRO, CTX, CTX-CLA, CAZ, CAZ-CLA, IPM	>256	>32	>32	
IDH 3077 (Recipient)	<i>Shigella flexneri</i> 2a	NA, STR, TET		0.75	0.25	0.38
CT-S. flexneri (Transconjugant)	<i>S. flexneri</i> 2a	NA, TET, STR, SXT, AMP, CXM, CRO, CTX, CTX-CLA, CAZ, CAZ-CLA, IPM	>256	32	12	
	Transfer frequency*: 3.6×10^{-6}					
	Transfer frequency*: 1.9×10^{-5}					
	Transfer frequency*: 1.5×10^{-8}					
	Transfer frequency*: 3.7×10^{-5}					

NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; OFX, ofloxacin; AZM, azithromycin; STR, streptomycin; AMP, ampicillin; CXM, cefuroxime; CRO, ceftazidime; CTX, cefotaxime; CAZ, ceftazidime; CTX-CLA, cefotaxime/clavulanate; CAZ-CLA, ceftazidime/clavulanate; IPM, Imipenem; SXT, sulfamethoxazole/trimethoprim; CHL, chloramphenicol; TET, tetracycline; AZD, azide; (I) = intermediate resistance.

*Frequency of transfer was calculated as number of transconjugants per donor cell.

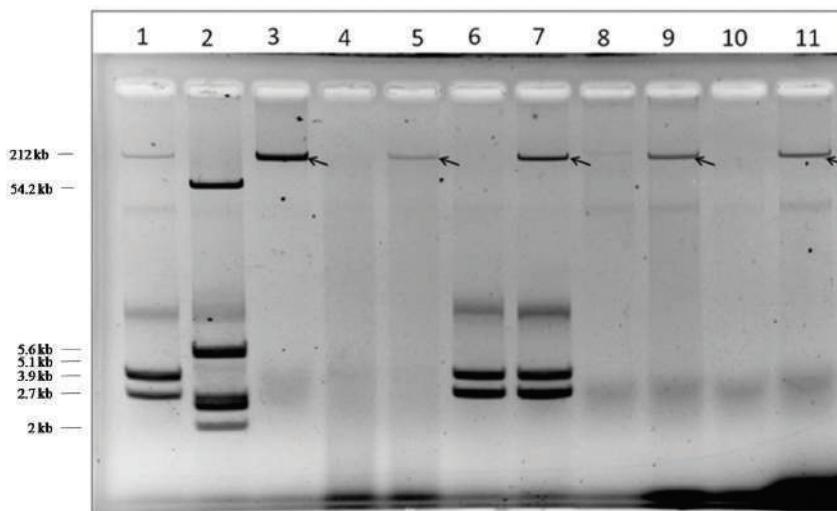


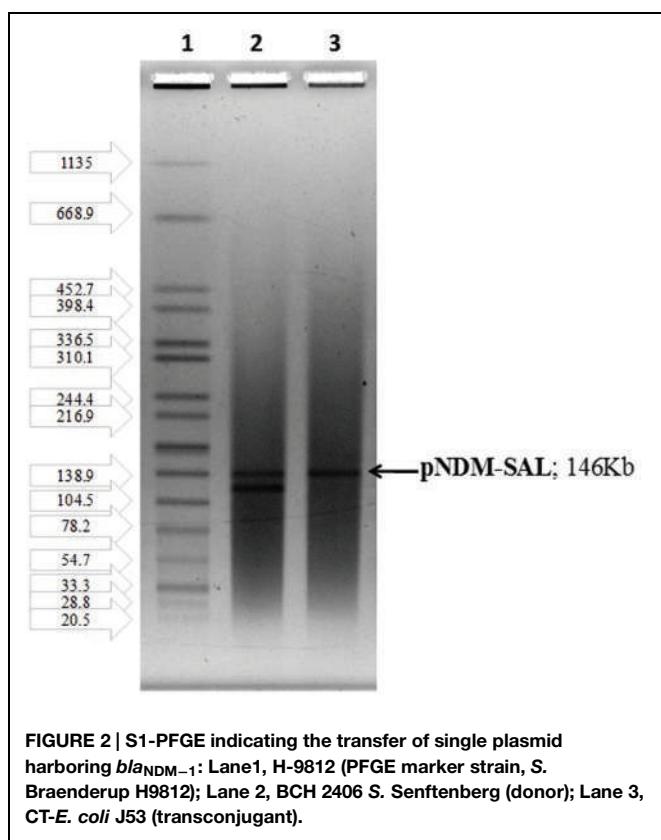
FIGURE 1 | Plasmid profile of donor, recipient, and transconjugants. Lanes 1 and 2, *Shigella flexneri* YSH 6000 and *Escherichia coli* K12 V517 were used as reference standards, respectively; Lane 3, BCH 2406: *Salmonella* Senftenberg (donor); Lane 4, IDH 5313: *Vibrio cholerae* O1 Ogawa (recipient); Lane 5, CT-V. cholerae (transconjugant); Lane 6, IDH 3077: *S. flexneri* 2a (recipient); Lane 7, CT-S. flexneri (transconjugant); Lane 8, XL1-Blue, *E. coli* (recipient); Lane 9, CT-E. coli XL1-Blue (transconjugant); Lane 10, *E. coli* J53 (recipient); Lane 11, CT-E. coli J53 (transconjugant).

the presence of *bla*_{NDM-1} in all the transconjugants. S1-PFGE clearly showed that the resistance phenotype was associated with the transfer of single plasmid of about 146-kb (Figure 2) and the NDM-encoding gene was detected by PCR on the plasmid isolated from the transconjugants. Plasmid pNDM-SAL was assigned to the IncA/C incompatibility group using the PCR-based PBRT method.

Sequence Analysis of Plasmid pNDM-SAL

Whole plasmid showed the plasmid pNDM-SAL to consist of 146.13-kb with an average %GC content of 51.7. In addition to its

own replication machinery with partitioning system, it has two separate gene clusters for *bla*_{NDM-1} and *bla*_{CMY-4} (Figure 3). It contained 155 predicted coding sequences (CDSs). This plasmid shared extensive homology (99% identity with 98% query coverage) with an IncA/C plasmid, pNDM-1_Dok01 (195.56-kb, described for *E. coli*, accession no. AP012208) including the complete array of genes for replication, type IV conjugative transfer machinery, partition and stabilization, except for the flanking region around *bla*_{NDM-1} and *bla*_{CMY-4}. In addition, it had sequence similarity with *Klebsiella* plasmid pNDM-KN (JN157804) and *Citrobacter* plasmid pNDM-CIT (JX182975)



showing 99% identity with 87% query coverage and 99% identity with 87% query coverage, respectively.

Analysis and Comparison of Gene Organization Around *bla*_{NDM-1} and between Other Plasmids

The *bla*_{NDM-1} gene was localized in a multidrug resistance region of 15.3-kb (Figure 4). This region was bracketed by two different copies of IS elements in inverted orientation, suggesting that the gene was acquired as a composite transposon. Unlike, pNDM-1_Dok01 and pGUE-NDM (87-kb, described in *E. coli*, accession no JQ36496) where *bla*_{NDM-1} gene was flanked by IS903 and IS26, respectively, pNDM-SAL possessed IS26 and IS4321 upstream and downstream of the *bla*_{NDM-1}. This orientation is much similar to that found in the pNDM-CIT. In between the IS26 element and the *bla*_{NDM-1} gene, a remnant of ISAb125 insertion sequence was identified which comprised –35 promoter sequences leading to the high expression of NDM.

Downstream of the *bla*_{NDM-1}, the *ble*_{MBL} gene encoding resistance to bleomycin followed by a truncated phosphorybosilanthranilate isomerase gene (*trpF*), and a twin-arginine translocation pathway signal protein gene (*tat*) was identified. Such an arrangement has been reported previously in other plasmid scaffolds (Sekizuka et al., 2011). Following this, a ISCR1 element was identified trailed by a class 1 integron containing *aac*(6')-Ib as its variable resistance gene cassette. Such genetic arrangement has been found in a complex class 1 integron containing ISCR1 element of ESBL positive *E. coli*

(EF450247) and *V. cholerae* (DQ310703). ISCR1 and IS26 are known to mediate transposition and/or expression of multiple resistance genes in their close proximity (Bae et al., 2007; Kiiur et al., 2013). Similarly, class 1 integron bearing ISCR1 was detected in the previously described NDM plasmids, namely pGUE-NDM (JQ364967) and pNDM-CIT (JX182975). The only difference being that the other plasmids harbored *dfrA12-ofrA-aadA2* as their integron resistance gene cassettes (Figure 4). However, in all the cases *qacEΔ1* and *sul1* gene are present in the integron structure. The *intI1* gene was followed by the *tnpR* and *tnpA*, the genes of transposon Tn21 and then by a copy of insertion element IS4321, as previously identified in pNDM-CIT (JX182975). Like pNDM-SAL, linkage between Tn21 transposon bearing class 1 integron and IS26 element has been seen in NDM negative *E. coli* of human and animal origin (Dawes et al., 2010) and in *E. coli* D22 (EU914098). From the *in silico* analysis, it was not possible to determine the genetic events that led to the formation of this heterogeneous genetic structure. However, it is likely that multiple genetic events contributed to the acquisition of the *bla*_{NDM-1} containing locus by the plasmid.

The *bla*_{CMY} Module Region

In addition to the *bla*_{NDM-1}, pNDM-SAL carried an additional β-lactam resistance gene, *bla*_{CMY-4}, distantly located from the NDM harboring composite transposon (Figure 5). The *bla*_{CMY} gene was preceded by the IS1 instead of IS*Ecp1*, as reported previously for other NDM plasmids, pNDM-1_Dok01 and pKP1-NDM-1 (KF992018). Remarkably, *bla*_{CMY} gene associated with IS1 element was detected before in NDM-negative *E. coli* (DQ173300) with inverted repeats (IRs) of IS*Ecp1* (Hopkins et al., 2006). This makes it tempting to speculate that an intact copy of IS*Ecp1* was responsible for the early transposition and mobility of *bla*_{CMY-4} followed by insertion of the IS1 element; a process similar to what has been reported previously for *E. coli* (DQ173300). Downstream of the *bla*_{CMY-4} gene, *blc* gene encoding outer membrane lipoprotein was detected followed by the *sugE* encoding quaternary ammonium compound resistance protein (Figure 5), a feature common in many of the *bla*_{CMY} regions in other NDM-positive plasmids (Kang et al., 2006; Sekizuka et al., 2011). This IS1-*bla*_{CMY} module was located within the *tra* locus. Similar insertion with IS*Ecp1* has been demonstrated before (Poole et al., 2009).

Discussion

The present study revealed EDR features of the pNDM-SAL isolated from *S. Senftenberg*. This finding highlights the ease with which the resistance determinants can move to other enteric pathogens. Firstly, the *bla*_{NDM-1} is located on a broad host range IncA/C plasmid, providing a possible explanation why the pNDM-SAL could be transferred by conjugation to *E. coli* (*E. coli* J53 and XL1-Blue) as well as to wild-type strains of *V. cholerae* O1 Ogawa and *S. flexneri* 2a easily. In repeated experiments, CT-*V. cholerae* and CT-*S. flexneri* showed a higher level of

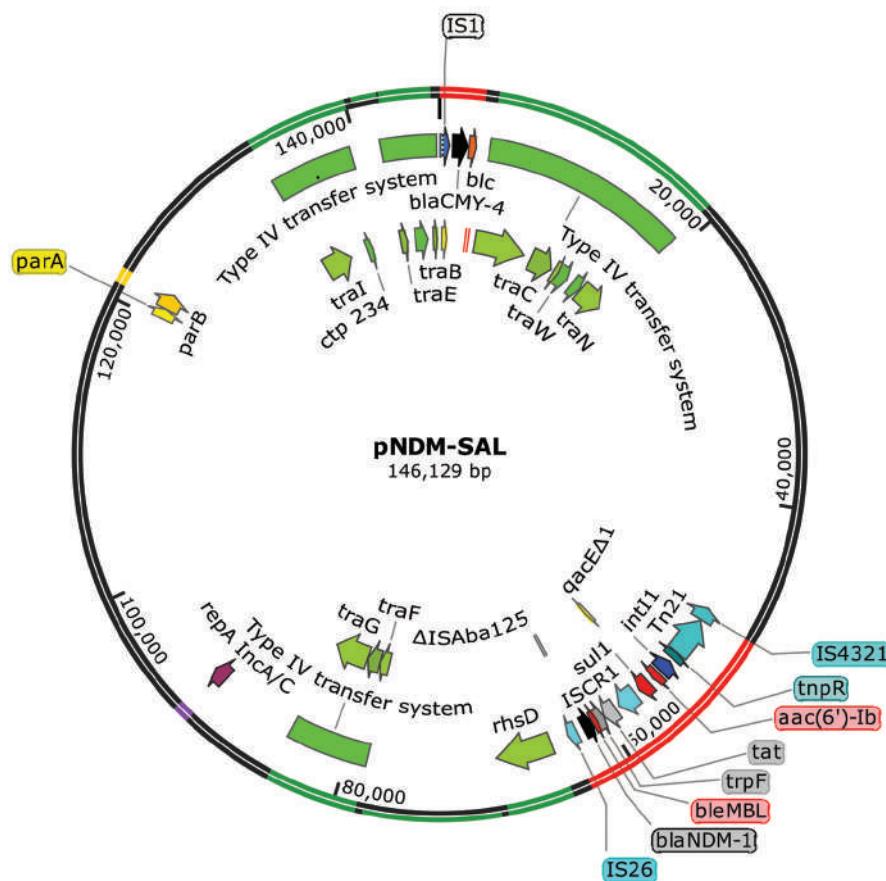


FIGURE 3 | Salient features of *bla*_{NDM-1} and *bla*_{CMY-4} encoding plasmid pNDM-SAL with transposons and type IV conjugative transfer system.

Starting from the outside, the first circle indicates the coordinate of the complete plasmid circle. Transfer machinery and variable region are showed in green and red, respectively. *rep* and *par* loci are showed in purple and yellow, respectively. The open reading frames (ORFs) were annotated in the inner circle with arrows representing the direction of transcription. β-lactam resistance genes are denoted by black color and other resistance genes as red color. All transposons and IS elements are indicated in shades of blue. ORFs responsible for conjugation are denoted by shades of green.

imipenem resistance compared to the donor strains. This could be hypothesized to be due to “possibly” a higher copy number of the plasmid carrying the gene in the recipient than in the donor strains. In other words due to “gene dosage” effect. There could be other possible explanations as well. Examples of such phenomenon, transconjugants showing higher drug resistance are documented in the literature (Ouellette et al., 1988; Petroni et al., 2002).

Since IncA/C plasmids possess highly mobile nature, it has commonly been found in NDM harboring bacteria, especially among the members of Enterobacteriaceae (Ghosh et al., 2014). The size of plasmid varies considerably, ranging from 35.9 to 400-kb (Ghosh et al., 2014) with certain shared genetic background.

A recent study has shown that the NDM carrying plasmids from Enterobacteriaceae at New Delhi, shared the replicon type IncA/C with 140-kb in size (Walsh et al., 2011). Large plasmids belonging to the IncA/C group have received increased attention, primarily due to their ability to confer resistance to a diverse group of antimicrobial agents (Fernandez-Alarcon et al., 2011). Transmission of pNDM-SAL was also found to be

closely associated with transfer of multi drug resistance. Since clinical strains of *Vibrio* or *Shigella* are typically resistant to some antimicrobials, transfer of pNDM-SAL carrying *bla*_{NDM-1} and other genes could make them multi resistance. This situation is of major concern in the clinical management of infections. Since the pNDM-SAL harbored antibiotic resistance genes are clustered in a few integration hotspots within complex genetic structures, they may be able to acquire novel antibiotic resistance genes through homologous recombination (Doublet et al., 2012). In the pNDM-SAL, *rhs* locus remains near *bla*_{NDM-1} with a phage-integrase and hence they are likely to be the hotspots for integration of accessory genes within the IncA/C plasmids (Carattoli et al., 2012).

In pNDM-SAL, *bla*_{NDM-1} was located between IS26 and IS4321. Similar genetic arrangements have previously been observed in other *bla*_{NDM-1} bearing plasmids (Sekizuka et al., 2011; Bonnin et al., 2012; Dolejska et al., 2013). Comparison of the flanking regions of *bla*_{NDM-1} present in different plasmids suggests that different genetic events may have supported acquisition of this gene in different plasmids (Ghosh et al.,

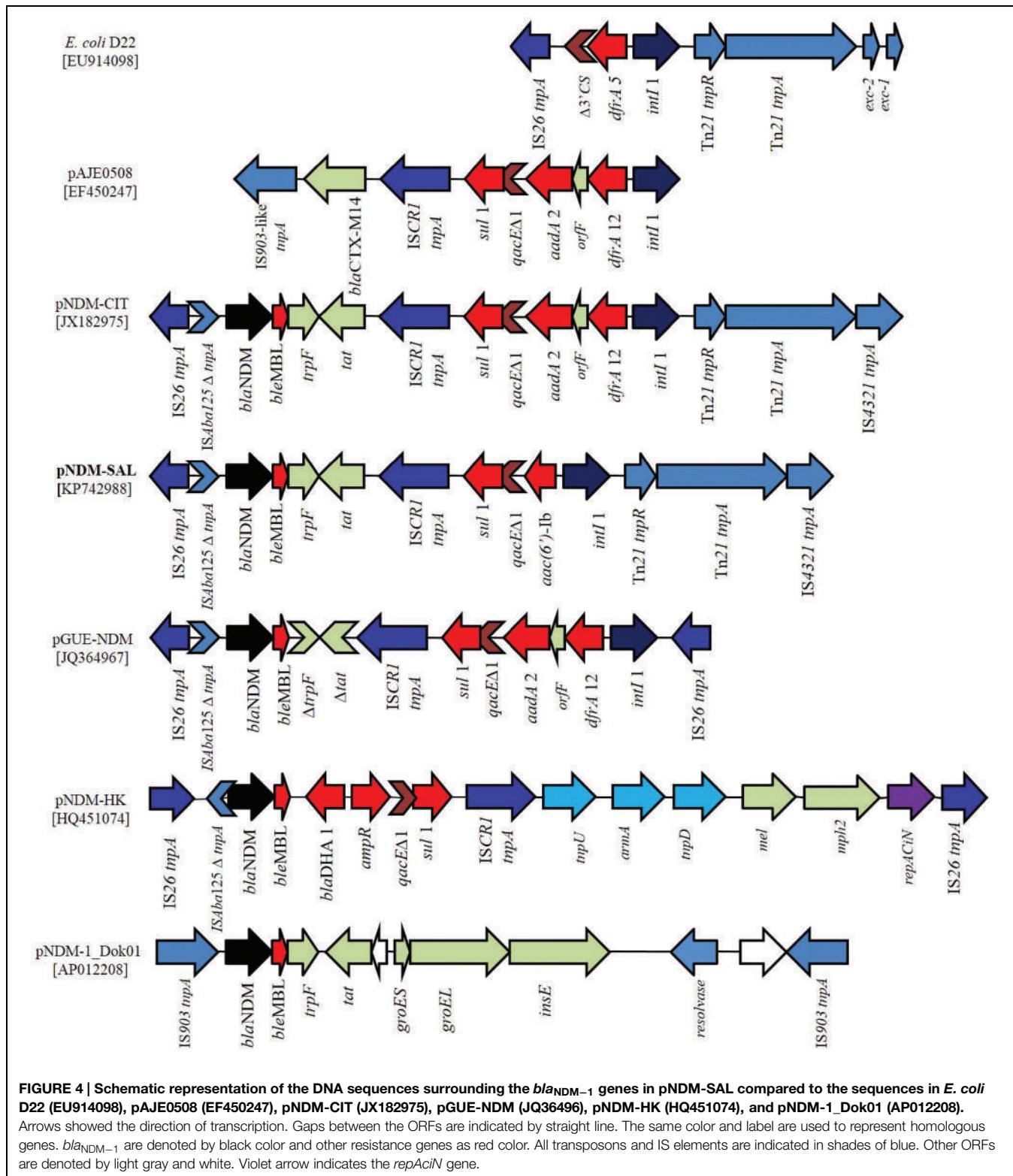


FIGURE 4 | Schematic representation of the DNA sequences surrounding the *bla_{NDM-1}* genes in pNDM-SAL compared to the sequences in *E. coli* D22 (EU914098), pAJE0508 (EF450247), pNDM-CIT (JX182975), pGUE-NDM (JQ364967), pNDM-HK (HQ451074), and pNDM-1_Dok01 (AP012208).

Arrows showed the direction of transcription. Gaps between the ORFs are indicated by straight line. The same color and label are used to represent homologous genes. *bla_{NDM-1}* are denoted by black color and other resistance genes as red color. All transposons and IS elements are indicated in shades of blue. Other ORFs are denoted by light gray and white. Violet arrow indicates the *repACIN* gene.

2014). In pNDM-SAL, the linkage of *bla_{NDM-1}* with IS26 creates a condition favorable for the mobilization of *bla_{NDM-1}*. Interestingly, in many NDM negative *E. coli* strains, arrangement of class 1 integron between Tn21 transposon and IS26 was

a characteristic feature (Dawes et al., 2010) as noticed in the pNDM-SAL. The IS26 element, a member of the IS6 family, is widespread among Enterobacteriaceae and exists adjacent to the β-lactamases region, which is a part of transposon-like structure

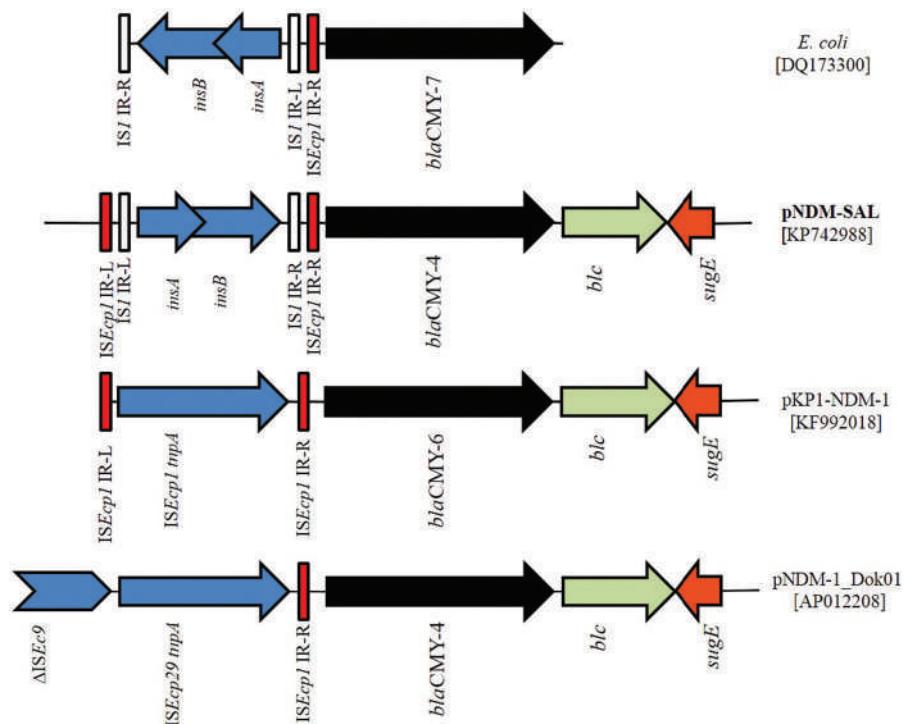


FIGURE 5 | Schematic representation of the DNA sequences surrounding the blaCMY-4 genes in pNDM-SAL and comparison with the sequences in *E. coli* (DQ173300), pKP1-NDM-1 (KF992018), and pNDM-1_Dok01 (AP012208). Arrows indicating blaCMY-4 (black), blc (gray) and sugE (orange) are showed according the direction of transcription. Gaps between the ORFs are indicated by straight line. The same color and label are used to represent homologous genes. IS elements are indicated in shades of blue. Red and white color indicates the inverted repeat (IR) of IS*Ecp*1 and IS1, respectively.

in many plasmids (Yu et al., 2006; Ho et al., 2011). Similar to the other plasmids such as *E. coli* DVR22 (JF922606), pNDM-HK (HQ451074), pKpANDM-1 (FN396877) and p271A (JF785549), a truncated IS*Aba*125 has been identified upstream of bla_{NDM-1} in pNDM-SAL. Interestingly, in *Acinetobacter baumanii* isolates of African origin, the NDM encoding gene was found within the Tn125-like or IS*Aba*125 (Bonnin et al., 2013). This suggests that the IS*Aba*125 insertion sequence has possibly played an important role in the mobilization of bla_{NDM-1} from a common progenitor and that event was followed by subsequent transfer events mediated by the other insertion elements like IS26 as seen in *Acinetobacter* spp. and Enterobacteriaceae (Ghosh et al., 2014). In pGUE-NDM (*E. coli*, JQ364967) and pNDM-MAR (*Klebsiella*, JN420336) NDM regions are fully bracketed by IS26. But pNDM-SAL possesses IS4321 and Tn21 transposases in one end similar to what is found in the pNDM-CIT (*Citrobacter*, JX182975). Instead of bla_{DHA-1} gene commonly found downstream of many NDM plasmids, e.g. pNDM-HK (*E. coli*, HQ451074) the pNDM-SAL ISCR1 bearing class 1 integron has been inversely located (Figure 4). The association between ble_{MBL} and bla_{NDM} appears to be strong as they are expressed by a common promoter. This may be the reason why both the genes transfer *en bloc* (Fu et al., 2012; Ghosh et al., 2014).

Based on the genetic environment of bla_{NDM-1} in pNDM-SAL, we hypothesize that (i) a common transposon structure

with IS*Aba*125 could be responsible for the early acquisition of bla_{NDM-1}, (ii) by extensive genetic rearrangement; it was captured in a Tn21 linked complex class 1 integron bearing ISCR1, (iii) later on it was bracketed by two IS elements, namely IS4321 preceding the Tn21 transposase and IS26 truncating the IS*Aba*125.

The plasmid pNDM-SAL has similarity to other NDM-plasmids, which harbor blaCMY-4 and the complex class 1 integron carrying several antibiotic resistance-conferring genes (Toleman et al., 2006a,b). AmpC-like cephalosporinase (blaCMY) genes that have been frequently mobilized by IncA/C-type plasmids, could be identified in *E. coli* and *Salmonella* isolates not only of human but also of animal origins in the United States, Canada, and Europe (Winokur et al., 2001; Carattoli et al., 2012). The evolutionary relationship between NDM-1-bearing plasmids and the IncA/C plasmids carrying blaCMY suggests that IncA/C blaCMY-carrying plasmid could have acquired the bla_{NDM-1} within its scaffold due to a secondary event (Carattoli et al., 2012).

From the data presented in this paper it can be seen that plasmid pNDM-SAL possesses many interesting features as it contains gene encoding the metallo-β-lactamase NDM-1, which is positively associated with multidrug resistance (Figure 3). Besides, pNDM-SAL harbors a large arsenal of genetic elements (integrons, transposons, and ISCRs), giving it the ability to acquire and disseminate antibiotic resistance

genes. However, the extent of carbapenem resistance due to the presence of NDM plasmid varied in different hosts. The trend of antimicrobial susceptibility is shifting toward old generation antibiotics, which are less commonly used in recent years (Meziane-Cherif and Courvalin, 2014). In many studies, it was shown that NDM producers were susceptible to old generation antibiotics such as chloramphenicol, tetracycline etc. (Abdul Rahim et al., 2015; Uppu et al., 2015). In our finding, excerpt for tetracycline, the NDM-positive *S. Senftenberg* were resistant to most of the old generation antibiotics such as ampicillin, trimethoprim, sulfamethoxazole, streptomycin, nalidixic acid, and chloramphenicol. It appears that the maintenance of resistance to any given antibiotic may vary from species to species.

From our data it can be inferred that the strain *S. Senftenberg* probably was not the natural host for this NDM plasmid but once acquired, the plasmid it had the ability to transfer it to a broad range of pathogenic and non-pathogenic Gram-negative bacteria, an observation which merits serious and important attention to our findings.

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AS, GP, and GC isolated and identified the pathogens, performed phenotypic and genetic analysis. AG and TR analyzed the data, conceived the idea, and wrote the manuscript. All authors were involved in the compilation of the report and approved the final version.

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Comparative Genomics of Two ST 195 Carbapenem-Resistant *Acinetobacter baumannii* with Different Susceptibility to Polymyxin Revealed Underlying Resistance Mechanism

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Acinetobacter baumannii is a Gram-negative nosocomial pathogen of importance due to its uncanny ability to acquire resistance to most antimicrobials. These include carbapenems, which are the drugs of choice for treating *A. baumannii* infections, and polymyxins, the drugs of last resort. Whole genome sequencing was performed on two clinical carbapenem-resistant *A. baumannii* AC29 and AC30 strains which had an indistinguishable Apal pulsotype but different susceptibilities to polymyxin. Both genomes consisted of an approximately 3.8 Mbp circular chromosome each and several plasmids. AC29 (susceptible to polymyxin) and AC30 (resistant to polymyxin) belonged to the ST195 lineage and are phylogenetically clustered under the International Clone II (IC-II) group. An AbaR4-type resistance island (RI) interrupted the *comM* gene in the chromosomes of both strains and contained the *bla_{OXA-23}* carbapenemase gene and determinants for tetracycline and streptomycin resistance. AC29 harbored another copy of *bla_{OXA-23}* in a large (~74 kb) conjugative plasmid, pAC29b, but this gene was absent in a similar plasmid (pAC30c) found in AC30. A 7 kb Tn1548::*armA* RI which encodes determinants for aminoglycoside and macrolide resistance, is chromosomally-located in AC29 but found in a 16 kb plasmid in AC30, pAC30b. Analysis of known determinants for polymyxin resistance in AC30 showed mutations in the *pmrA* gene encoding the response regulator of the two-component *pmrAB* signal transduction system as well as in the *lpxD*, *lpxC*, and *lpsB* genes that encode enzymes involved in the biosynthesis of lipopolysaccharide (LPS). Experimental evidence indicated that impairment of LPS along with overexpression of *pmrAB* may have contributed to the development of polymyxin resistance in AC30. Cloning of a novel variant of the *bla_{AmpC}* gene from AC29 and AC30, and its subsequent expression in *E. coli* also indicated its likely function as an extended-spectrum cephalosporinase.

Keywords: *Acinetobacter baumannii*, carbapenem-resistant, polymyxin, whole genome sequencing, resistance island, resistance mechanisms

INTRODUCTION

Acinetobacter baumannii is a significant nosocomial pathogen that infects immunocompromised patients, including patients with underlying diseases, especially those who are warded in intensive care units (ICU; Bergogne-Bérénin and Towner, 1996; Nordmann and Poirel, 2008; Camp and Tatum, 2010). These nosocomial infections include ventilator-associated pneumonia, secondary meningitis, endocarditis, urinary tract infections, surgical site infections, and blood stream infections (Camp and Tatum, 2010; Huang et al., 2012). *A. baumannii* is intrinsically resistant to commonly used antibiotics such as aminopenicillins, first- and second-generation cephalosporins and chloramphenicol (Dijkshoorn et al., 2007). The notoriety of this pathogen stems from its ability to develop and acquire resistance to almost all antimicrobial drugs as well as its tolerance to desiccation and ability to survive on inanimate surfaces for prolonged periods of time (Camp and Tatum, 2010; Roca et al., 2012; Doi et al., 2015). In depth analyses of the resistance mechanisms in *A. baumannii* revealed that its multidrug resistance phenotype is mediated by all the major resistance mechanisms that are known to occur in bacteria, including modification of target sites, enzymatic inactivation, active efflux, and decreased influx of drugs (Dijkshoorn et al., 2007).

Carbapenem resistance in *A. baumannii* is usually acquired with the most significant mechanism being the production of carbapenemases (Poirel and Norman, 2006). *A. baumannii* naturally produces the OXA-51-group carbapenemase at a low level. The transposition of an insertion sequence (usually ISAbal or ISAbal9) upstream of the *bla*_{OXA-51} gene provides a strong promoter for expression of the carbapenemase leading to elevation of carbapenem MICs and thus, resistance (Higgins et al., 2010). *A. baumannii* also readily acquires several OXA-group β -lactamases usually through transposons and plasmids with OXA-23 being the most prevalent (Poirel and Norman, 2006; Roca et al., 2012).

A. baumannii naturally produces the AmpC-type β -lactamase and overexpression of the *bla*_{AmpC} gene has been associated with the presence of ISAbal providing a strong promoter leading to cephalosporin resistance (Segal et al., 2005; Tian et al., 2011). These *Acinetobacter*-derived cephalosporinases (ADCs) typically hydrolyze penicillins, narrow- and extended-spectrum cephalosporins but not cefepime or carbapenems (Rodríguez-Martínez et al., 2010). Recently, several ADC variants have been identified that are capable of hydrolyzing cefepime and these are termed as extended-spectrum AmpCs (ESACs) (Rodríguez-Martínez et al., 2010; Tian et al., 2011).

It is of great concern when carbapenems, which are the drugs of choice for treatment, are increasingly compromised. As the efficacy of these drugs decreases, polymyxins were re-introduced as the drug of “last resort” (Landman et al., 2008). The renewed interest in polymyxins (polymyxin B and colistin, or polymyxin E) as therapeutic agents was due to the pathogen’s outer

membrane, polyanionic lipopolysaccharide (LPS) having high affinity toward the cationic polymyxin molecules (Landman et al., 2008). Binding of the LPS and polymyxin molecules resulted in a detergent-like effect that disrupts the outer membrane thereby causing cytoplasm leakage of the pathogen (Landman et al., 2008; Arroyo et al., 2011). Although polymyxins were once discarded due to their neurotoxicity and nephrotoxicity, polymyxins have a more effective antibacterial activity against most Gram negative pathogens, including *A. baumannii* (Landman et al., 2008). Previous studies from Garnacho-Montero et al. (2003) and Moffatt et al. (2010) showed that intravenous polymyxins were safe to use as an effective treatment to *Acinetobacter* infections. However, uncontrolled use or overuse of polymyxins in the hospital environment may lead to the development of polymyxin-resistance in *A. baumannii* (Arroyo et al., 2011).

Polymyxin resistance in *A. baumannii* appeared to develop intrinsically as a result of exposure to this class of drugs. Two major mechanisms of polymyxin resistance have been described for *A. baumannii*. The first is the modification of the lipid A moiety of LPS with phosphoethanolamine as a result of mutations in the *pmrA/pmrB* two-component signal transduction system which leads to the up-regulated expression of the *pmrCAB* operon. Overexpression of *pmrC* which encodes the enzyme responsible for phosphoethanolamine addition to lipid A, impairs the binding of polymyxin to the outer membrane thereby leading to resistance (Adams et al., 2009; Arroyo et al., 2011; Beceiro et al., 2011; Park et al., 2011). The second mechanism is the complete loss of the LPS caused either by mutations or the insertional inactivation of the lipid A biosynthesis genes, namely *lpxA*, *lpxC*, and *lpxD* (Moffatt et al., 2010, 2011). Mutations in the *lpsB* gene that encodes a glycosyltransferase involved in the biosynthesis of the LPS core have also been implicated in polymyxin resistance (Hood et al., 2013).

We have previously characterized 54 *A. baumannii* strains obtained from a tertiary hospital in Terengganu, Malaysia (Lean et al., 2014). Out of these, 39 were carbapenem- and multidrug-resistant (MDR). Among the 39 carbapenem resistant strains, 14 were also resistant to polymyxin B and categorized as extensively-drug resistant (XDR). Two strains, *A. baumannii* AC29 and AC30 were isolated from the wounds of different patients, and shared an identical *Apal* pulsotype. However, AC29 is susceptible to polymyxin B whereas AC30 was resistant with an MIC value of 128 μ g/ml. Here, we report the comparative genomic analyses of these two *A. baumannii* strains, AC29 and AC30, to show that despite sharing an identical pulsotype, there are significant changes in the genome structure particularly in the resistance islands and the plasmid content of the two isolates. We also present experimental evidence to elucidate possible mechanisms for the development of polymyxin resistance in *A. baumannii* AC30 and the likely implication of a novel *bla*_{AmpC} cephalosporinase gene in resistance to extended-spectrum cephalosporins as well as imipenem in both AC29 and AC30.

MATERIALS AND METHODS

Whole Genome Sequencing

Strains Selection, Antibiotic Resistance Profiles, and DNA Extraction

Two strains of *A. baumannii* AC29 and AC30 from a tertiary hospital in Terengganu, Malaysia were selected for this study (Lean et al., 2014). Both strains were obtained from the wounds of different patients using standard microbiology procedures. *A. baumannii* AC29 and AC30 shared an identical *Apal* pulsotype and showed resistance to gentamycin, tobramycin, amikacin, ciprofloxacin, levofloxacin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, ampicillin-sulbactam, tetracycline, and doxycycline. Both isolates were also resistant to carbapenem with MIC values of $>32\text{ }\mu\text{g/ml}$ for both imipenem and meropenem (Clinical and Laboratory Standards Institute, 2013). However, AC29 was susceptible to polymyxin B whereas AC30 was resistant with an MIC value of $128\text{ }\mu\text{g/ml}$ (Magiorakos et al., 2011; Lean et al., 2014).

Genomic DNA of AC29 and AC30 were extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Extracted DNA was quantified using the spectrophotometer at OD₂₆₀ and the purity was determined by OD₂₆₀/OD₂₈₀ ratio (Ausubel et al., 2002).

Genomic Sequencing, Assembly, and Annotations

Whole genome sequencing of *A. baumannii* AC29 and AC30 was carried out by a commercial vendor using the Illumina Genome Analyzer IIx platform. CLC Bio software package was used to assemble the genome sequence data. Open reading frame (ORF) prediction and gene functional assignments were done using Prodigal 2.60 (Hyatt et al., 2010), RNAmmer 1.2 (Lagesen et al., 2007), and tRNAscan-SE (Lowe and Eddy, 1997). Functional annotation of the genome was performed using Blast2Go and the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008).

MLST and Phylogenetic Analysis

To determine the sequence types, multilocus sequence typing (MLST) of AC29 and AC30 was performed according to the Bartual or Oxford scheme using the seven housekeeping genes *cpn60*, *gdhB*, *gltA*, *gpi*, *gyrB*, *recA*, and *rpoD* (Bartual et al., 2005) and the Pasteur scheme using the genes *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* (Diancourt et al., 2010). The gene sequences were compared to the PubMLST database for *A. baumannii* (<http://pubmlst.org/abaumannii/>) and assigned to the appropriate sequence types.

The phylogenetic relationship of AC29 and AC30 to sequenced *A. baumannii* strains was inferred using composition vector tree (CVTree) version 2 (Xu and Hao, 2009) based on the concatenated nucleotide sequences of the seven reference genes used in the Bartual MLST scheme. The CVTree web-based server (<http://tlife.fudan.edu.cn/cvtree/>) generates phylogenetic trees based on *k*-tuple values (Xu and Hao, 2009). The completed *A. baumannii* genomes used in the analysis were downloaded via

the NCBI FTP site and the reference gene sequences extracted for phylogenetic analysis.

Comparative Genomics Analyses

Whole genome sequences of these two *A. baumannii* AC29 and AC30 strains were compared to our previously reported *A. baumannii* AC12 genome (Lean et al., 2015) and the completed *A. baumannii* genomes available from NCBI FTP site using Mauve (Darling et al., 2004). Circular chromosome and plasmid map of these strains were constructed using CGView Server developed by the Stothard Research Group (Grant and Stothard, 2008).

Accession Numbers

Genome sequences of *A. baumannii* strains AC29 and AC30 were deposited under the accession number CP007535 and CP007577, respectively. Plasmid sequences of pAC29a, pAC29b, pAC30a, pAC30b, and pAC30c were deposited under the accession number CP008850, CP008851, CP007578, CP007579, and CP007580, respectively. Accession numbers for our previously reported *A. baumannii* strain AC12 (CP007549) and pAC12 (CP007550) were as in (Lean et al., 2015).

Quantitative Real-Time PCR Of *pmrAB*

One of the gene loci that have been implicated in polymyxin resistance is the *pmrCAB* operon with *pmrAB* encoding the two-component signal transduction system and *pmrC* encoding the enzyme that catalyzes the addition of phosphoethanolamine to the lipid A moiety of lipopolysaccharide (LPS; Arroyo et al., 2011; Park et al., 2011; Hood et al., 2013). To determine the transcript levels of *pmrAB* from the polymyxin-susceptible AC29, and the polymyxin-resistant isolates AC30 as well as AC12 (Lean et al., 2015), quantitative real-time PCR (qRT-PCR) was employed. *A. baumannii* ATCC19606 was used as the polymyxin-susceptible control. Total RNA was extracted from *A. baumannii* strains grown in LB broth using Qiagen RNeasy Mini Kit (Qiagen).

The extracted total RNA was subjected to reverse transcription using the Quantitect Reverse Transcription Kit (Qiagen) in a T-Gradient PCR machine from Biometra/Applied Biosystems. The resulting cDNA produced was used as template for quantitative real-time PCR using the SYBR Green PCR Kit (Qiagen) performed in a Rotor-Gene 6000 Real-Time PCR Machine (Corbett Life Science/Qiagen). Prior to the actual amplification cycling conditions, the mixture was subjected to initial heat activation at 95°C for 5 min followed by 35 cycles of the two-step cycling condition of denaturation at 95°C for 10 s and annealing at 60°C for 30 s. The melting curve profile for each amplification reaction and the relevant C_T value were automatically generated using the software provided (Corbett Life Science/Qiagen). The *rpoB* gene was used as the housekeeping gene for normalization (Park et al., 2011). Relative quantification using the ΔΔC_T method (Pfaffl, 2001) was then applied to quantify the expression of *pmrAB*.

Lipopolysaccharide Analysis

Lipopolysaccharide (LPS) analysis was carried out for the polymyxin resistant strains AC30 and AC12 (Lean et al.,

2015), the polymyxin susceptible strain AC29 along with *A. baumannii* ATCC19606 as the polymyxin-susceptible control. LPS extraction was carried out according to Hood et al. (2013). Briefly, bacteria were swabbed from LB agar plates into 154 mM NaCl, and the cell density was adjusted to an OD₆₀₀ of 1.5, pelleted and resuspended in lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl, pH 6.8). Samples were then boiled for 10 min, cooled to 60°C and treated with proteinase K for 1 h. These samples were electrophoresed through a 15% polyacrylamide gel using standard methods and stained with Pro-Q Emerald 300 LPS stain according to the manufacturer's recommendations (Invitrogen). Molecular weight marker used in the experiment was the Precision Plus Protein Dual Xtra Standards (BioRad). The stained minigels were then viewed and photographed under UV light using a UV Gel Imager (Alpha-Innotech).

Cloning and Expression of the Novel Variant *bla*_{AmpC} Gene

The novel variant *bla*_{AmpC} gene was PCR-amplified from the genomes of *A. baumannii* AC29 and AC30 using specific primers ampC_BamHI: 5'-GGATCCATGGCTGTGGGTATTCAA-3' and ampC_HindIII: 5'-AAGCTTTATTCTTTATTGCATT CAGCAC-3' with the following conditions—initial hold at 95°C for 3 min, followed by 40 cycles of 95°C for 50 s, 55°C for 50 s, 72°C for 1 min, and final extension at 72°C for 90 s. Purified PCR products were then cloned into pGEM-T Easy (Promega, USA) according to the manufacturer's instructions and transformed into *E. coli* JM109. Transformants were selected on LB agar supplemented with 100 mM ampicillin with 100 mM IPTG and 50 mg/ml X-gal. Plasmids were prepared from white colonies and screened by digestion with *Bam*HI and *Hind*III which cut at the restriction sites incorporated into the primers used for amplification. Plasmids with the expected restriction fragments were validated by conventional Sanger dideoxy sequencing prior to subcloning into the pET30a expression vector. The resulting pET30a-ampC recombinant plasmids were then transformed into *E. coli* BL21 (DE3/pLysS) and expression of the cloned *bla*_{AmpC} gene carried out by inducing the cells with 0.1 mM IPTG. After that, the *E. coli* BL21 recombinant clones were tested for their MIC values for extended-spectrum cephalosporins (i.e., ceftazidime and cefepime) as well as aztreonam and imipenem at concentrations of 2, 4, 8, 16, and 32 µg/ml using the agar dilution method.

RESULTS

Strain Characteristics and Genome Analyses

Basic Genome Features and Sequence Types

Analyses of the whole genome sequencing indicated that the *A. baumannii* AC29 and AC30 genomes were 3,935,134 and 3,925,274 bp, with GC content of 38.84 and 38.98%, respectively. Predicted ORFs from the genomes of *A. baumannii* AC29 and AC30 were 3728 and 3646, respectively. Both strains contained only one chromosome with varying number of plasmids, as

TABLE 1 | General genomic features of the whole genome sequences of *A. baumannii* strains AC29 and AC30.

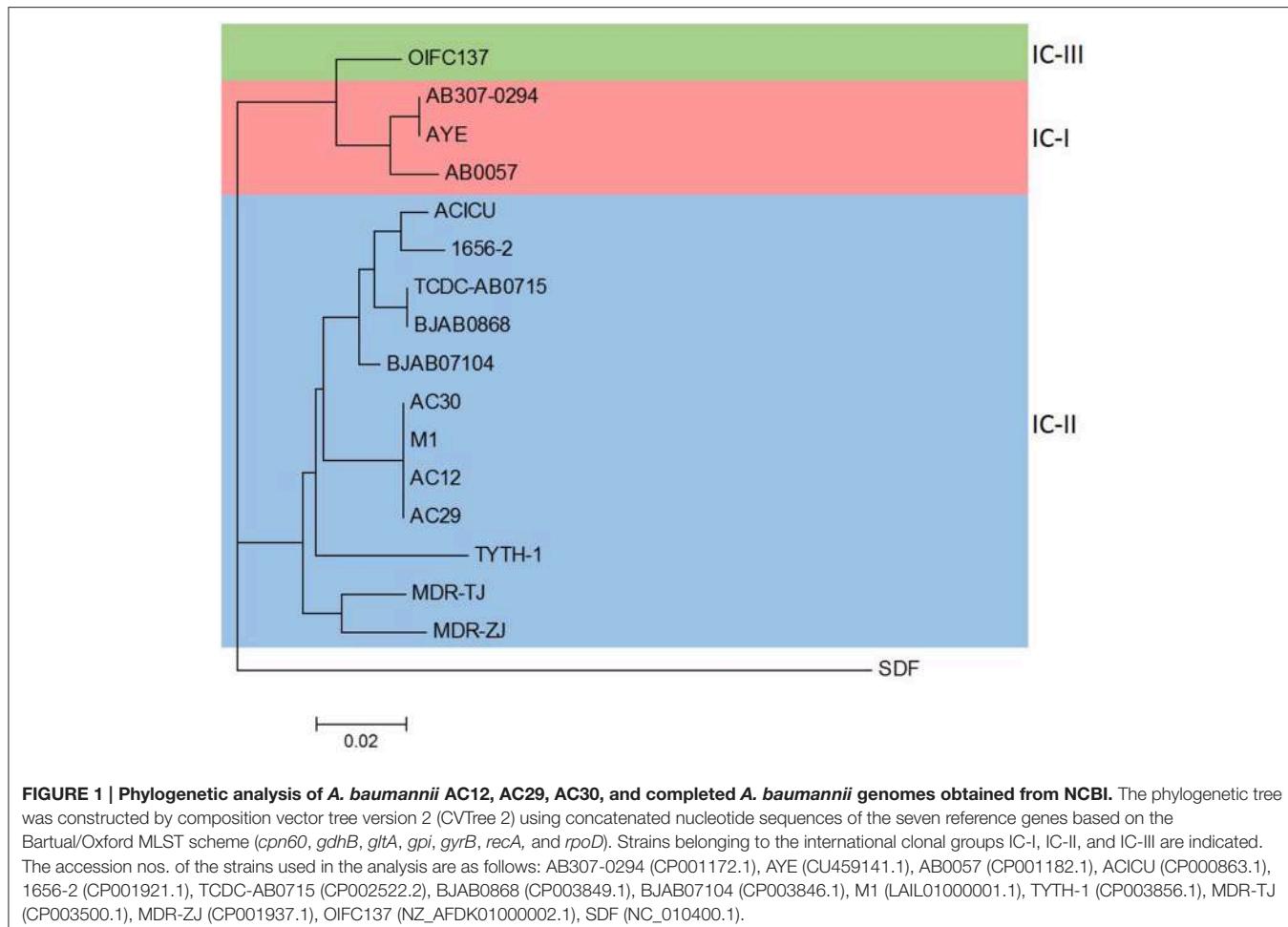
Feature	Strains	
	AC29	AC30
Accumulated length	3,935,134 bp	3,925,274 bp
Average GC content	38.84%	38.98%
Number of contigs	102	91
Number of ORF	3728	3646
Number of tRNA	66	58
Number of rRNA	4	3
Number of plasmid	2	3
Plasmid size	pAC29a (8737 bp) pAC29b (74,749 bp)	pAC30a (8729 bp) pAC30b (16,236 bp) pAC30c (71,433 bp)

featured in **Table 1**. The Bartual or Oxford scheme for MLST utilizes seven housekeeping genes *cpn60*, *gdhB*, *gltA*, *gpi*, *gyrB*, *recA*, and *rpoD* to determine the sequence type (ST) of *A. baumannii* (Bartual et al., 2005). Both AC29 and AC30 were designated ST195 (derived from *cpn60*-2, *gdhB*-3, *gltA*-1, *gpi*-96, *gyrB*-3, *recA*-2, and *rpoD*-3) whereas using the Pasteur MLST scheme (Diancourt et al., 2010), both *A. baumannii* strains were designated ST2 (*cpn60*-2, *fusA*-2, *gltA*-2, *pyrG*-2, *recA*-2, *rplB*-2, and *rpoB*-2). Thus, AC29 and AC30 were categorized as belonging to the International Clone-II (or IC-II, also referred to as Global Clonal 2, or GC2) group of strains.

Phylogenetic analysis of the *A. baumannii* AC29 and AC30 genomes were performed with other available *A. baumannii* genomes from the NCBI's FTP site and using *A. baumannii* SDF as an outgroup to root the phylogenetic tree based on the concatenated nucleotide sequences of the seven reference genes utilized in the Bartual MLST scheme (Farrugia et al., 2013). Based on the phylogenetic tree (**Figure 1**), *A. baumannii* AC29 and AC30 were closely related to *A. baumannii* AC12 which was also isolated from the same hospital in Terengganu and was also typed as ST195 (Lean et al., 2015) along with *A. baumannii* M1 which was also isolated from Malaysia (accession no. LALI0100000). These strains were clustered together with other *A. baumannii* isolates of the IC-II group and this was also reflected in their genome grouping based on genomic BLAST (as indicated at <http://www.ncbi.nlm.nih.gov/genome/genomegroups/403?> accessed on 19 August 2015).

The AbaR4-Type Resistance Island

The genomes of *A. baumannii* AC29 and AC30 contained an AbaR4-type resistance island (RI) which interrupts the *comm* gene (Nigro and Hall, 2012). The island found in AC29 is 23 kb and designated AC29-RI1 whereas in AC30, it is 26 kb and designated AC30-RI1. Tn6022 and/or Δ Tn6022 is part of the important features for this type of RI; in AC29-RI1, only the entire *tnc*C gene is deleted in Δ Tn6022 whereas AC30-RI1 carries a complete copy of Tn6022 (**Figure 2**). In AC29-RI1, the Δ Tn6022 is missing the *tnc*C gene on its far left end, but full-length *tnc*D and *tnc*E genes are present in the transposon.



AC30-RI1 contains the full length Tn6022. In both AC29-RI1 and AC30-RI1 islands, only one copy of either Δ Tn6022 or Tn6022 is found.

Drug resistance genes found in the AC29-RI1 and AC30-RI1 islands are *sulI* (conferring sulphonamide resistance), *bla_{OXA-23}* (conferring carbapenem resistance), *tetA* and *tetB* (conferring tetracycline resistance), and *strA* with *strB* (conferring streptomycin resistance). The *bla_{OXA-23}* gene is flanked by two copies of the *ISAbal* insertion element in a composite transposon structure similar to Tn2006 (Figure 2). Tn2006 comprises of the *bla_{OXA-23}* carbapenemase-encoding gene, a DEAD/DEAH box helicase-like gene and an ATPase gene (*yeeA*) flanked by two copies of *ISAbal*.

Plasmids of *A. baumannii* AC29 and AC30

A Small 8.7 kb Plasmid Found in Both AC29 and AC30

Sequence analysis revealed the presence of a small 8.7 kb plasmid in both the genomes of *A. baumannii* AC29 and AC30, designated pAC29a and pAC30a, respectively (Table 1). These plasmids contained a total of nine coding sequences (CDS) (Figure 3). No antibiotic resistance gene was found on these plasmids. Two plasmid replication genes, designated *repA* and *repB*, were found on these plasmids along with an iteron sequence made up of four direct repeats of 5'-ATA TGT CCA CGT TTA

CCT TGC A-3' located 53 nucleotides upstream of the *repB* gene. Other features on this cryptic plasmid are a *Sel1* repeat protein-encoding gene (*sel1*) which is flanked by XerC/XerD-like recombination sites in an inverted repeat formation, an outer membrane TonB-dependent receptor gene, a gene encoding for putative septicolysin and two hypothetical protein-encoding genes. A toxin-antitoxin (TA) system designated AbkB/AbkA (Mosqueda et al., 2014) was also encoded on these 8.7 kb plasmids.

pAC30b, a 16.2 kb Resistance Plasmid Found in *A. baumannii* AC30

Plasmid pAC30b is 16,236 bp in size and is found only in *A. baumannii* AC30. pAC30b contained 11 CDS and is a resistance plasmid, as indicated by the presence of the 16S rRNA methylase gene (*armA*) and aminoglycoside 3'-phosphotransferase gene (*aphA1*) which confer resistance to aminoglycosides, along with macrolide 2'-phosphotransferase (*mph2*) and macrolide efflux protein-coding (*mel*) genes which confer resistance to macrolides (Figure 4; Zhou et al., 2011). The *armA*, *mph2*, and *mel* genes along with adjacent putative transposase genes *tnpD* and *tnpU* make up a small 7 kb RI designated Tn1548::*armA* which have been reported in several other plasmids from *Enterobacteriaceae* (Dolejska et al., 2013).



FIGURE 2 | Structures of resistance islands present in *A. baumannii* AC12, AC30, and AC29 as compared to similar islands in *A. baumannii* MDR-ZJ and MDR-TJ. (A) Structure of resistance island ACRI12-1 present in *A. baumannii* AC12. Δ Tn6022, Tn6022, and Tn2006 are indicated in the red dotted-line box. Directions of genes and ORFs are indicated by arrows above the central thick line and the names are given above. Green boxes represent truncated Δ comM, turquoise blue boxes represent insertion element, ISAb1, and orange boxes represent genes conferring antibiotic resistance. Numbered boxes in gray represent genes encoding for hypothetical proteins. Comparison between structures of (A) AC12-RI1, (B) AC30-RI1, (C) AC29-RI1, (D) AbaR22, and (E) RI_{MDR-TJ}, revealed similarities in the islands, as indicated by the same colors and red dotted-line box.

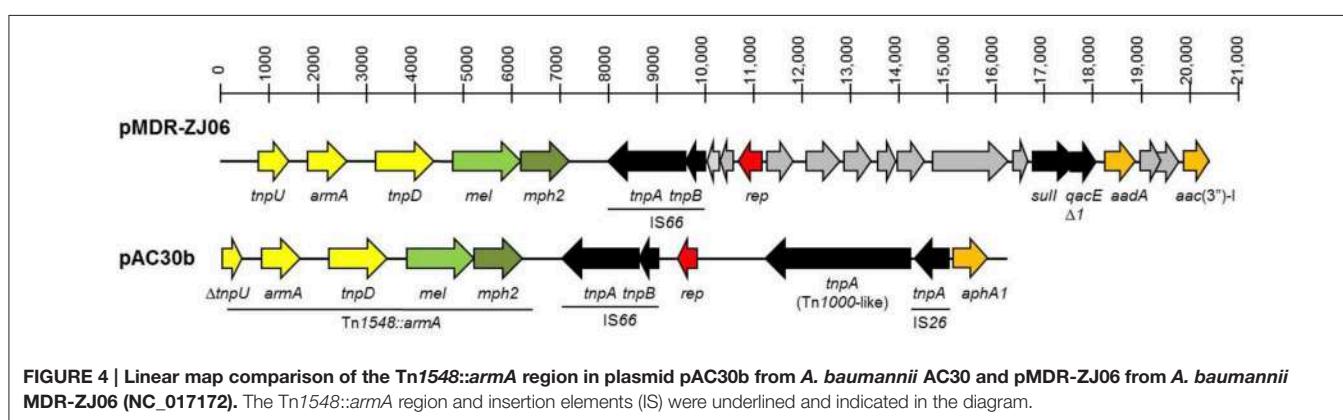
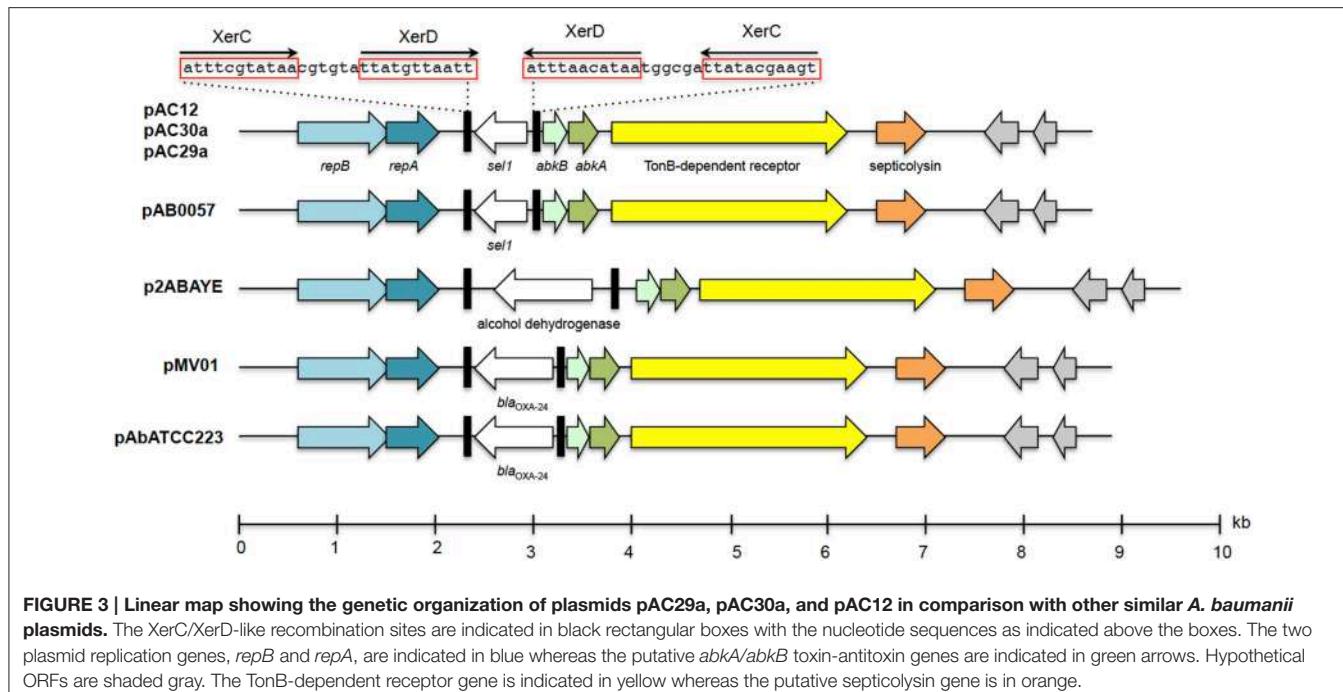
The *tnpU* putative transposase gene located at the 3'-end of Tn1548::*armA* is truncated in pAC30b whereby only the last 480 bp of the 836-bp gene could be found and was thus designated Δ *tnpU*. The pAC30b plasmid encodes a number of putative transposases: Δ *tnpU* and *tnpD* from Tn1548::*armA*, *tnpA*, and *tnpB* transposases from IS66, a 2888-bp *tnpA* from the Tn3 family of transposases (or Tn1000-like) and immediately adjacent to that, another smaller 704-bp *tnpA* transposase belonging to IS26. The aminoglycoside resistance gene *aphA1* is found downstream from IS26.

A Large ca. 70 kb Conjugative Plasmid in the Genomes of *A. baumannii* AC29 and AC30

Both *A. baumannii* AC29 and AC30 were found to harbor similar conjugative plasmids of ca. 70 kb in size designated pAC29b and pAC30c, respectively. There are a total of 101 CDS in the 74,749 bp pAC29b whereas pAC30b is 71,433 bp and contains a total of 96 CDS. Both pAC29b and pAC30c encode a complete *tra* locus (Figure 5) similar to the *tra* locus

found in pACICU2 (Iacono et al., 2008), p2ABTCDC0715 (Chen et al., 2011), pAb-G7-2 (Hamidian and Hall, 2014), and pA85-3 (Hamidian et al., 2014). Genes for the T4SS in pAC30c and pAC29b are clustered in two separate regions: the main *tra* region responsible for mating pair formation spans approximately 20 kb from *traL* to *traG*, and a second smaller region containing *traD* and *traI* (or *trwB* and *trwC*) responsible for plasmid mobilization. In pAC30c, the space between these two regions contains several hypothetical ORFs and a solitary *relE* toxin gene without the corresponding *relB* antitoxin gene (Figure 5).

Plasmid pAC29b is larger by 3316 bp when compared to pAC30c. However, there is a 14,374 bp fragment in pAC30c which has no homology with pAC29b. The additional fragment found in pAC29b included the β -lactamase gene *bla*_{OXA-23} and two transcriptional regulators, *tetR* and *asnc*. Other identifiable genes within this pAC29b-unique fragment include *yeeA* (encoding DNA methyltransferase), *yeeB* (encoding ATP-dependent helicase), *yhbS* (encoding N-acetyltransferase),



mscM (encoding mini-conductance mechanosensitive channel), *iutA* (encoding ferric aerobactin receptor), *lrp* (encoding leucine-responsive regulatory protein), and *aroQ* (encoding monofunctional chorismate mutase). Thus, AC29 harbors two copies of *bla*_{OXA-23}—one within the AbaR4-like AC29RI-1 island in the chromosome, and another encoded on the pAC29b plasmid. However, plasmid pAC30c does not encode for any known resistance determinant.

Investigations into the Possible Contributors of Polymyxin Resistance in *A. baumannii* AC30

To investigate if differential expression of *pmrAB* occurred in the polymyxin-resistant AC30 as well as AC12 strains, relative quantification of the *pmrAB* transcript levels were determined by qRT-PCR with *A. baumannii* ATCC19606 as the

polymyxin-susceptible control. When compared to ATCC19606, the relative expression levels of *pmrA* in AC30 was surprisingly lower at about 0.2-folds but levels of *pmrB* were higher at 4.8-folds (Figure 6). In contrast, the other polymyxin-resistant strain, AC12 displayed about 8.5-folds higher *pmrA* expression levels and about two-folds higher *pmrB* levels in comparison with ATCC19606. The polymyxin-susceptible AC29 showed dramatically lower *pmrAB* expression, at about 0.05- and 0.03-folds, respectively.

LPS from the two polymyxin-resistant strains, AC12 and AC30, along with two polymyxin-susceptible strains AC29 and ATCC19606 were extracted and analyzed on 15% SDS-polyacrylamide gels (Figure 7). SDS-PAGE of the extracted LPS yielded a band of ~10 kD, which was within the expected molecular weight (between 6 and 10 kDa) for LPS. Results indicated that the LPS in the polymyxin resistant strains AC12 and AC30 were not totally absent as had been previously reported

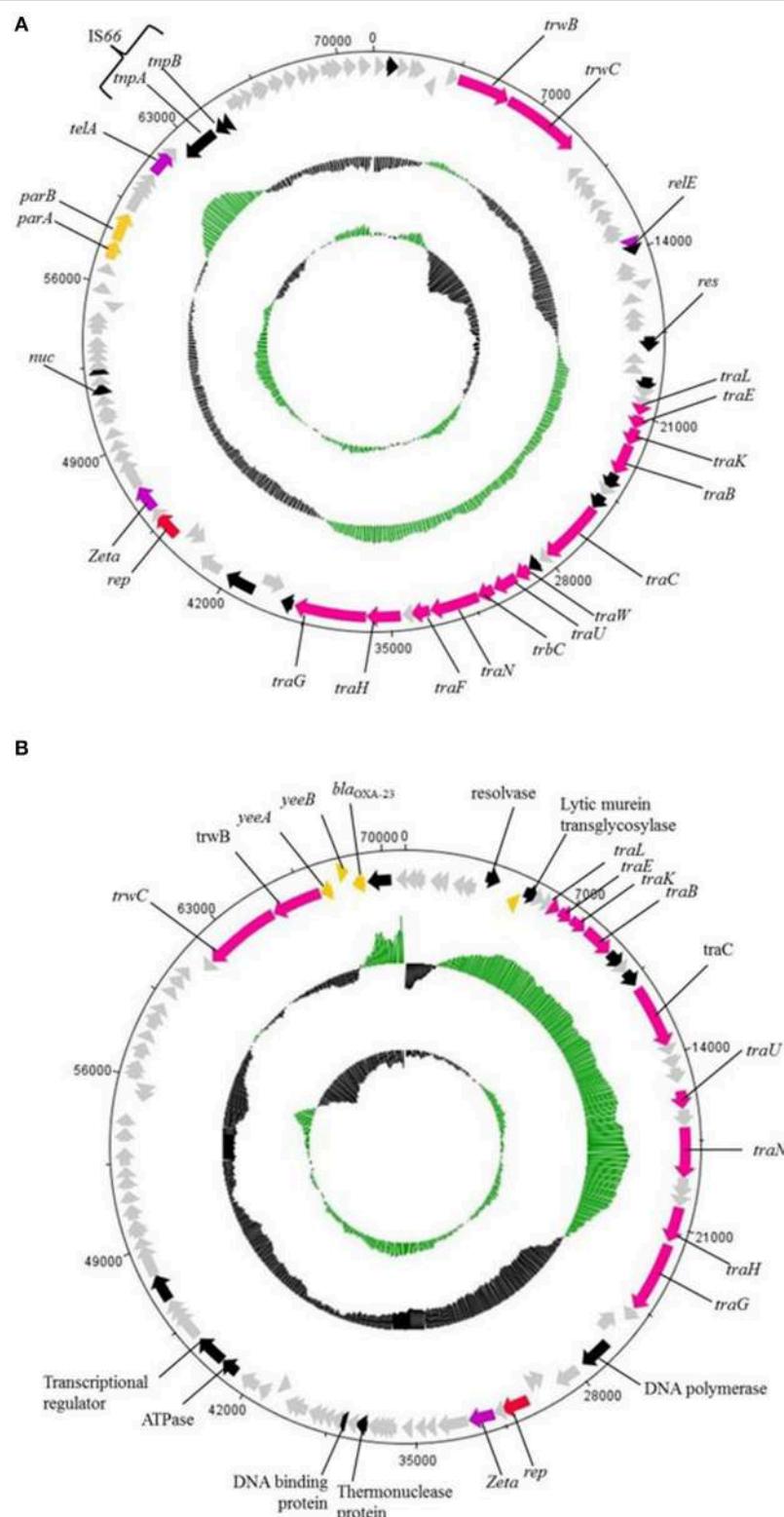


FIGURE 5 | Circular map of the ~70 kb plasmids pAC30c (A) and pAC29b (B). Outer circle of the map represents ORFs found in the plasmid, gray ORFs are genes encoding for hypothetical proteins. The two inner circles represent GC plot and GC skew, whereby the green circle stands for above average and black circle stands for below average G+C content. Red colored arrow represents plasmid replication gene, *rep*; orange colored arrow represents resistant determinants; pink colored arrow represents *tra* genes; black colored arrow represents genes encoding proteins with known functional homologs; purple colored arrow represents putative toxin gene homologs of toxin-antitoxin systems; and gray colored arrow represents genes encoding hypothetical proteins.

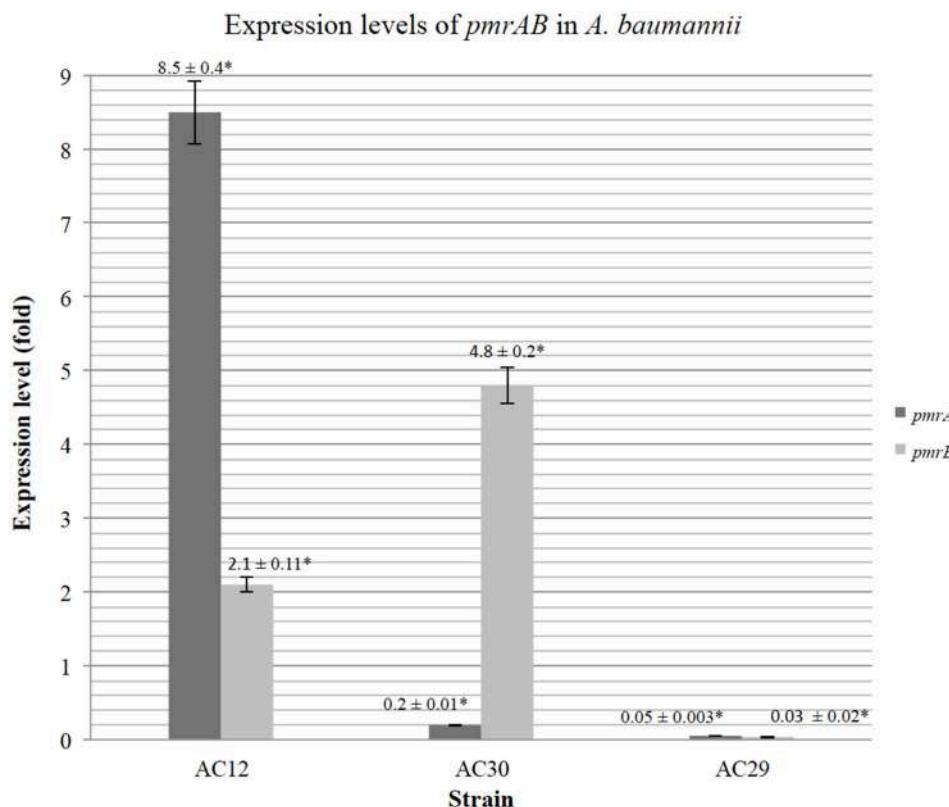


FIGURE 6 | Relative expression levels of *pmrAB* of the polymyxin-resistant *A. baumannii* AC12 and AC30 as compared to the polymyxin-susceptible *A. baumannii* AC29 as determined by Quantitative Real-Time reverse transcriptase-PCR (qRT-PCR). The data represents the mean fold change \pm standard deviation (SD; indicated as error bars in the graph) taken from three replicates performed for each qRT-PCR reaction. Asterisk (*) indicate statistical significance, as determined by using two-tailed, unpaired Student's *t*-test with $p < 0.05$.

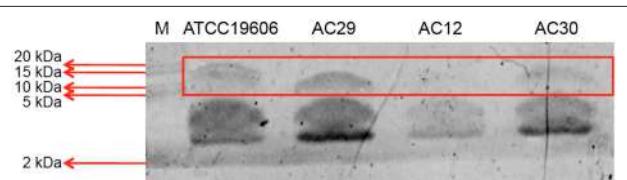


FIGURE 7 | SDS-PAGE analysis of the extracted lipopolysaccharide (LPS) layer of the *A. baumannii* ATCC19606 (control; polymyxin susceptible strain), AC29 (polymyxin susceptible strain), AC12 (polymyxin resistant strain), AC29 (polymyxin susceptible strain), and AC30 (polymyxin resistant strain). M stands for the protein marker Precision Plus Protein Dual Xtra Standards (BioRad) with the sizes as indicated in kDa.

in other polymyxin-resistant strains (Moffatt et al., 2010, 2011; Hood et al., 2013) but the intensity of the LPS band was considerably less when compared with the LPS band of the polymyxin-susceptible strains.

AmpC-Mediated Resistance to Extended-Spectrum Cephalosporins

The AmpC cephalosporinase encoded by *A. baumannii* AC29 and AC30 displayed identical non-synonymous mutations,

TABLE 2 | MIC values of the *ampC* clones in IPTG-induced *E. coli* BL21 toward selected antibiotics.

Clones	MIC value ($\mu\text{g/mL}$)			
	Aztreonam	Ceftazidime	Imipenem	Cefepime
BL21_ampC-AC12	16	16	32	16
BL21_ampC-AC30	16	16	32	16
BL21_ampC-AC29	16	16	32	16
Control (BL21_pET30a)	2	2	2	2

R80S and G246S (with reference to ADC-7, the reference *A. baumannii*-encoded AmpC; Hujer et al., 2005; Rodríguez-Martínez et al., 2010) to that found in *A. baumannii* AC12 (Lean et al., 2015) but the effects of these mutations on cephalosporin resistance have not been reported. To investigate if the mutations in the *bla*_{AmpC} gene harbored by *A. baumannii* AC29 and AC30 has any effect on resistance against β -lactams especially cephalosporins, these genes were cloned and expressed in *E. coli* BL21 through the IPTG-inducible T7 promoter of the pET30a vector. Recombinant *E. coli* BL21 carrying the *bla*_{AmpC} from AC29 and AC30 displayed resistance to ceftazidime, cefepime, aztreonam, and even imipenem (Table 2). Thus, the *bla*_{AmpC}

encoded by AC29 and AC30 is likely an extended-spectrum *Acinetobacter*-derived AmpC (ESAC).

Other Resistance Determinants and Efflux Pumps

Besides the *bla*_{AmpC}-encoded cephalosporinase (belonging to Ambler Class C β -lactamases) and the Class D carbapenemases encoded by *bla*_{OXA-23} and *bla*_{OXA-51} that were described in the previous sections, two other β -lactamase genes were found in the genomes of AC29 and AC30 (**Table 3**). These are the *bla*_{TEM} gene which encodes class A extended-spectrum β -lactamase and a gene identified as belonging to the metallo- β -lactamase family

(Ambler Class B). The contributions of these genes to the β -lactam resistance phenotype of AC12 and AC30 are currently unknown.

One of the main mechanisms of fluoroquinolone resistance is mutations that alter the drug targets. The targets of fluoroquinolone action are the bacterial enzymes DNA gyrase (encoded by *gyrA* and *gyrB*) and DNA topoisomerase IV (encoded by *parC* and *parE*), both of which work together in replication, transcription, recombination and repair of DNA (Jacoby, 2009). The AC29 and AC30-encoded *gyrA* and *parC* showed the Ser \rightarrow Leu amino acid substitutions at positions 83 and 80, respectively, which have been implicated in fluoroquinolone resistance (Wisplinghoff et al., 2003; Fournier

TABLE 3 | Genes conferring antibiotic resistance found in *A. baumannii* AC29 and AC30.

Antibiotics	Genes	Contig location		Products
		AC29	AC30	
Aminoglycosides	<i>aadA</i>	3_40	10_40	Adenylyltransferase
	<i>aadA1a</i>	3_40	10_40	Aminoglycoside adenylyltransferase
	<i>aphA1b</i>	88_1	91_1	Aminoglycoside 3'-phosphotransferase
	<i>strA</i>	54_7	38_7	Phosphotransferase
	<i>strB</i>	54_6	38_6	Phosphotransferase
Beta-lactams	<i>ampC</i>	14_1	37_1	Beta-lactamase Class C
	<i>bla</i> _{TEM}	85_1	87_1	Beta-lactamase TEM
	Class A beta-lactamase	34_58	6_58	Beta-lactamase Class A
	MBL	16_33	3_184	Metallo-beta-lactamase family protein
	<i>bla</i> _{OXA-23}	60_16	40_16	Beta-lactamase OXA-23
	<i>bla</i> _{OXA-51}	53_6	60_1	Beta-lactamase OXA-51
Carbapenems	<i>carO</i>	16_123	6_123	Putative porin protein associated with imipenem resistance
Chloramphenicol	<i>cmlA</i>	35_12	47_5	Major facilitator superfamily permease
	<i>cmlA</i>	35_12	47_5	<i>cmlA</i> transporter
Fluoroquinolones	<i>parC</i>	45_19	49_19	DNA topoisomerase IV subunit A
	<i>parE</i>	17_100	1_100	DNA topoisomerase IV subunit B
	<i>gyrA</i>	16_68	3_149	DNA gyrase subunit A
	<i>gyrB</i>	40_38	23_38	DNA gyrase subunit B
Tetracyclines	<i>tetA</i> , Class A	54_1	38_1	Tetracycline resistance protein
	<i>tetR</i>	54_2	38_2	Tetracycline repressor protein
Trimethoprim	<i>dhfrl</i>	33_27	5_27	Dihydrofolate reductase
Sulfonamides	<i>sull</i>	16_16	57_14	Dihydropteroate synthase
Polymyxin B	<i>pmrA</i>	15_53	34_55	Polymyxin resistance component, PmrA
	<i>pmrB</i>	15_54	34_54	Polymyxin resistance component, PmrB
	<i>pmrC</i>	15_55	43_1/39_1	Polymyxin resistance component, PmrC
Colistin	<i>lpxA</i>	29_1	13_136	UDP-N-acetylglucosamine acyltransferase
	<i>lpxB</i>	38_1	8_113	Lipid-A-disaccharide synthase
	<i>lpxC</i>	12_51	1_129	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase
	<i>lpxD</i>	19_75	13_138	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
	<i>lpxH</i>	17_129	35_87	UDP-2,3-diacylglycosamine hydrolase
	<i>lpxK</i>	12_53	8_19	Tetraacyldisaccharide 4'-kinase

et al., 2006; Maragakis and Perl, 2008). However, four additional novel point mutations (G145D, S118G, L644P, and T872A) were observed in the AC29 and AC30-encoded *gyrA*. Whether these mutations contribute to fluoroquinolone resistance in *A. baumannii* AC29 and AC30 would require further investigations.

Multidrug efflux pumps and porins also play important roles in *A. baumannii* antimicrobial resistance (Vila et al., 2007). Among the five major families of bacterial efflux pumps (i.e., RND, MFS, APC, ABC, and MATE; **Table 4**), the RND family is widely disseminated in Gram-negative bacteria (Poole, 2004; Bonomo and Szabo, 2006; Nordmann and Poirel, 2008;

Wieczorek et al., 2008). The genome analysis of *A. baumannii* AC29 and AC30 showed the presence of the complete *adeABC* genes along with its two-component regulatory genes, *adeRS*. Another member of the RND family encoded by *adeIJK* was also present in AC29 and AC30. However, a third RND efflux pump encoded by the *adeFGH* operon was absent in both isolates. Overexpression of *adeABC* and to a certain extent, *adeIJK*, has also been associated with the multidrug resistance phenotype in *A. baumannii* (Vila et al., 2007) and it would be of interest to investigate if this is likewise in *A. baumannii* AC29 and AC30.

TABLE 4 | Drug transporter and efflux pumps found in *A. baumannii* AC29 and AC30.

Drug transporters	Genes	Contig location		Gene products
		AC29	AC30	
APC family transporter	<i>cycA</i>	16_60	3_157	D-serine/D-alanine/glycine transporter
	<i>cycA2</i>	3_70	10_70	amino acid APC transporter
		3_71	10_71	amino acid APC transporter
	<i>mmuP</i>	67_1	22_32	S-methylmethionine APC transporter
RND family transporter	<i>proY</i>	17_59	1_59	proline-specific permease ProY
	<i>adeA</i>	31_11	16_59	membrane-fusion protein
	<i>adeB</i>	31_10	16_60	cation/multidrug efflux pump
	<i>adeC</i>	31_9	16_61	multidrug efflux protein AdeC
	<i>adel</i>	15_38	34_39	multidrug efflux protein Adel
	<i>adeJ</i>	15_39	34_40	multidrug efflux protein AdeJ
	<i>adeK</i>	15_40	34_41	multidrug efflux protein AdeK
	<i>adeR</i>	31_21	16_58	AdeR
	<i>adeS</i>	31_13	16_57	AdeS
	<i>fusE</i>	9_83	19_57	Putative FusE-MFP/HlyD membrane fusion protein
	<i>mdtA</i>	20_59	14_55	multidrug ABC transporter
	<i>nolF</i>	35_29	4_13	NolF secretion protein
MFS family transporter	RND efflux pumps	35_29	4_13	NolF secretion protein
	<i>emrB</i>	26_33	27_212	major facilitator superfamily multidrug resistance protein
	<i>fsr</i>	25_59	27_36	major facilitator superfamily permease
	<i>bcr</i>	35_12	47_5	MFS superfamily bicyclomycin/multidrug transport protein
	MFS superfamily protein	12_101	13_186	transporter, major facilitator superfamily protein
	MFS transporter	19_98	8_90	major facilitator superfamily permease
	<i>ygaY</i>	8_35	18_33	transporter, major facilitator superfamily protein
	<i>norM</i>	9_13	19_5	multidrug ABC transporter
ABC family transporter	<i>cmr</i>	22_155	22_42	major facilitator superfamily multidrug/chloramphenicol efflux transporter
	ABC efflux pump	9_13	19_5	multidrug ABC transporter
Na ⁺ driven transporters	Na ⁺ driven efflux pump	17_88	1_88	Na ⁺ -driven multidrug efflux pump
Efflux pumps	<i>abeM</i>	9_13	19_5	multidrug efflux pump AbeM
	<i>dmt</i>	16_81	3_136	EamA-like transporter family protein
	<i>ywfM</i>	56_27	12_128	DMT family permease
	MATE efflux pump	17_88	1_88	MATE efflux family protein
SMR	<i>smr</i>	22_155	14_62	multidrug resistance protein, SMR family
		20_66	14_1	Smr protein/MutS2

DISCUSSIONS

Whole Genome Sequencing and Sequence Types

In this study, we applied comparative genomics on two *A. baumannii* strains that are indistinguishable by PFGE but have different antibiograms to better understand their mechanism of resistance. Although these two strains only differed by their susceptibility toward polymyxin B (AC29 was susceptible whereas AC30 was resistant), detailed genomic analysis indicated a number of differences in the RI structures and the plasmid contents. Both *A. baumannii* AC29 and AC30 were typed as ST195 and, not surprisingly, their closest neighbor was *A. baumannii* AC12 which was also another ST195 strain as previously reported from (Lean et al., 2015). Another close neighbor was *A. baumannii* M1, an isolate from Malaysia that was recently deposited and from its genome sequence, was deduced to be also ST195. All these strains were clearly clustered in the IC-II group of global *A. baumannii* clones (Figure 1). Strains that were typed as ST195 under the Bartual MLST scheme are grouped into the clonal complex 92 (CC92) which are characterized as under IC-II (Kim et al., 2013; Zarrilli et al., 2013).

The AbaR4-Type Resistance Island

One of the known hotspots for the insertion of resistance islands in *A. baumannii* is the *comM* gene (Post et al., 2010; Zhou et al., 2011; Huang et al., 2012; Kim et al., 2013). The genomes of *A. baumannii* AC29 and AC30 also contained a similar island within *comM* but with slight differences. Previous analysis of the *A. baumannii* AC12 genome showed the presence of a 23 kb AbaR4-type island designated AC12-RI1 within *comM* (Lean et al., 2015) which consisted of a backbone of Tn6167 and a truncated version of Tn6022 designated Δ Tn6022. In all three islands (i.e., AC12RI-1, AC29RI-1, and AC30RI-1), only one copy of either Δ Tn6022 or Tn6022 is found whereas other similar resistance islands usually contained two copies of Δ Tn6022 and/or Tn6022. For instance, in RI_{MDR-TJ} and AbaR22, another copy of the complete Tn6022 was located next to the *tetA(B)* gene (Figure 2). Besides that, the *blaOXA-23* gene in AC12-RI1, AC29-RI1, and AC30-RI1 was found flanked by two copies of the ISAbal insertion element in a composite transposon structure similar to Tn2006 (Figure 2). The presence of ISAbal upstream of *blaOXA-23* may contribute to carbapenem resistance by increasing the expression level of the gene by virtue of its outward-directed promoter (Zhu et al., 2013). The Tn2006-like structure was inserted immediately downstream from Δ Tn6022 or Tn6022, and interestingly in AC12-RI1 and AC29-RI1, the *orf4* gene found at the right-most end of Δ Tn6022 was duplicated, resulting in the Δ Tn2006-like structure being flanked by *orf4* on either end (Figure 2). Such an arrangement has not been reported in similar resistance islands before. Besides that, comparison between the Tn2006 present in the three RIs and Tn6167 showed some differences. Tn6167 harbors Tn2006, but at a different location whereas both RI_{MDR-TJ} and AbaR22 do not contain Tn2006. However, the right end of the islands were identical with Tn6167, RI_{MDR-TJ} and AbaR22 and comprises of *tetA(B)-tetR(B)*, the small mobile

element CR2, *stra-strB* and *orf4b*, a hypothetical ORF related to *orf4* of Tn6022 and Tn6022 Δ 1 (Figure 2).

The structures of AC29-RI1 and AC30-RI1 indicated that they are novel variants of the AbaR4-type RIs that were recently reported in a survey of RIs found in *A. baumannii* strains throughout Asia (Kim et al., 2013). Kim et al. (2013) also reported that AbaR4-type RIs were commonly found among carbapenem-resistant CC92 strains which included the ST195 lineage.

Plasmids of *A. baumannii* AC29 and AC30

A Small 8.7 kb Plasmid Found in Both AC29 and AC30

Plasmid pAC29a and pAC30a were almost identical to pAC12 (Lean et al., 2015), pAB0057 (Adams et al., 2008), p1ABTCDC0715 (Chen et al., 2011), and pPKAB07 (Saranathan et al., 2014). A similar *A. baumannii* plasmid, pABVA01 (8963 bp), was found to harbor the *blaOXA-24* carbapenese gene flanked by XerC/XerD-like recombination sites (D'Andrea et al., 2009) and this arrangement was also subsequently reported in the 8771 bp plasmid pMMC1 (Merino et al., 2010). The XerC/XerD-like recombination sites were present in pAC29a/pAC30a but with the *sel1* gene instead of *blaOXA-24* within the potential recombination region. The pAC12 plasmid from *A. baumannii* AC12 (Lean et al., 2015) and pAB0057 from *A. baumannii* AB0057 (Adams et al., 2008) also contained the *sel1* gene in between the XerC/XerD sites. The function of the *sel1* gene is currently unknown. Other similar *Acinetobacter* plasmids harbored different sized fragments between the XerC/XerD recombination sites with p2ABAYE from *A. baumannii* AYE (Fournier et al., 2006) for example, harboring a putative alcohol dehydrogenase gene (Figure 3). The *blaOXA-24* gene is so far reported only in strains from Italy and Spain (D'Andrea et al., 2009; Merino et al., 2010). This was corroborated in a recent multicenter study which showed that the *blaOXA-24/blaOXA40*-like genes in *A. baumannii* strains isolated from Spain was predominantly carried by small 8–12 kb plasmids with two of the sequenced plasmids, pAbATCC223 and pAbATCC329, harboring the *blaOXA-24* gene in between the XerC/XerD sites (Mosqueda et al., 2013). Xer recombination is a site-specific recombination mechanism involved in events such as the integration of phage CTX-Φ at the *dif1* site in the *Vibrio cholerae* chromosome (D'Andrea et al., 2009). Moreover, the proteins required for Xer recombination, such as the XerC and XerD recombinases and PepA are reportedly encoded in the chromosome of several *A. baumannii* strains (Merino et al., 2010). Thus, the XerC/XerD-like sites on these 8.7 kb plasmids could act as site-specific recombination targets responsible for mobilization of discrete gene modules such as *blaOXA-24* and *sel1* within *Acinetobacter* plasmids (D'Andrea et al., 2009; Merino et al., 2010).

Other highlights of this small plasmid are the presence of a toxin-antitoxin (TA) system designated AbkB/AbkA (Mosqueda et al., 2014) and a gene encoding a possible TonB-dependent receptor protein. Most TA systems are usually characterized by two co-transcribed genes with the antitoxin gene preceding the toxin gene (Chan et al., 2012; Hayes and Kêdzierska, 2014). AbkB/AbkA seems to differ from canonical TA systems as the *abkB* toxin gene precedes the *abkA* antitoxin gene. To date, only

three characterized TA loci have been reported to display this unusual genetic arrangement: the *mqsRA* (Brown et al., 2009), the *higBA* (Tian et al., 1996), and *hicAB* (Jørgensen et al., 2009) modules. AbkB/AbkA was previously identified as one of the four functional TA systems in *A. baumannii* (it was designated SpT/SplA or DUF497/COG3914) whereby overexpression of the toxin was shown to inhibit growth in *E. coli* and this was overcome by co-expression of the cognate antitoxin (Jurenaite et al., 2013). The AbkB (or SpT) toxin was shown to inhibit translation when overexpressed in *E. coli* with cleavage of *lpp* mRNA and transfer-messenger RNA (tmRNA) demonstrated, thus indicating that the AbkB toxin likely functions as an endoribonuclease or RNA interferase. The AbkB/AbkA locus was found to be highly prevalent in small plasmids of *A. baumannii* clinical strains (88.6% prevalence among 476 clinical isolates from Lithuania; Jurenaite et al., 2013). The presence of a TA system on these plasmids would explain their stability in the absence of any apparent selection pressure, particularly for the small plasmids without the *bla_{OXA-24}*/*bla_{OXA40}*-like gene such as pAC30a and pAC29a.

Some TonB-dependent receptors, in particular BauA, play important roles in the acquisition of iron in *A. baumannii* (Dorsey et al., 2004; Mihara et al., 2004) with recent transcriptomic and proteomic analyses indicating approximately 20 TonB-dependent receptors in *A. baumannii*, some of which are regulated by iron (Antunes et al., 2011; Eijkelkamp et al., 2011; Nwugo et al., 2011). TonB-dependent receptors are usually found in the outer membrane where they interact with TonB and associated inner membrane proteins (ExbB and ExbD) that provide energy needed to transport host iron-carrier and iron-siderophore complexes into the periplasm once these complexes are bound to the TonB-dependent receptors (Zimbler et al., 2013). Thus, along with TonB, the TonB-dependent receptors play an important role in the virulence of *A. baumannii*. Whether the TonB-dependent receptor protein encoded by these small plasmids plays a similar role in iron acquisition and hence, virulence, awaits further experimentation. Another possible virulence-associated gene encoded in these small plasmids is found downstream of the gene encoding the TonB-dependent receptor. This gene is predicted to encode a 152-amino acids-residue protein homologous to septiclysin, a putative virulence factor (Mosqueda et al., 2014). Septiclysin is a member of thiol-activated cytolsins which have been implicated in the pathogenesis of infections by several Gram-positive pathogens such as *Clostridium perfringens*, *Listeria monocytogenes*, and *Streptococcus pneumoniae* and are characterized by their cytolytic activity for eukaryotic cells (Billington et al., 2000). With two putative virulence factors encoded on these small plasmids, it would therefore be of interest to investigate if these plasmids play a role in the virulence and pathogenesis of *A. baumannii* infections, especially in view of their prevalence among clinical *A. baumannii* isolates.

pAC30b, a 16.2 kb Resistance Plasmid Found in *A. baumannii* AC30

Both pAC30b and pMDR-ZJ06 are resistance plasmids, as indicated by the presence of genes which confer resistance to

aminoglycosides and macrolides within the 7 kb Tn1548::armA resistance island (Zhou et al., 2011). Interestingly, Tn1548::armA is located in the chromosome of *A. baumannii* AC12 (Lean et al., 2015) as well as in *A. baumannii* AC29. In both cases, Tn1548::armA was found downstream of a cluster of five genes encoding proteins annotated as paraquat-inducible protein A and protein B. The pMDR-ZJ06 plasmid harbors a class 1 integron encoding the aminoglycoside acetyltransferase (*aacC1*) and adenyltransferase (*aadA1*) along with *sull* that confers sulphonamide resistance (Zhou et al., 2011) but this integron was absent in pAC30b.

Besides pMDR-ZJ06, pAC30b also shared sequence identity with p3BJAB0868 from *A. baumannii* BJAB0868 and p2BJAB07104 from *A. baumannii* BJAB07104 (Zhu et al., 2013) and this is mainly an approximately 10 kb fragment that spanned Tn1548::armA, IS66 and the *rep* gene. Two other parts of pAC30b that were identical with pMDR-ZJ06, p3BJAB0868, and p2BJAB07104 are IS26 and the *aphA1* gene, both of which were in different locations in pAC30b when compared with pMDR-ZJ06 (Figure 4), p3BJAB0868, and p2BJAB07104. The *aphA1* gene is flanked by two copies of IS26 in a composite transposon-like structure designated Tn6210 in p3BJAB0868 and p2BJAB07104 (Zhu et al., 2013) as well as pMDR-ZJ06 but in pAC30b, only one copy of IS26 is found adjacent to *aphA1*. It is possible that a deletion had occurred in pAC30b that took out the other copy of IS26 as well as part of the *tnpU* gene of Tn1548::armA which is located adjacent to the IS26-*aphA1* structure. All three plasmids pMDR-ZJ06, p3BJAB0868, and p2BJAB07104 which were isolated from *A. baumannii* strains in China, harbored a class 1 integron but this was absent in pAC30b.

It is noteworthy that in contrast to pAC30a and pAC29a, pAC30b has very few similar plasmids in other *A. baumannii* isolates besides pMDR-ZJ06, p3BJAB0868, and p2BJAB07104, suggesting that these plasmids are not so prevalent. What the four *A. baumannii* plasmids (namely, pAC30b, pMDR-ZJ06, p3BJAB0868, and p2BJAB07104) have in common are (1) a number of transposases encoded by IS elements and transposons, and (2) a lack of any plasmid stability genes such as toxin-antitoxin systems. Thus, there is a likelihood that these plasmids are much less stable as compared to the smaller pAC29a/pAC30a plasmid which is endowed with the AbkB/AbkA toxin-antitoxin system. Hence, in *A. baumannii* AC29 (and AC12), portions of pAC30b are chromosomally-located and there is no widespread occurrence of pAC30b-like plasmids in other *A. baumannii* strains.

A Large ca. 70 kb Conjugative Plasmid in the Genomes of *A. baumannii* AC29 and AC30

The *tra* locus in pAC29b and pAC30c encodes for a type IV secretion system (T4SS) which is related to the bacterial conjugation machinery and mediates horizontal gene transfer (Juhas et al., 2008). Plasmids pAb-G7-2, pA85-3, and even pACICU2 have been demonstrated to be transmissible by conjugation to other *A. baumannii* strains (Hamidian and Hall, 2014; Hamidian et al., 2014). It is thus very likely that pAC29b and pCA30c are also conjugative as the *tra* locus in these

plasmids is almost identical. In pAb-G7-2, the space between these two regions contained the aminoglycoside resistance transposon, *TnaphA6* (Hamidian and Hall, 2014) whereas in pA85-3, the AbaR4 resistance island is located in the same area (Hamidian et al., 2014). In pAC30c, this region contained several hypothetical ORFs and a solitary *relE* toxin gene without the corresponding *relB* antitoxin gene. However, the two genes flanking *relE* could perhaps function as the antitoxin as they encode for hypothetical proteins of about the same size as the putative RelE toxin. Toxin-antitoxin pairs are usually about the same size with a few exceptions such as the Zeta toxin which is much larger (~270 amino acids) as compared to their cognate Epsilon antitoxin (~90 amino acids; Chan et al., 2012; Jurenaite et al., 2013). Although toxins usually interact with their cognate antitoxin pair, sometimes mixing and matching between different toxin and antitoxin families do occur (Chan et al., 2012; Hayes and Kedzierska, 2014). Nevertheless, the functionality of the plasmid pAC30c and pAC29b-encoded *relE* toxin gene needs to be ascertained.

Both pAC30c and pAC29b carry the *rep* gene designated *repAci6*, similar to pACICU2, pAb-G7-2, and pA85-3. Another solitary toxin, this time from the Zeta family, is located downstream from the *repAci6* gene in both plasmids. Experimental evidence had suggested that its overexpression is non-toxic to *E. coli* and thus, may not function as a typical toxin (Jurenaite et al., 2013). Its putative antitoxin partner, located upstream of its reading frame, does not bear any homology to the Epsilon antitoxin. These solitary Zeta-like toxins have been observed in several other plasmids in diverse bacterial species and their function is currently unknown (Chan et al., 2012; Jurenaite et al., 2013). In the absence of typical canonical toxin-antitoxin systems, the only other identifiable genes that could contribute to plasmid stability in pAC30c and pAC29b are the *parAB* genes which likely encode proteins that are involved in plasmid partitioning.

Plasmid pAC29b harbors a *bla_{OXA-23}* gene but unlike the *A. baumannii* A85 plasmid pA85-3 (Hamidian et al., 2014), the gene is not located within an AbaR4 island. Both pAb-G7-2 and pACICU2 harbor the aminoglycoside resistance gene *aphA6* within a composite transposon designated *TnaphA6* (Hamidian and Hall, 2014; Hamidian et al., 2014). Plasmid pAC30c however, does not encode any known resistance determinant.

Investigations into the Possible Contributors of Polymyxin Resistance in *A. baumannii* AC30

The mechanism for polymyxin resistance in *A. baumannii* has only recently been elucidated and the main mechanism appeared to be either covalent modification of the lipid A portion of LPS (Arroyo et al., 2011; Beceiro et al., 2011) or disruption of LPS biosynthesis (Moffatt et al., 2010; Park et al., 2011). By modification and/or mutations in the amino acid sequences, negative charges on the outer membrane can be reduced, leading to reduction in the affinity of the positively-charged polymyxin component, hence giving rise to polymyxin resistance (Adams et al., 2009; Arroyo et al., 2011; Beceiro

et al., 2011). Overexpression of the two-component signal transduction system *pmrAB* and mutations within these genes, especially *pmrB*, were reported to contribute to polymyxin resistance (Adams et al., 2009; Arroyo et al., 2011; Park et al., 2011). These two genes are part of an operon along with *pmrC*, which encodes the enzyme responsible for the covalent addition of phosphoethanolamine to lipid A. Sequence analysis of *pmrCAB* from the polymyxin-resistant AC30 in comparison with the polymyxin-susceptible AC29 and other susceptible strains in the database (including ATCC19606 and ATCC17978) showed a P102H mutation within *pmrA*. Identical mutations were found within *pmrA* of AC12 (Lean et al., 2015) and other polymyxin-resistant isolates from Terengganu (Lean et al., 2014). Polymyxin-resistant isolate AC12 displayed higher *pmrA* expression levels (8.5-folds) and also higher *pmrB* levels (about two-folds) in comparison with ATCC19606. In contrast, the polymyxin-susceptible AC29 isolate showed dramatically lower *pmrAB* expression (0.05- and 0.03 folds). It should be noted that in this case, no isogenic polymyxin-susceptible strains for the polymyxin-resistant strains were available for comparison. Thus, the expression levels for AC29, AC30, and AC12 were compared with the non-isogenic reference strain ATCC19606 and therefore may not yield an accurate picture for the *pmrAB* levels. Nevertheless, the results do indicate upregulation particularly for *pmrB* in both the polymyxin-resistant isolates AC30 and AC12.

The *lpxA*, *lpxC*, and *lpxD* genes encode the first three enzymes in the lipid A biosynthesis pathway (Moffatt et al., 2010, 2011). No mutation was found in *lpxA*. In contrast, *lpxD* showed three amino acid mutations (S102T, V141I, R173G) in AC30 while *lpxC* had a K141R substitution. The *lpsB* gene encodes a glycosyltransferase is involved in the biosynthesis of the LPS core and was recently implicated in *A. baumannii* colistin resistance (Hood et al., 2013). Comparison of *lpsB* sequences indicated a H181Y substitution in AC30. Identical mutations in *lpxD*, *lpxC*, and *lpsB* were reported in the polymyxin-resistant *A. baumannii* AC12 (Lean et al., 2015); likewise, these and other mutations had been previously reported in the polymyxin-resistant *A. baumannii* strains from Terengganu (Lean et al., 2014). Results from the LPS analysis indicated that the LPS in the polymyxin resistant strains AC12 and AC30 were considerably less when compared with the LPS band of the polymyxin-susceptible strains, which has been previously reported in other polymyxin-resistant strains (Moffatt et al., 2010, 2011; Hood et al., 2013). This suggested that the mutations found in the *lpxD*, *lpxC*, and *lpsB* genes in the two polymyxin-resistant strains may have led to impairment but not a total loss of the LPS. It is likely that in the case of AC12 and AC30, polymyxin resistance could be the result of a combination of increased *pmrAB* expression leading to covalent modification of the lipid A moiety of LPS and possibly impaired LPS synthesis as well. Nevertheless, it should be noted that in a recent study (Hood et al., 2013), screening of transposon mutant libraries led to the identification of more than 20 genes that may be involved in inducible colistin resistance in *A. baumannii*. Most of these genes converged on pathways involved in osmotolerance, cell envelope biosynthesis along with protein folding (Hood et al., 2013). The role that these factors may play in the development of polymyxin resistance

in the Terengganu *A. baumannii* strains would also need to be investigated.

AmpC-Mediated Resistance to Extended-Spectrum Cephalosporins

Resistance to β -lactam antibiotics via synthesis of β -lactamase encoded by the chromosome and/or plasmids is the most common resistance mechanism observed in *A. baumannii* (Bou and Martínez-Beltrán, 2000). Resistance to broad-spectrum cephalosporins in *A. baumannii* are usually related to the over production of extended spectrum β -lactamases (ESBL), especially AmpC-type β -lactamases designated ADCs (*Acinetobacter*-derived cephalosporinases) (Rodríguez-Martínez et al., 2010). ADCs typically hydrolyze penicillins, narrow- and extended-spectrum cephalosporins but not zwitterionic cephalosporins such as cefepime or carbapenems (Rodríguez-Martínez et al., 2010). However, extended-spectrum AmpCs (ESACs) have been reported in *A. baumannii* that confer reduced susceptibility to all cephalosporins and this includes ADC-33 (Rodríguez-Martínez et al., 2010) and ADC-56 (Tian et al., 2011).

Since the recombinant *E. coli* BL21 carrying the *bla*_{AmpC} from AC29 and AC30 displayed resistance to ceftazidime, cefepime, aztreonam, and even imipenem, this strongly suggests that the AmpC from AC29 and AC30 is an ESAC cephalosporinase. ADC-33 possessed a P210R substitution together with a duplication of the Ala residue at position 215 within the Ω loop, both of which are required for extended spectrum activity (Rodríguez-Martínez et al., 2010). ADC-56 possessed an R148Q mutation also within the Ω loop, that enabled the enzyme to hydrolyze cefepime (Tian et al., 2011). Thus, the G246S mutation within the Ω loop of the AmpC from AC29 and AC30 could be responsible for the extended spectrum activity. This could be examined and verified by site-directed mutagenesis of the pET30a recombinant clones. Likewise, the contribution of the R80S mutation toward extended spectrum activity should be investigated even though it is located in a non-active site.

CONCLUSIONS

In this study, we presented the comparative genome analyses of two Malaysian *A. baumannii* strains AC29 and AC30 that belonged to the ST195 lineage and had identical *Apal* pulsotype but different susceptibilities to polymyxin. Their

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MLST profiles and phylogenetic clustering based on their concatenated MLST sequences clearly showed these strains belonging to the International Clone II (IC-II) group. Novel resistance island (RI) variants and plasmids were discovered from the genome sequence of these strains. Both strains shared a similar AbaR4-type RI of approximately 22 kb interrupting the *comM* gene insertional hotspot and which contains the carbapenem resistance gene, *bla*_{OXA-23} within a composite transposon, Tn2006. The island also encodes genes conferring resistance to tetracyclines, sulphonamides and streptomycin. Both *A. baumannii* strains harbored a small ~8 kb cryptic plasmid which encode putative virulence determinants (TonB-dependent receptor and septicolysin) as well as a XerC/XerD recombination site. Plasmid pAC30b is found only in AC30 but not AC29 and contained the Tn1548::*armA* island that confers resistance to aminoglycosides and macrolides. Interestingly, this island was found in the chromosome of AC29. Both AC30 and AC29 harbored a ~70 kb conjugative plasmid designated pAC30c and pAC29b with pAC29b containing a copy of *bla*_{OXA-23}. Thus, genomic islands and, to a lesser extent, conjugative plasmids, appeared to play an important role in the dissemination and acquisition of antibiotic resistance determinants in the Terengganu *A. baumannii* strains. Experimental evidence also indicated that polymyxin resistance in AC30 may have developed through a combination of *pmrAB* upregulation and partial impairment of the lipopolysaccharide layer. The *bla*_{AmpC} variant encoded by both AC29 and AC30 was also shown to be likely an extended-spectrum *Acinetobacter*-derived AmpC (ESAC) conferring resistance to cefepime as well as imipenem. Whole genome sequencing of the two Terengganu *A. baumannii* clinical strains and subsequent experiments enabled a detailed characterization of their genetic repertoire of resistance, thereby giving us an insight into the genetic blueprint of Malaysian isolates of this increasingly important and deadly nosocomial pathogen.

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Genotypic and Antimicrobial Susceptibility of Carbapenem-resistant *Acinetobacter baumannii*: Analysis of ISAb elements and bla_{OXA-23-like} Genes Including a New Variant

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Carbapenem-resistant *Acinetobacter baumannii* (CR-AB) causes serious nosocomial infections, especially in ICU wards of hospitals, worldwide. Expression of bla_{OXA} genes is the chief mechanism of conferring carbapenem resistance among CR-AB. Although some bla_{OXA} genes have been studied among CR-AB isolates from Iran, their bla_{OXA-23-like} genes have not been investigated. We used a multiplex-PCR to detect Ambler class A, B, and D carbapenemases of 85 isolates, and determined that 34 harbored bla_{OXA-23-like} genes. Amplified fragment length polymorphism (AFLP) genotyping, followed by DNA sequencing of bla_{OXA-23-like} amplicons of CR-AB from each AFLP group was used to characterize their bla_{OXA-23-like} genes. We also assessed the antimicrobial susceptibility pattern of CR-AB isolates, and tested whether they harbored insertion sequences ISAb1 and ISAb4. Sequence comparison with reference strain *A. baumannii* (NCTC12156) revealed five types of mutations in bla_{OXA-23-like} genes; including one novel variant and four mutants that were already reported from China and the USA. All of the bla_{OXA-23-like} genes mutations were associated with increased minimum inhibitory concentrations (MICs) against imipenem. ISAb1 and ISAb4 sequences were detected upstream of bla_{OXA-23} genes in 19 and 7% of isolates, respectively. The isolation of CR-AB with new bla_{OXA-23} mutations including some that have been reported from the USA and China highlights CR-AB pervasive distribution, which underscores the importance of concerted national and global efforts to control the spread of CR-AB isolates worldwide.

Keywords: *Acinetobacter baumannii*, bla_{OXA-23-like} gene, carbapenemase, novel mutations

INTRODUCTION

Carbapenem-resistant *Acinetobacter baumannii* (CR-AB) can cause severe nosocomial infections particularly among patients in intensive care units (ICUs) around the world (Safari et al., 2013). Inadequate antimicrobial management of CR-AB infections often gives rise to highly resistant strains leading to prolonged hospitalization, treatment failures, and increased mortality (Higgins et al., 2010a). Epidemics of multi-, extensively-, and pandrug-resistant (MDR, XDR, and PDR) CR-AB have been reported from several countries (Kempf and Rolain, 2011; Bahador et al., 2013a; Moradi et al., 2015). In developing countries, such as Iran, challenges in the treatment of CR-AB infections are often exacerbated by widespread nosocomial outbreaks of OXA-type β -lactamase producing MDR-AB (for review see, Moradi et al., 2015). CR-AB are usually resistant to several β -lactams through the expression of chromosomal and plasmid-encoded carbapenemases including Ambler class A (*bla*_{GES}, and *bla*_{KPC}), class B (*bla*_{IMP}, *bla*_{NDM-1}, *bla*_{SPM-1}, and *bla*_{VIM}), and class D (*bla*_{OXA-23,40}, and 58-like; Siroy et al., 2005; Lu et al., 2009; Abbott et al., 2013). While the production of OXA-23 by *A. baumannii* is sufficient to confer resistance to carbapenems, insertion sequence (IS) elements ISAb_{a1} and/or ISAb_{a4} upstream of *bla*_{OXA-23-like} genes enhance the *bla*_{OXA}-mediated carbapenem resistance of CR-AB (Turton et al., 2006; Lee et al., 2012; Evans and Amyes, 2014). Although there are a few reports from Iran regarding the distribution and/or frequency of the *bla*_{OXA-51-like} genes among CR-AB, data about characterization of their *bla*_{OXA-23} genes and ISAb_a elements is not available.

In this study, we have characterized *bla*_{OXA} genes in CR-AB isolates from Iran, and report new variants that harbor novel mutations in their *bla*_{OXA-23-like} carbapenemase genes. In addition to analyzing the distribution and frequency of *bla*_{OXA-23-like} genes, we have determined the antimicrobial susceptibility patterns of isolates and the presence of ISAb_{a1} and ISAb_{a4} enhancer elements upstream of their *bla*_{OXA-23-like} genes. Characterization of *bla*_{OXA} genes and assessment of carbapenemase-mediated antibiotic resistance among *A. baumannii* isolates can help efforts to develop databases, which are essential to a comprehensive national surveillance program in Iran, toward the local and global control of CR-AB outbreaks.

MATERIALS AND METHODS

Specimens and Bacterial Isolates and Cultures

A total of 85 non-repetitive clinical specimens were collected during 2011 from the intensive care units (ICUs) of Imam Khomeini Medical Center (IKMC) and Children's Medical Center (CMC) in Tehran, Iran. IKMC and CMC are affiliated with Tehran University of Medical Sciences (TUMS), and both are large referral centers that provide tertiary health care to patients from all over Iran. Specimens were collected from ICUs in surgical (S), internal medicine (M), emergency (E), pediatrics (P), and kidney transplantation (T) wards. Clinical isolates

were initially identified as *A. baumannii* using the API20NE system (bioMérieux, Marcy-l'Etoile, France), and were further confirmed by *gyrB* multiplex PCR, as described previously (Higgins et al., 2010b). Specimen sources for *A. baumannii* isolates were as follows: respiratory tract ($n = 51$), urine ($n = 16$), blood ($n = 11$), wound ($n = 5$), and cerebral spinal fluid (CSF; $n = 2$). Twenty six of the *A. baumannii* isolates were part of a previous molecular epidemiologic study (Bahador et al., 2014). Brain heart infusion (BHI) agar plates and Mueller-Hinton broth (MHB; both from Merck, Germany) were used to culture the bacterial isolates.

Antimicrobial Susceptibility Testing

To assess susceptibility of *A. baumannii* clinical isolates, the disk agar diffusion (DAD) method (CLSI, 2015) was carried out according to the Clinical and Laboratory Standards Institute (CLSI) procedures and breakpoint interpretations, using antimicrobial disks containing 19 different antimicrobial agents (Mast Diagnostics, Bootle, UK; Table 2). The CLSI guideline for broth microdilution test for minimum inhibitory concentrations (MICs) was used to assess the susceptibility of MDR-AB isolates to colistin (CST), imipenem (IPM), rifampicin (RIF), and tigecycline (TGC). For tigecycline susceptibility tests, the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *Enterobacteriaceae* were used, in which an MIC of $<1 \mu\text{g/mL}$ was defined as susceptible and $>2 \mu\text{g/mL}$ was considered resistant (EUCAST, 2015). Rifampicin susceptibility was interpreted according to CLSI criteria using breakpoint values suggested for *Staphylococcus aureus*, in which susceptible and resistant were defined as $\leq 1 \mu\text{g/mL}$ and $\geq 4 \mu\text{g/mL}$, respectively (CLSI, 2015). *A. baumannii* isolates were defined as MDR, XDR, and PDR according to the definitions provided by Magiorakos (Magiorakos et al., 2012). The MIC geometric mean (MIC_{gm}) of imipenem against *bla*_{OXA-23-like}⁺ CR-AB isolates were also compared with the MIC_{gm} of non-mutant isolates, and fold-increase calculations were measured against MIC_{gm} of non-mutant strains.

Detection of Carbapenemase Gene ISAb_{a1} and ISAb_{a4} Insertion Sequences

The overall strategy for the identification of the 34 *bla*_{OXA-23-like}⁺ CR-AB isolates is shown in Supplemental Figure 1. Briefly, we tested all 85 *A. baumannii* isolates for carbapenemase production by the modified Hodge test (Lee et al., 2012), and their chromosomal DNA were tested by two different confirmatory multiplex-PCR assays to identify the most common carbapenemase encoding genes. The criteria to include isolates in this study were the presence of PCR-specific amplicons, confirmed by agarose gel electrophoresis analysis (Supplemental Figure 1). A novel in-house multiplex-PCR, referred to as AB-hexaplex-PCR was optimized for the rapid and simultaneous detection of the most common carbapenemase genes, including Ambler class A and B (*bla*_{KPC}, *bla*_{GES}, *bla*_{IMP-1}, *bla*_{VIM-2}, *bla*_{NDM-1}, and *bla*_{SPM-1}) in *A. baumannii* using Primer 3 software (version 4.0; <http://primer3.wi.mit.edu/>; accessed June 05, 2011). Reference gene sequences were accessed from GenBank [<http://www.ncbi.nlm.nih.gov/GenBank>] (*bla*_{KPC}:

GQ140348, bla_{GES}: GU207844, bla_{IMP-1}: EF375699, bla_{VIM-2}: GQ288396, bla_{NDM-1}: JN794561, and bla_{SPM-1}: HM370523; accessed June 04, 2011)], as shown in **Table 1**. The Ambler class D type carbapenemase genes (bla_{OXA-23,24,51,58} like) were detected using the Woodford multiplex PCR assay method (Woodford et al., 2006). Additionally, the AB-hexaplex-PCR distinguished amplicons corresponding to the bla_{IMP-1}, bla_{SPM-1}, bla_{GES} and

bla_{KPC}, bla_{NDM-1}, and bla_{VIM-2} genes, and isolates that harbored these genes were excluded from our study (representative gel; **Supplemental Figure 2**). The frequency of ISAb_{a1} and ISAb_{a4} elements upstream of bla_{OXA-23-like} and bla_{OXA-51-like} genes were assessed using a series of PCR amplifications. A set of primers, referred to as ISAb_{a1}F/OXA-23R, ISAb_{a4}F/OXA-23R, and ISAb_{a1}F/OXA-51R, is shown in **Table 1**. After our serial

TABLE 1 | Primer sequences and adaptors (and their corresponding reference) utilized in order to identify most common carbapenemase genes in our isolates, and to generate amplicons for AFLP genotyping analysis.

Assay		Primer	Sequence (5'-3') [†]	Size of amplicon	References
Detection of carbapenemase in the molecular class D	Multiplex PCR	bla _{OXA-51} likeF	TAATGCTTGATCGGCCCTG	353	Woodford et al., 2006
		bla _{OXA-51} likeR	TGGATTGCACCTCATCTTGG	501	"
		bla _{OXA-23} likeF	GATCGGATTGGAGAACCGAGA	240	"
		bla _{OXA-23} likeR	ATTCTGACCGCATTCCAT	590	"
		bla _{OXA-24} likeF	GGTTAGTTGGCCCCCTTAAA	263	Present study
		bla _{OXA-24} likeR	AGTTGAGCGAAAAGGGGATT	395	"
		bla _{OXA-58} likeF	AAGTATTGGGCTGTGCTG	118	"
		bla _{OXA-58} likeR	CCCTCTGCGCTACATAC	640	"
Detection of carbapenemase in the molecular classes A and B	hexaplex PCR (h-PCR)	IMP-1F	AACATGGTTGGTGGTCTTGT	439	Present study
		IMP-1R	TCCGCTAAATGAATTGTGGCT	567	"
		VIM-2F	CAATGGTCTATTGCGTGT	439	"
		VIM-2R	AAATCGCACACCACCATAGAG	227	"
		NDM-1F	CTGGATCAAGCAGGAGATCAAC	439	"
		NDM-1R	ATTGGCATAAGTCGCAATCCC	590	"
		KPCF	CGCTAAACTCGAACAGGACTT	263	"
		KPCR	ATAGTCATTTGCCGTGCCATAC	314	"
		blaGESF	GAAAACTTTCATATGGCCGGA	327	"
		blaGESR	GACCGACAGAGGCACTAATTC	590	"
		SPM-1F	CCATTGCTGCAAAAAGTTGG	640	"
		SPM-1R	AAACATTATCCGCTGGAACAGG	227	"
ISAb _{a1} detection upstream of bla _{OXA-51}	IsAba-1 F/OXA-51 R	IsAba-1 F	AAGCATGATGAGCGCAAAG	227	"
		OXA-51 R	GGTGAGCAGGCTGAAATAAAA	314	"
ISAb _{a1} detection upstream of bla _{OXA-23}	IsAba-1 F/OXA-23 R	IsAba-1 F	TGAGATGTCTAGTATTCT	327	"
		OXA-23 R	AGAGCATTACCATATAGATT	590	"
ISAb _{a4} detection upstream of bla _{OXA-23}	IsAba-4 F/OXA-23 R	IsAba-4 F	CACAATTCTGATAAAGATA	263	"
		OXA-23 R	TTTATTAAATTATGCTGAAC	314	"
AFLP	Adaptors	adp Mbl	GTAGCGCGACGGCCAGTCGCG	No amplicon	Bahador et al., 2013b
		ADP Mbl	GATCCCGCAGTGGCCGTGCGCTAC	"	"
		adp Msl	GTAGCGCGACGGCCAGTCGCGT	"	"
		ADP Msl	TAACCGCAGTGGCCGTGCGCTAC	"	"
	Pre-amplification	PreAmp Mbo	ACGGCCAGTCGCGGATC	Multiple and variable	"
		PreAmp Mse	CGACGGCCAGTCGCGTTAA	"	"
	Selective primers	Mbo1	PreAmp Mbo + A	Multiple and variable	"
		Mbo2	PreAmp Mbo + T	"	"
		Mbo3	PreAmp Mbo + C	"	"
		Mbo4	PreAmp Mbo + G	"	"
		Mse1	PreAmp Mse + A	"	"
		Mse2	PreAmp Mse + T	"	"
		Mse3	PreAmp Mse + C	"	"
		Mse4	PreAmp Mse + G	"	"

[†] Nucleotide.

screening of isolates, 34 isolates were identified that harbored *bla*_{OXA-23-like} gene as their sole acquired carbapenemase gene.

AFLP Genomic Fingerprint Analysis

Amplified fragment length polymorphism (AFLP) genotyping of *bla*_{OXA-23-like}⁺ and *bla*_{OXA-51-like}⁺ isolates was carried out by a modified Vos method (Vos et al., 1995), as described previously (Bahador et al., 2013b). AFLP typing was carried out prior to sequence analysis to ensure thorough examination of the diversity of CR-AB isolates. Briefly, chromosomal DNA was size-verified and double-digested with MboI and MseI (Fermentas, Lithuania). Then DNA fragments were ligated to corresponding adapters using T4 DNA ligase (350 U/μ L, Takara Bio, Japan) followed by the preliminary PCR using PreAmp-Mbo and PreAmp-Mse primers (Table 1). Preliminary PCR amplicons served as templates for selective PCR, which generated AFLP genotype profiles upon agarose gel analysis. Initial testing of 36 combinations of primers, including PreAmp Mbo (PreAmp Mbo+A, +T, +C, +G), and PreAmp Mse (PreAmp Mse+A, +T, +C, +G) and *A. baumannii* NCTC12156 DNA as a normalization reference showed that the Mbo4-Mse4 combination generated the clearest AFLP profiles when analyzed using BioNumerics version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity between band patterns was calculated using the Dice coefficient, with an optimization of 0.5% and a position tolerance of 1%. The AFLP types were grouped at the 90% similarity cutoff on a dendrogram constructed by the unweighted-pair group method using average linkages (UPGMA).

DNA Sequencing of *bla*_{OXA-23-like} Genes

To evaluate an association between changes in the chromosomal carbapenemase gene sequence of isolates and their antimicrobial resistance pattern, a two-step approach was adopted. An initial AFLP assay was carried out on *bla*_{OXA-23-like}⁺ CR-AB, followed by DNA sequence analysis of the *bla*_{OXA-23-like} gene of a representative isolate from each AFLP genotype group.

Briefly, we used a high fidelity Pfu DNA polymerase (Fermentas, Lithuania) to generate *bla*_{OXA-23-like} specific amplicons, which were purified using a AccuPrep® PCR Purification Kit (Bioneer, Daejeon, Korea) and cloned into pTZ57R (InsT/A Clone PCR product cloning kit, Fermentas, Vilnius, Lithuania). DNA was then transferred into competent *E. coli* TOP10 cells, which were then isolated using Luria-Bertani (LB) agar supplemented with ampicillin (100 μg /mL). Plasmid DNA was prepared with the AccuPrep Plasmid MiniPrep DNA Extraction Kit, (Bioneer, Daejeon, Korea) and sequenced using an ABI3730 automatic sequencer (Applied Biosystems, CA, USA). The sequences were analyzed using a BLAST algorithm against the NCBI GenBank database [<http://www.ncbi.nlm.nih.gov/guide/dna-rna/> (accessed 05.06.11)].

Iodometric Assay of β-lactamase Activity

Bacterial β-lactamase enzymatic activity was determined by an iodometric assay, as described previously (Sawai et al., 1978). Briefly, crude lysates of 16 isolates that represented AFLP groups were extracted using the Saino method (Saino et al., 1982).

Briefly, overnight bacterial growth were diluted in MHB broth to a concentration of 10⁷ cfu/ml and incubated in a shaker for 2 h at 35°C. As an inducer, imipenem was added at 0.25 of the isolate MIC and incubated for an additional 2 h (Clark, 1996). Bacterial cells were harvested, centrifuged at 4°C, washed twice with 50 mM phosphate buffer saline (PBS; pH 7.0), and re-suspended in 0.1 M PBS (pH 7.0). The suspension was sonicated in an ultrasonic disrupter (Branson Ultrasonics Co., Shanghai, China) at 75 W for 3 min in an ice bath; afterward the disrupted cell suspension was centrifuged at 13,000 × g for 30 min at 4°C. The β-lactamase enzymatic activity of the supernatant fluid (i.e., bacterial lysate) was measured against imipenem using iodometric method described by Doust (Daoust et al., 1973) using reagents prepared, as described previously (Onishi et al., 1974; Sawai et al., 1978; Minami et al., 1980). Briefly, iodine reagent (40 μmol in 0.5 M acetate buffer, pH 4.0) was added to lysate supernatant fluids, after 5 min incubation with imipenem (50 μg/mL) at 30°C. Ten minutes later, samples' absorbance was measured at 620 nm, and imipenem hydrolysis was determined. Activity was reported as the mean of triplicate samples in micromoles of imipenem degraded per minute per milligram of protein in each bacterial extract. Protein concentrations were measured by Bradford assay kit (Pierce™ Coomassie Plus Assay Kit, Thermo Scientific, Ottawa, Canada).

In Silico Analysis and Nucleotide Sequence Accession Numbers

In silico analysis was carried out using GenBank nucleotide database. Predict Protein software (hosted by Rostlab) was also used to predict changes as a result of a frameshift mutation. The nucleotide sequence data were deposited in the GenBank nucleotide database under accession numbers: JQ343842.1, JQ343840.1, JQ343838.1, JQ343836.1, JQ343841.1, JQ343839.1, JQ343837.1, JQ360584.1, JQ360582.1, JQ360580.1, JQ360578.1, JQ360583.1, JQ360581.1, JQ360579.1, JQ360577.1, and JQ061320.1. The novel DNA sequence of *bla*_{OXA 23} genes, with "No Full-Match" by GenBank; as well as its corresponding peptide amino acid sequence was submitted to Lahey database (lahey.org/Studies).

RESULTS

Antimicrobial Susceptibility Profiles and AFLP Genomic Fingerprint Analysis

Table 2 shows the susceptibility profiles of all 85 CR-AB isolates against CLSI groups of antimicrobial agents. Overall, CR-AB isolates were most resistant to CLSI group A (51–96%), followed by group B (25–97%) antimicrobials. Overall, up to 96% of isolates were resistant to 12 of the tested antimicrobials; while the rates of resistance to tigecycline, imipenem, and doripenem were 34, 65, and 94%, respectively. The lowest resistance rates among isolates were against colistin (12%), minocycline (25%), and doxycycline (31%). Interestingly, all colistin-resistant isolates were susceptible to tigecycline and/or tobramycin. The frequency of MDR, XDR and PDR isolates were 69, 24, and 0%, respectively; and broadly-resistant CR-AB isolates were most

TABLE 2 | *In vitro* antimicrobial susceptibility results of 85 non-replicate clinical *A. baumannii* isolates according to CLSI antimicrobial grouping of A, B, and O, as according to the frequency of *A. baumannii* isolation from the type of ICU ward.

ICU Ward (No.)	% Resistant to CLSI antimicrobial groups ^a																O						
	A						B						O				O						
	IPM ^b	DOR	SAM	CRO	CAZ	TOB	GEN	CIP	AMK	MIN	DOX	TET	TGC	CTX	PIP	LVX	FEP	TZP	TIM	SXT	CST	NET	RIF
Emergency (12)	8	13	9	14	14	7	12	11	12	5	5	9	8	6	14	14	13	11	14	14	1	6	13
Medical Care (27)	25	30	20	31	32	22	27	22	27	7	7	16	5	19	27	27	33	27	33	32	4	12	27
Pediatrics (8)	6	9	5	9	9	5	9	6	5	1	1	6	4	6	8	7	9	9	8	9	1	5	8
Surgical (31)	22	34	20	34	34	13	29	28	31	11	14	21	13	19	33	33	35	29	33	33	5	14	33
Transplantation (7)	4	8	5	8	7	4	6	7	7	1	4	5	5	5	7	8	7	4	8	8	1	2	7
Total (85)	65	94	59	96	96	51	82	74	81	25	31	58	34	56	89	89	97	80	96	96	12	39	88

^a Criteria in the assignment of agents to Groups A, B, and C included clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA clinical indications for usage, and current consensus recommendations for first-choice and alternative drugs. **Group A** are considered appropriate for inclusion in a routine, primary testing panel, as well as for routine reporting of results for the specific organism. **Group B** comprises agents that may warrant primary testing. **Group O (Other)** includes agents that have a clinical indication for the organism. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used for quality control of antimicrobial susceptibility testing and included in each run.
^b CSF, cerebrospinal fluid; AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftazoxine; CST, colistin; CTX, cefotaxime; DOR, doripenem; DOX, doxycycline; GEN, gentamicin; IPM, imipenem; MIN, minocycline; NET, netilmicin; LVX, levofloxacin; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin-sulbactam; SXT, trimethoprim/sulfamethoxazole; TZP, tigecycline; TIM, ticarcillin/clavulanic acid; TOB, tobramycin.

frequently recovered from the surgical and internal medicine ICU wards. However, the frequency of resistant isolates was generally proportional to the number of specimens from each ICU (**Table 2**).

Our analysis revealed that 34 (40%) isolates harbored bla_{OXA-23-like} genes, and 51 isolates were resistant to carbapenems but did not harbor bla_{OXA-23-like} genes (**Supplemental Figure 1**). AFLP genotype analysis of bla_{OXA-23-like}⁺ isolates generated 16 distinct AFLP genotypic groups, labeled genotype A through P. Group C (*n* = 6) was the predominant AFLP type, followed by genotype I (*n* = 4), and genotypes B, K, L, and N (*n* = 3 in each group). While each AFLP group consisted of 1 to 6 isolates, 50% (8/16) of the groups consisted of a single isolate, indicative of a high diversity among CR-AB isolates. Despite this diversity, the antimicrobial susceptibility patterns among 13 (82%) genotypes were similar, with the exception of genotypes A, M, and N (**Figure 1**).

Detection of ISAb_{a1} and ISAb_{a4}

Overall, ISAb_{a1} and ISAb_{a4} sequences were present in 61% and 7% of all tested CR-AB isolates, respectively. Among 34 bla_{OXA-23-like}⁺ isolates, 67% were ISAb_{a1}⁺, and 18% were ISAb_{a4}⁺ (**Figure 1**); whereas 10 (63%) and 3 (19%) of AFLP genotypes harbored ISAb_{a1} and ISAb_{a4} elements, respectively. ISAb_{a4} was only present among AFLP types G, E, and K isolates, while genotypes A, M, and N has neither ISAb_{a1}, nor ISAb_{a4} element (**Table 3**; **Figure 1**). Interestingly, even though the ISAb_{a1}⁺ isolates of genotype B (*N* = 3) exhibited an XDR profile, all genotype B isolates remained susceptible to tobramycin and ampicillin-sulbactam.

Among the bla_{OXA-23-like}⁺ isolates that harbored either ISAb_{a1} or ISAb_{a4} elements, a majority (82%) displayed a distinctive profile of resistance to 9 antimicrobial agents, namely, CAZ, CRO, CTX, DOR, FEP, IPM, PIP, SAM, and TZP (**Figure 1**). The presence of either ISAb_{a1}, or ISAb_{a4} imparted resistance to imipenem and doripenem among bla_{OXA-23-like}⁺ genotypes. However, the genotype A isolate (which was ISAb_{a1}⁻ and ISAb_{a4}⁻) also showed resistance to imipenem (MIC = 16 µg/mL).

Sequence Analysis of bla_{OXA-23-like} Genes

Table 3 demonstrates sequence differences between the bla_{OXA-23-like} specific amplicon among members of AFLP groups vs. the bla_{OXA-23} sequence of the *A. baumannii* reference strain (referred to as “wild type”). The bla_{OXA-23} gene sequences of six (37%) AFLP genotypes did not differ from the wild-type strain; however, isolates from 10 (63%) AFLP types had mutations in bla_{OXA-23} genes. Five different bla_{OXA-23} gene mutations were detected, and a Genbank search revealed that one of the mutant sequences had been recently reported from the USA, namely bla_{OXA-366}, and three were reported from China (i.e., bla_{OXA-422}, 481, and 482-like genes). One novel bla_{OXA-23-like} gene sequence was thus submitted to the Lahey database and assigned as the bla_{OXA-495}-like gene.

At the points of carbapenemase gene mutations, comparison of a 21-nucleotide sequence of PCR amplicons obtained from isolates in mutant AFLP groups that had >2 members, i.e.,

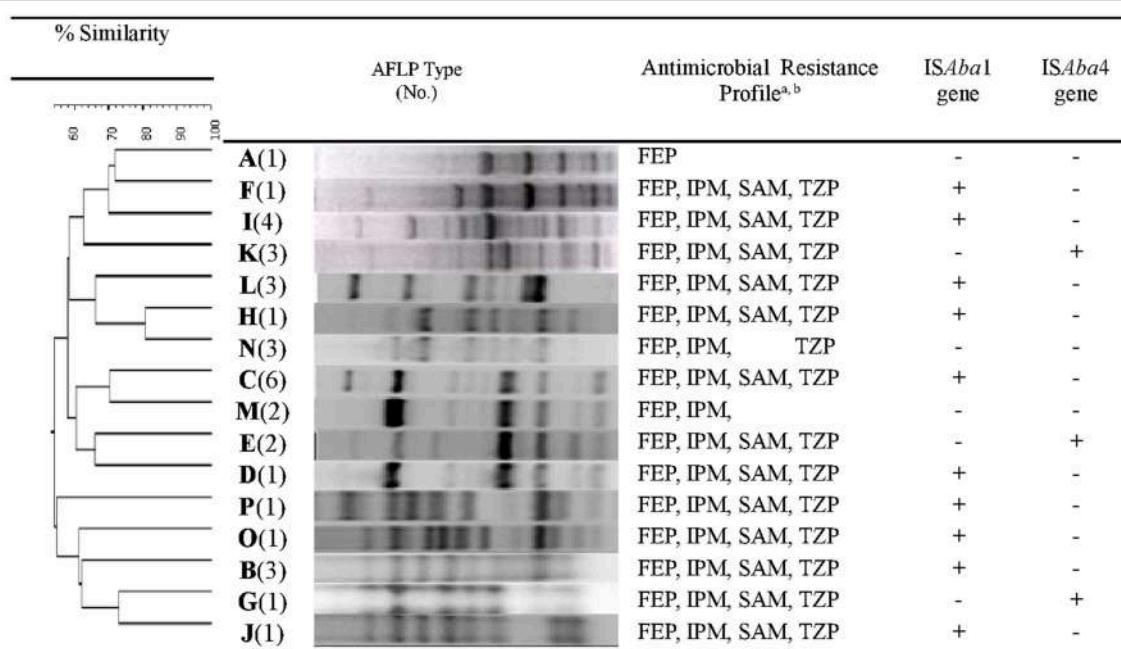


FIGURE 1 | Dendrogram of amplified fragment length polymorphism (AFLP) analysis of genomic DNA extracted from *bla*_{OXA-23-like}⁽⁺⁾ CR-AB isolates.

Susceptibility to select antimicrobials and ISAb_a presence is also indicated. Dice coefficient with 0.5% optimization and 1% position tolerance was used. Dendrogram was constructed by unweighted-pair group method using average linkages (UPGMA); and AFLP type identification was defined by groups formed at 90% similarity cutoff. ^aFEP, cefepime; IPM, imipenem; SAM, ampicillin-sulbactam; TZP, Piperacillin/tazobactam. ^bAll 16 AFLP types were resistant to the following beta-lactam antimicrobial agents: CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; PIP, piperacillin; TIM, ticarcillin/clavulanic acid.

groups L, E, C, and K, showed strong homologies within these AFLP types (**Supplemental Figure 3**). Additionally, **Table 3** displays the AFLP type, imipenem MIC, and ISAb_a status among various *bla*_{OXA-23}⁺ isolates according to their specimen source. The absence of mutation(s) in *bla*_{OXA-23} genes was associated with lower imipenem MICs as compared to mutant isolates. Imipenem MIC_{gm} for all non-mutant *bla*_{OXA-23} gene isolates was 16 µg/mL (range = 8 – 32 µg/mL), whereas the mean MIC for mutants was almost 50 µg/mL (range = 16 – 128 µg/mL). Conversely, alterations of the *bla*_{OXA-23} gene were associated with an increased MIC to imipenem among *A. baumannii* isolates. While 16 (47%) of *bla*_{OXA-23-like}⁺ isolates harbored ISAb_{a1} sequences upstream of their carbapenemase gene, only six (18%) isolates were ISAb_{a4}⁺. ISAb_{a1} elements were detected upstream of either *bla*_{OXA-23} or *bla*_{OXA-51} genes, or both these genes. Since, none of the *bla*_{OXA-23-like}⁺ isolates were both ISAb_{a1}⁺ and ISAb_{a4}⁺, the presence of these elements appeared mutually exclusive among isolates (**Table 3**). The ISAb_{a4} sequence was absent among *bla*_{OXA-23-like} genes of non-mutant CR-AB isolates, whereas six of mutant isolates, namely genotypes G, E, and K, were ISAb_{a4}⁺ with moderate MICs, ranging from 16 to 64 µg/mL (**Table 3**). Although mutant isolates of genotypes C and D were ISAb_{a1}⁺ had the highest imipenem MICs (128 µg/mL), the imipenem MIC of ISAb_{a1}⁺ mutants among genotypes O, P, J, and L was 16 µg/mL. Furthermore, all ISAb_{a1}[–] and ISAb_{a4}[–] CR-AB isolates had non-mutant *bla*_{OXA-23}⁺ genes, namely genotypes A, M, and N isolates,

which showed the lowest imipenem MICs (8–16 µg/mL), as well. Surprisingly, three *bla*_{OXA-23-like}⁺ isolates (genotypes A and M) had imipenem MICs of 16 µg/mL, but harbored neither ISAb_{a1}, nor ISAb_{a4} elements upstream of the *bla*_{OXA-23-like} gene, suggestive of other resistance mechanisms in these isolates (**Table 3**).

As shown in **Table 3**, among mutations of the *bla*_{OXA-23-like} genes, insertions/deletions at nucleotide positions 335 and 336 were most frequent (40%), followed by a single substitution at position 771 (30%). Isolates with F, G, O, and P genotypes showed the insertion/deletion mutations at position 335, with imipenem MICs of 16–32 µg/mL, whereas isolates with genotypes E, C, and K had the substitution at position 771 and showed the highest imipenem MICs of 64–128 µg/mL (**Table 3**). Further analysis revealed that three other single substitutions also occurred at positions 376, 625, and 766 among genotypes J and L, but their imipenem MICs were not higher than non-mutants (16 µg/mL). In addition, three isolates with the substitution at position 771 had a 3–4-fold increase in imipenem MIC over that of the non-mutants. A frame-shift mutation at position 355, which corresponds to a change at aa112–118, in genotype D isolate (strain TUMS/BTRF 661) was associated with a four-fold increase in imipenem MIC_{gm} (128 µg/mL) over that of non-mutant isolates. Software prediction showed that the frame-shift mutation may change the subcellular localization of carbapenemase and enhance its secretion rate, which can explain the high MIC of the isolate against carbapenems. *In silico*

TABLE 3 | Comparison of *bla*_{OXA-23-like} gene sequences among CR-AB isolates belonging to various AFLP genotype groups, as compared to *bla*_{OXA-23} gene sequence of *A. baumannii* reference strain, according to the specimen source, MIC against imipenem, β-lactamase activity, and ISAb element status.

No.	Isolates	AFLP type (No.)	Specimen (ICU) ^a	Nucleotide Change(s)	Amino Acid Change(s)	MIC of IMP (mg/L)	Mean of β-lactamase activity ^d (SD) ^e	IS ^b type	
								ISAb 1	ISAb 4
1	<i>bla</i> _{OXA-23}	N (3)	Urine (S)	None	None	8	ND	—	—
2	"	M (2)	Urine (S)	"	"	16	1.05(0.10)	—	—
3	"	A (1)	Urine (S)	"	"	16	0.87(0.04)	—	—
4	"	H (1)	Blood (P)	"	"	32	2.63(0.15)	+	—
5	"	I (4)	Blood (E)	"	"	16	1.35(0.09)	+	—
6	"	B (3)	CSF (S)	"	"	16	ND	+	—
7	<i>bla</i> _{OXA-482}	F (1)	Urine (M)	Insertion of A at position 335 and deletion of A at position 336	Ser → Tyr at aa 112	32	0.42(0.04)	+	—
8	"	G (1)	Urine (T)	"	"	16	5.58 (0.91)	—	+
9	"	O (1)	Urine (E)	"	"	16	2.11(0.63)	+	—
10	"	P (1)	Sputum (E)	"	"	16	2.35(0.87)	+	—
11	<i>bla</i> _{OXA-481}	E (2)	Wound (S)	G → A at position 771	Met → Ile at aa 257	64	1.67(0.09)	—	+
12	"	C (6)	Urine (S)	"	"	128	7.96(0.92)	+	—
13	"	K (3)	Blood (M)	"	"	64	7.75(1.03)	—	+
14	<i>bla</i> _{OXA-495}	D (1)	Sputum (S)	Frame-shift due to insertion of A at position 335 and deletion at A at position 354	Change in aa112-aa118 motif ^c	128	14.37(1.33)	+	—
15	<i>bla</i> _{OXA-366}	J (1)	Blood (M)	A → C at position 376, and G → A at position 625	Met → Ile at aa 126, and Glu → Lys at aa 209	16	2.62(0.53)	+	—
16	<i>bla</i> _{OXA-422}	L (3)	Sputum (S)	G → A at position 766	Glu → Lys at aa 256	16	0.37(0.03)	+	—

^aICUs: E, Emergency; M, Medical Care; P, pediatric; S, surgical; T, transplantation.

^bIS: Insertion sequence.

^cSFTAWE → YYRLG.

^dμmole imipenem hydrolyzed per min per mg of protein.

^estandard deviation.

ND, No detectable activity.

comparison of carbapenemase binding domains of wild-type vs. the frame-shift mutant also showed that the binding domain changed from "aa15–24 and aa28–29" to "aa15–22 and aa24–25" motif, which may lead to higher affinity of mutant OXA-23 enzyme for the binding cleft of carbapenems.

Five *bla*_{OXA-23} mutants and one non-mutant isolate showed imipenem MICs of >16 μg /mL. Further *in silico* analysis revealed that the *bla*_{OXA-23-like} gene mutations would lead to up to six amino acid changes in the carbapenemase protein. However, the highest imipenem MICs were associated with a single substitution at position 771 of *bla*_{OXA-23-like} gene, corresponding to aa 257 substitution in carbapenemase among genotypes E, C, and K, which represented 55% of the mutant isolates. All AFLP types with non-mutant *bla*_{OXA-23} genes showed MICs of ≤16 μg/mL for imipenem, except the genotype H isolate (MIC = 32 μg /mL); however, all *bla*_{OXA-23-like} mutants showed high imipenem resistance (MIC = 16–128 μg/mL).

As shown in **Table 3**, we compared the β-lactamase activity of the 16 representative isolates from each AFLP group. Overall, the range of β-lactamase activity of non-mutant

isolates was lower (not detectable–2.63 μmoles/min/ mg protein) than the *bla*_{OXA-23-like} mutants (0.37–14.37 μmoles/min/mg protein). Mutant isolates, such as the frame-shift mutant genotype D, showed the highest imipenem MICs (i.e., 64 and 128 μg/mL), as well as the highest β-lactamase activities, i.e., 7.96 and 14.37 μmoles/min/mg protein for genotype C and K, respectively. In contrast, lysates from isolates with no *bla*_{OXA-23-like} gene mutation that had the lowest MICs (e.g., genotypes N and B), and showed no detectable β-lactamase activity. The β-lactamase activity of a imipenem-susceptible (MIC < 4 μg/ml), clinical *A. baumannii* isolate, was also below assay's detection level (data not shown). Among the 4 isolates that had the same mutation at position 335, the β-lactamase activity was between 0.42 and 5.58 μmoles/min/ mg protein, while imipenem their MIC was 16–32 μg/mL. However, with the exception of genotype E, the β-lactamase activity of mutants with position 771 mutation, was increased concomitant with high MICs in these isolates. The majority (70%) of mutant isolates were recovered from two ICU wards; namely, the surgical (*n* = 4), and the internal medicine (*n* = 3) ICU; however, no mutant isolates were recovered from the pediatric ICU ward. Among the

*bla*_{OXA-23-like} mutant isolates, eight (80%) were recovered from either urine or sputum specimens (**Table 3**).

Table 4 shows the distribution of ISAb_{a1} or ISAb_{a4} sequences upstream of various *bla*_{OXA-23-} and *bla*_{OXA-51-like} genes among the isolates that harbored ≥ 1 carbapenemase genes; and also their resistance rate against carbapenems. The presence of ISAb_a upstream of the *bla*_{OXA-51-like} and *bla*_{OXA-23-like} genes was associated with high rate of carbapenem resistance. Among all *bla*_{OXA-51-like}⁺ or *bla*_{OXA-23-like}⁺ isolates, almost 32% ($n = 27$) lacked either ISAb_{a1}, or ISAb_{a4} sequences. CR-AB isolates were consistently more resistant to doripenem than to imipenem, regardless of their *bla*_{OXA-} genes (**Table 4**). There was no marked difference in carbapenem resistance rates whether the isolates harbored the “*bla*_{OXA-51-like} gene alone,” or “*bla*_{OXA-51-like} plus *bla*_{OXA-24-like}” genes. Overall, the ISAb_{a1} element was more often associated with *bla*_{OXA-51-like} gene (20–100%) than with *bla*_{OXA-23-like} genes; and ISAb_{a4}⁺ isolates showed high rates of carbapenem resistance, especially against doripenem. Despite this high resistance rate, 13% of *bla*_{OXA-23}⁺/*bla*_{OXA-51}⁺ isolates that harbored both ISAb_{a1} and ISAb_{a4} were imipenem susceptible (**Table 4**). Among ISAb_{a4}⁺ isolates, these elements were upstream of the *bla*_{OXA-51-like} gene in 60% (31/52) of the isolates, whereas only 31% harbored ISAb_{a1} upstream of the *bla*_{OXA-23-like} gene. All 13 (15%) CR-AB isolates with *bla*_{OXA-51-like} gene as their sole carbapenamase gene had ISAb_{a1} elements. Interestingly, even though test isolates showed an overall high resistance rate against carbapenems, 32% of isolates did not harbor either ISAb_{a1} or ISAb_{a4} elements. By and large, there was no marked change in resistance rate among isolates that harbored the *bla*_{OXA-24-like} gene in combination with other carbapenemase genes (**Table 4**).

DISCUSSION

Infections caused by carbapenem-resistant *A. baumannii* are among the most difficult to treat, especially among ICU patients (Alfandari et al., 2014). In several countries, including Iran, clinicians face serious challenges in choosing an effective combination of antimicrobial agents while treating patients with severe nosocomial CR-AB infections. Efforts to control MDR-AB outbreaks have prompted widespread use of antimicrobials, such as tigecycline and colistin, as therapeutic measures to combat severe infections (Garnacho-Montero et al., 2015). However, appropriate treatment and effective infection control measures require local susceptibility patterns, as well as molecular epidemiologic data, such as the *bla*_{OXA} gene status of CR-AB isolates. Several surveillance studies have reported widespread nosocomial outbreaks of OXA-type producing *A. baumannii*, and a high prevalence of *bla*_{OXA} gene-carrying CR-AB in Iran, but data regarding their *bla*_{OXA-23-like} gene is not available (Moradi et al., 2015).

In the present study, we have genetically evaluated *bla*_{OXA-23-like}⁺ CR-AB isolates and found high genotypic diversity among the isolates, including variants with new *bla*_{OXA-23-like} gene mutations. These mutations were associated with up to four-fold increases in MIC levels against imipenem, as compared to non-mutant isolates. Mutations in certain codons associated with a high degree of resistance to imipenem. For instance, substitutions at position 355 of the *bla*_{OXA-23-like} gene (i.e., the frame-shift mutation) were associated with high-level resistance, whereas position 256 mutations were associated with low-level resistance. Surprisingly, newly-found mutations correspond to regions of the carbapenemase molecule that

TABLE 4 | Frequency of ISAb_{a1} or ISAb_{a4} sequences upstream of various *bla*_{OXA-} genes among test CR-AB isolates that harbored ≥ 1 carbapenemase genes, and the comparison of percent resistance against carbapenems among CR-AB isolates according to the isolate's *bla*_{OXA-} gene combination.

No.	Carbapenemase gene(s) of CR-AB isolates (Total=85)	% of isolates with insertion sequences					% Carbapenem Resistance ^a	
		ISAb _{a1} on <i>bla</i> _{OXA-51-like} ($n = 31$)	ISAb _{a1} on <i>bla</i> _{OXA-23-like} ($n = 16$)	ISAb _{a1} on <i>bla</i> _{OXA-51-like} and <i>bla</i> _{OXA-23-like} ($n = 5$)	ISAb _{a4} on <i>bla</i> _{OXA-23-like} ($n = 6$)	Without ISAb _a ($n = 27$)	DOR ^b	IPM
1	<i>bla</i> _{OXA-51-like} ($n = 13$; 15%)	38	—	—	—	62	77	46
2	<i>bla</i> _{OXA-51-like} and <i>bla</i> _{OXA-23-like} ($n = 54$; 63%)	37	30	9	9	15	100	72
3	<i>bla</i> _{OXA-51-like} and <i>bla</i> _{OXA-24-like} ($n = 10$; 12%)	30	—	—	—	70	90	40
4	<i>bla</i> _{OXA-51-like} , <i>bla</i> _{OXA-23-like} and <i>bla</i> _{OXA-24-like} ($n = 5$; 6%)	20	0	0	20	60	100	80
5	<i>bla</i> _{OXA-51-like} , <i>bla</i> _{OXA-23-like} , <i>bla</i> _{OXA-24-like} and <i>bla</i> _{OXA-58-like} ($n = 1$; 1%)	100	0	0	0	0	100	100
6	<i>bla</i> _{OXA-51-like} and <i>vim-2</i> ($n = 2$; 3%)	50	0	0	0	50	50	50

^aMIC $\leq 8 \mu\text{g/ml}$.

^bDOR, doripenem; IPM, imipenem.

are outside the standard “S-T-F-K, S-X-I, Y-G-N” and “K-S-G” oxacillinase motifs (Couture et al., 1992), suggesting that configurational changes due to novel mutations may also affect oxacillinase activity against carbapenems.

Although a high frequency of MDR (69%) and XDR (24%) *A. baumannii* isolates from this region is consistent with previous reports (D’Arezzo et al., 2011; Potron et al., 2011; Sung et al., 2011), our finding of 26 and 6% increases in resistance to tigecycline and colistin, respectively, (Bahador et al., 2014) is quite worrisome. Fortunately, while all isolates harboring mutations in *bla*_{OXA-23-like} genes showed resistance to carbapenem-class antibiotics; they were susceptible to tigecycline and/or tobramycin, which concurs with a recent report that shows potential activity of a number of combinations against MDR *A. baumannii* (Garnacho-Montero et al., 2015). Our data regarding to a high prevalence of *bla*_{OXA-23-like} genes among CR-AB from Tehran confirms previous reports (Shahcheraghi et al., 2011), and implies that extra efforts should be focused on controlling the spread of *bla*_{OXA-23-like}⁺ *A. baumannii* in this area. Clonal outbreaks of OXA-23-producing CR-AB have been reported from several countries (Mugnier et al., 2010). Interestingly, isolates in this study did not harbor any NDM-1 metallo-β-lactamase genes, nor the “*bla*_{SPM-1} and *bla*_{GES-1}” genes, which have recently been reported from India and Pakistan (Jones et al., 2014; Sartor et al., 2014), and Tehran (Shahcheraghi et al., 2011), respectively. However, we detected three mutant *bla*_{OXA-23-like} genes with identical sequences reported from China.

The data on the presence of ISAbal sequences upstream from *bla*_{OXA} genes and enhancement of OXA-enzyme expression confirms previous reports (Sung et al., 2011); however, these findings are in contrast with a report from northwestern Iran, which detected no ISAbal sequences upstream of the *bla*_{OXA} gene (Peymani et al., 2012). Together, these results suggest that the CR-AB populations in various parts of Iran are diverse and distinct, which may hint on probable differences in antimicrobial management of *A. baumannii* infections in various regions. Moreover, most of the *bla*_{OXA-23} mutant *A. baumannii* isolates were obtained from urine and sputum samples, suggesting that specific infection control protocols regarding urinary catheters and ventilators are possible primary sources of CR-AB transmission.

Although increased imipenem resistance due to a mutation in the *bla*_{OXA-23} gene has been reported previously (Lin et al., 2011), to the best of our knowledge, this is the first report of CR-AB *bla*_{OXA-23} gene mutants from Iran. It is noteworthy that the TUMS/BTRF661 strain showed the highest MIC (128 μg/mL), implying a greater influence of the frame-shift mutation on carbapenem resistance than any of the substitution mutations. Our future studies will focus on exploring the difference in the MICs of the various mutants (16 vs. 128 μg/mL) and the potential complex interactions between antimicrobial agents and carbapenemase at the molecular level, where the position of the affected motif plays a critical role. While production of carbapenemase remains to be the chief mechanism of carbapenem-resistance in *A. baumannii*, whether additional

factors, such as alterations in outer membrane permeability, efflux pumps as with AdeABC (Potron et al., 2015), or OprD porin (Potron et al., 2015), contribute to carbapenem-resistance among mutants. The variability in the β-lactamase activity and MIC values of variants that share a mutation, suggests that other factors play a role in high MIC levels among mutant CR-AB isolates, and they remain to be explored.

Assuming future confirmation of the correlation of specific mutations with high MICs, carbapenem resistance levels may be predictable by DNA sequence-based detection methods. Also, determination of predominant *bla*_{OXA-23} genotype(s) of isolates in various areas and their *bla*_{OXA-23-like} gene mutations may serve as a tool for molecular epidemiologic investigations to control the spread of CR-AB infections. While the present study focused on the chromosomal OXA-encoding genes in CR-AB, we also plan to explore the role of mutations in plasmid-encoded *bla*_{OXA-23-like} genes among carbapenem-resistant *A. baumannii*, since many of these genes are plasmid-borne.

In conclusion, we report the identification of CR-AB variants that harbor *bla*_{OXA-23-like} gene mutations, which are associated with an increased MIC against imipenem. Several *bla*_{OXA-23-like} mutant isolates are widespread and have been reported from the USA, and China. The detection of new *bla*_{OXA-23} mutant isolates from Iran highlights the importance of concerted efforts, at the national and global levels, toward the control of carbapenem-resistance among *A. baumannii* isolates worldwide.

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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.2015.01249>

Supplemental Figure 1 | Presentation of study strategy to select *bla*_{OXA-23}⁺ *A. baumannii* clinical isolates for AFLP genotype determination and DNA sequence analysis. Step-wise selection of *bla*_{OXA}⁺ isolates was carried out using 2 sets of multiplex PCR assay, followed by AFLP genotypic analysis and DNA sequencing of *bla*_{OXA-23-like} gene amplicons.

Supplemental Figure 2 | Agarose gel electrophoresis analysis of PCR amplicons specific for Ambler Class A and B carbapenemases. Genomic DNA from clinical *A. baumannii* isolates were analyzed by uniplex and multiplex PCR assay as described (M&M). Lanes 1 through 6; specific bands for (1) NDM1, (2) IMP1, (3) VIM2, (4) SPM1, (5) *bla*_{GES}, and (6) KPC encoding genes. Lane 7; AB-hexplex (AB-h) PCR products of the above genes. Lane 8; 100 bp DNA markers.

Supplemental Figure 3 | Comparison between DNA sequences of AB0057Ref.seq and PCR amplicon sequences obtained from mutant CR-AB isolates in genotype groups with >2 members showing a 10-nucleotide span in each direction of the point of mutation (total 21 nucleotides). (A) Alignment of amplicons from three isolates of AFLP group L (i.e., TUMS/BTRF443, L2, and L3) as compared to the AB0057Ref.seq sequence, with the mutation at position 766. (B) Alignment of AFLP groups E, C, and K amplicons with the AB0057Ref.seq reference sequence, with the mutation at position 771. In both panels (*) indicates sequence identity (or homology), and (–) shows the position of carbapenemase gene mutation.

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Tracking Cefoperazone/Sulbactam Resistance Development *In vivo* in *A. baumannii* Isolated from a Patient with Hospital-Acquired Pneumonia by Whole-Genome Sequencing

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Cefoperazone/sulbactam has been shown to be efficacious for the treatment of infections caused by *Acinetobacter baumannii*; however, the mechanism underlying resistance to this synergistic combination is not well understood. In the present study, two *A. baumannii* isolates, AB1845 and AB2092, were isolated from a patient with hospital-acquired pneumonia before and after 20 days of cefoperazone/sulbactam therapy (2:1, 3 g every 8 h with a 1-h infusion). The minimum inhibitory concentration (MIC) of cefoperazone/sulbactam for AB1845 and AB2092 was 16/8 and 128/64 mg/L, respectively. Blood samples were collected on day 4 of the treatment to determine the concentration of cefoperazone and sulbactam. The pharmacokinetic/pharmacodynamic (PK/PD) indices (%T_{>MIC}) were calculated to evaluate the dosage regimen and resistance development. The results showed that %T_{>MIC} of cefoperazone and sulbactam was 100% and 34.5% for AB1845, and 0% and 0% for AB2092, respectively. Although there was no available PK/PD target for sulbactam, it was proposed that sulbactam should be administered at higher doses or for prolonged infusion times to achieve better efficacy. To investigate the mechanism of *A. baumannii* resistance to the cefoperazone/sulbactam combination *in vivo*, whole-genome sequencing of these two isolates was further performed. The sequencing results showed that 97.6% of the genome sequences were identical and 33 non-synonymous mutations were detected between AB1845 and AB2092. The only difference of these two isolates was showed in sequencing coverage comparison. There was a 6-kb amplified DNA fragment which was three times higher in AB2092, compared with AB1845. The amplified DNA fragment containing the *bla*_{OXA-23} gene on transposon *Tn*2009. Further quantitative real-time PCR results demonstrated that gene expression at the mRNA level of *bla*_{OXA-23} was >5 times higher in AB2092 than in AB1845. These results suggested that the *bla*_{OXA-23}

gene had higher expression level in AB2092 via gene amplification and following transcription. Because gene amplification plays a critical role in antibiotic resistance in many bacteria, it is very likely that the *bla_{OXA-23}* amplification results in the development of cefoperazone/sulbactam resistance *in vivo*.

Keywords: cefoperazone/sulbactam, *Acinetobacter baumannii*, whole-genome sequencing, resistance development, PK/PD indices

INTRODUCTION

Acinetobacter baumannii is a non-fermentative, gram-negative opportunistic pathogen that can cause hospital-acquired pneumonia (HAP), blood infections and urinary tract infection, among others (Perez et al., 2007). The rapid spread of the multi-drug-resistant *A. baumannii* has resulted in very limited therapeutic options in the clinic. Sulbactam, a β -lactamase inhibitor, has intrinsic antimicrobial effects against *A. baumannii* by binding to penicillin-binding protein 2 (Peleg et al., 2008). It is commercially available in a combined formulation of ampicillin and cefoperazone. The clinical efficacy of cefoperazone/sulbactam has been shown in previous work (Xin et al., 2013; Sipahi et al., 2014; Xia et al., 2014). This combination has recently been applied to treat critically ill patients receiving continuous venovenous hemofiltration, and 11 of 14 patients survived (Gao et al., 2016). This combination was also administrated to treat neurosurgical patients in a pilot study, with the results showing that cerebrospinal fluid penetration of cefoperazone/sulbactam could be enhanced after neurosurgical impairment of the blood-brain barrier (Wang et al., 2015). In a retrospective review of the outcomes for patients with cefoperazone/sulbactam treated *A. baumannii* bacteremia, 77% of the patients (27/35) presented successful clinical efficacy (Choi et al., 2006). Because the combination has been widely used in the clinic, the resistance rate was monitored and observed to increase from 25.0 to 37.7% from 2004 to 2010 in China (Hu et al., 2016). However, the underlying mechanism is not well understood *in vitro* or *in vivo*, for sulbactam alone or the combination. Sulbactam alone has been reported to cause resistance in *A. baumannii* via PBP3 mutation *in vitro*, despite a lack of information regarding natural pbp3 mutations in clinical isolates (Penwell et al., 2015). The detection and expression of *blaTEM-1* has been suggested to relate to the minimum inhibitory concentration (MIC) of sulbactam in *A. baumannii* (Waltner-Toews et al., 2011). The combination resistance in *Klebsiella pneumoniae* in an *in vitro* study was reported owing to two different mechanisms, loss of a 39-kDa outer membrane protein and presence of TEM-2 β -lactamase (Rice et al., 1993).

Whole-genome sequencing is powerful and can reveal a vast amount of DNA information from a global perspective. The published complete genome sequences for many bacteria are beneficial and efficient for strain-to-reference sequencing and bioinformatics analysis (Mardis, 2008). They can be used to identify resistance genes as well as minor changes in the genome due to mutation, gene transfer, gene duplication, and amplification. It has been seen as a basis for whole-genome

sequencing revealing the mechanism of bacterial pathogen resistance that develops in patients (Snitkin et al., 2011; Wright et al., 2014; Holt et al., 2015). Wright et al. (2016) sequenced 136 strains of *A. baumannii* isolated from patients and showed the genome changes occurred mainly due to the single nucleotide variance in protein coding regions and IS element. Genome sequenced *A. baumannii* showed the colistin resistance was due to the mutations in transcriptional regulatory genes (Cheah et al., 2016). Susceptible *S. aureus* evolved resistance via a 35-point mutation in 31 loci in a patient with a bloodstream infection receiving vancomycin therapy (Mwangi et al., 2007). Tigecycline resistance to *A. baumannii* has been reported to be due to the deletion of three contigs *in vivo* (Hornsey et al., 2011). Additionally, gene amplification was frequently detected due to the antibiotic pressure. The evolution of β -lactamase resistance was studied by exposing *Salmonella typhimurium*, which contain low level β -lactamase resistance, in an environment of progressively increasing concentrations of cephalosporin. The results showed an amplification of *blaTEM-1* gene copy number followed by acquisition of gene mutations (Sun et al., 2009). A genome-wide assay of *E. coli* strain in 78 different antibiotic environments found that 56 genes were amplified to fit the environment and reproducible increasing in MIC for their corresponding antibiotics was detected (Soo et al., 2011). The amplification of aminoglycoside resistance gene *aphA1* in *A. baumannii* has been considered to be the mechanism underlying the development of tobramycin *in vivo* (McGann et al., 2014).

In the present study, two *A. baumannii* isolates, AB1845 and AB2092, were collected before and after cefoperazone/sulbactam therapy from a patient with HAP. Pharmacokinetics/pharmacodynamics (PK/PD) was applied to evaluate the dosage regimen and development of resistance. Moreover, whole-genome sequencing was employed to investigate the mechanism of cefoperazone/sulbactam resistance of the two isolates developed *in vivo*.

MATERIALS AND METHODS

Medical Records and Treatment

This study was approved by the institutional review board of Huashan Hospital affiliated to Fudan University. Written informed consent was obtained from the patient. A 19-year-old male patient with serious brain trauma was admitted to Huashan Hospital (Shanghai, China). The patient had a high fever with white blood cell counts out of the normal range after being checked into bed for 48 h. Both chest

X-Ray radiophotography and computed tomography showed pneumonia. *A. baumannii* (AB1845) was isolated from the sputum of the patient, revealing bacterial infection. Hence, the patient was treated with meropenem 0.5 g q8h without clinical efficacy. On day 6, the dosage regimen was changed to cefoperazone/sulbactam (2:1, 3 g every 8 h with a 1-h infusion, SULPERAZON®, batch no. 95839116) for 20 days. The treatment was stopped when body temperature and white blood cell counts returned to the normal range; additionally, *A. baumannii* was not detected in the sputum. However, the patient had a fever, and *A. baumannii* (AB2092) was again isolated 4 days after cefoperazone/sulbactam treatment. The cefoperazone/sulbactam treatment was finally considered to have failed both clinically and microbiologically efficacy.

Pharmacokinetics of Cefoperazone/Sulbactam Treatment

Blood samples were collected before the first infusion, immediately after the infusion (0 h), and at 0.5, 1, 2, 4, 6 h afterward on day 4 (i.e., steady state) of the cefoperazone/sulbactam treatment. The drug concentration was determined using the LC-MS/MS method (Zhou et al., 2010), which was validated according to the CFDA guidelines on bioanalytical method validation. The pharmacokinetic parameters of the patients were calculated by Winnonlin (v6.0, phoenix). The patient's pharmacokinetic profiles of cefoperazone and sulbactam were fit into a well-developed population PK model (Chen et al., 2015). The PK/PD indice of cefoperazone and sulbactam was %T_{>MIC}, namely the percentage of the dosing interval for which the plasma concentration of cefoperazone or sulbactam above the MIC. It was calculated based on the drug pharmacokinetic profile and MICs of the combination (Lips et al., 2014).

Bacterial Isolates and Susceptibility Testing

Acinetobacter baumannii AB1845 and AB2092 were isolated from the sputum of the patient before and after cefoperazone/sulbactam therapy. Both isolates were stored at -80°C. The MIC was determined in cation-adjusted Mueller-Hinton broth (Becton-Dickinson, Sparks, MD, USA) using a broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) standards. The MICs of the β-lactam/β-lactamase inhibitor combination, including cefoperazone/sulbactam, carbapenems, colistin, and polymyxin B, were determined.

Genome Sequencing and Analysis

Genomic DNA was extracted using a Genomic DNA Purification Kit (Tiangen, Beijing, China) according to the instruction manual and stored at -80°C before sequencing. A 300-bp paired-end library was constructed for the purified DNA sample following the standard Illumina paired-end protocol. Cluster generation was performed in C-bot, and sequencing was performed on the Illumina Hiseq2500 with 150 cycles. The sequence reads

were cleaned using the FASTX toolkit¹. Genome assembly was performed using Velvet (Ver 1.0.15) (Zerbino and Birney, 2008). Putative protein-coding sequences were determined by combining the prediction results of the glimmer 3.02 (Delcher et al., 2007) and Z-Curve (Guo and Zhang, 2006) program. The phylogenetic tree was constructed using PHYML (Guindon and Gascuel, 2003) by concatenating orthologs based on the protein sequences in the published genome. Functional annotation of CDS was performed by searching the NCBI non-redundant protein database and the KEGG protein database (Kanehisa et al., 2016). Gene annotation was mapped to the *A. baumannii* MDR-ZJ06 genome (from the phylogenetic tree) using Bowtie2 with default settings (Langmead and Salzberg, 2012). Single Nucleotide Polymorphisms (SNPs) from alignments were called using Samtools-0.1.16 (Li et al., 2009), and the output was generated in the pileup format. Gene amplification was detected by comparing the coverage of the reads in AB1845 and Ab2092 using the read depth method (Redon et al., 2006).

Quantitative Real-Time PCR Analysis of bla_{OXA-23}

The mRNA expression level of bla_{OXA-23} was quantified by real-time PCR. AB1845 and AB2092 were cultured in cation-adjusted Mueller-Hinton broth and collected during the early log phase. Total RNA was extracted using a TaKaRa Mini BEST Universal RNA Extraction Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. Quantitative real-time PCR (qPCR) was performed using a two-step process. RNA was first reverse-transcribed to cDNA (TaKaRa Biotechnology, Dalian, China), and real-time PCR was conducted on an ABI Vii7 (Applied Biosystems, Carlsbad, CA, USA) using a TaKaRa SYBR® FAST qPCR Kit with 40 cycles of denaturation for 5 s at 95°C, annealing for 30 s at 50°C, and extension for 20 s at 72°C. The PCR primers for the bla_{OXA-23} gene were F: CCGAGTCAGATTGTTCAAGGA and R: TGTAGAGGCTGGCACATATTC; and those for 16S rRNA were F: GGCGGCTTATTAAGTCGGATG and R: TTCGTACCTCAGCGTCAGTATT. The melting curve analysis was performed immediately after amplification to verify the specificity of the PCR amplification products.

Fluorescence was measured at the end of the annealing-extension phase of each cycle. A threshold value for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeded this fluorescence threshold was identified as the threshold cycle (CT). The relative quantitation was calculated by the 2^{-ΔΔCT} method (Schmittgen and Livak, 2008). The student *t*-test was applied to evaluate the significance of the gene expression level of the two isolates.

RESULTS

Antibiotic Susceptibility

Minimum inhibitory concentrations were obtained for cefoperazone/sulbactam combinations, carbapenems, colistin

¹http://hannonlab.cshl.edu/fastx_toolkit/

and polymyxin B for the pre-therapy isolate AB1845 and the post-therapy isolate AB2092 (**Table 1**). The MICs of the cefoperazone/sulbactam combination were 16/8 mg/L for AB1845; and the MICs of mono cefoperazone and sulbactam were >128 mg/L and 32 mg/L. The results supported a synergistic effect of the combination (FICI < 0.5, MIC of cefoperazone was set at 128 mg/L for the FICI calculation). However, the MICs of the combination and mono drugs were 128/64, >128, and 128 mg/L for AB2092. The combination did not display a synergistic effect on AB2092.

Interestingly, AB1845 and AB2092 also developed meropenem resistance with MIC increased from 16 to 64 mg/L, although the bacteria only exposed to meropenem for a short term (0.5 g every 8 h for 4 days). The isolates also developed resistance to imipenem and doripenem. Since both cefoperazone and meropenem are β -lactam antibiotics, the increased MIC in both cefoperazone/sulbactam and carbapenems may imply the similar underlying resistance mechanism. The isolates were only sensitive to colistin and polymyxin B, indicating an alternative choice for the AB2092 treatment. The MICs of the antibiotics are shown in **Table 1**.

TABLE 1 | The minimum inhibitory concentrations (MICs) of different antibiotics for AB1845 and AB2092.

Antibiotics	MIC (mg/L)	
	AB1845	AB2092
Cefoperazone/sulbactam	16/8*	128/64
Cefoperazone	>128	>128
Sulbactam	32	128
Meropenem	16	64
Imipenem	16	64
Doripenem	32	128
Colistin	2	2
Polymyxin B	1	1

*FICI = 0.375; FICI = $MIC_{(A)}/MIC_{(A \text{ in combination})} + MIC_{(B)}/MIC_{(B \text{ in combination})}$. The bold values showed the increasing MIC of AB1845 compared with AB2092.

Pharmacokinetics and Pharmacodynamics (PK/PD)

Plasma concentrations of cefoperazone and sulbactam at steady state were determined using the validated LC-MS/MS method, and the data were fitted into a developed population PK model (**Figure 1**). The pharmacokinetic parameters of cefoperazone and sulbactam are summarized in **Table 2**. The $T_{1/2}$ of cefoperazone was 4.1 h, which was a bit longer than the reported 1.8 h (Reitberg et al., 1988). This could attribute to liver dysfunction (Boscia et al., 1983) according to the medical records of the patients. The $T_{1/2}$ of sulbactam was 1.3 h, which was comparable to the published data (Reitberg et al., 1988).

Pharmacokinetic/pharmacodynamic indices were calculated to evaluate the drug dosage regimen. The %T_{> MIC} was the most predictive PK/PD index for cefoperazone and sulbactam (Cooper et al., 2011; Sy et al., 2015), and the MIC of the combination was used for the %T_{> MIC} calculation. In this study, the %T_{> MIC} was 100% for cefoperazone and 34.5% for sulbactam for AB1845; and the %T_{> MIC} was 0% for both cefoperazone and sulbactam for AB2092.

Whole-Genome Sequencing of AB1845 and AB2092

The two isolates were initially typed by PFGE of *Apal*-digested genomic DNA and shown to be the same strain. Whole-genome

TABLE 2 | Pharmacokinetic parameters of cefoperazone and sulbactam.

Parameters	Units	Cefoperazone	Sulbactam
$T_{1/2}$	h	4.1	1.3
T_{\max}	h	1.4	1.3
C_{\max}	mg/L	129.6	37.2
AUC_{0-8}	mg h/L	529.1	66.5
$AUC_{0-\infty}$	mg h/L	766.0	68.3
V_d	L	15.5	26.6
CLt	L/h	2.6	14.6
MRT_{0-8}	h	2.6	1.5
$MRT_{0-\infty}$	h	5.7	1.6

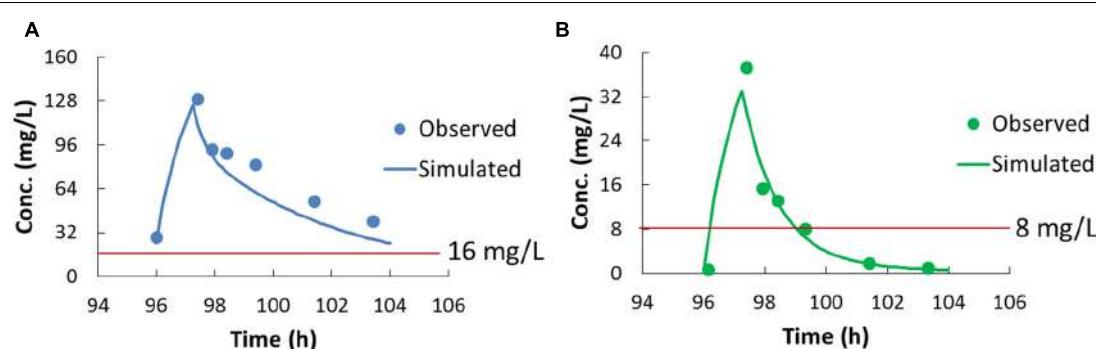
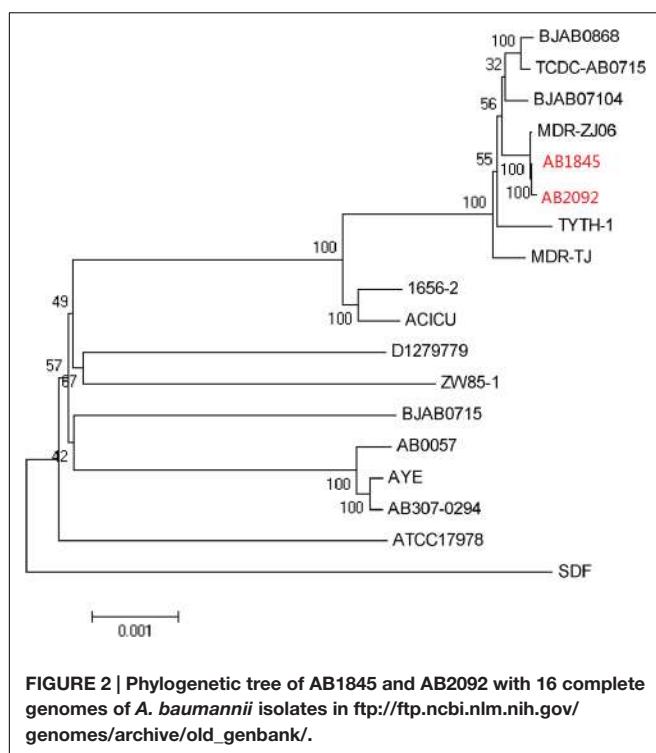


FIGURE 1 | The pharmacokinetic profiles of cefoperazone (A) and sulbactam (B). The dots in (A,B) are the concentration determined by LC-MS/MS (before the first infusion on Day 4 and 0.5, 1, 2, 4, 6 h after the infusion), and the curves are the profiles fitted to the population pharmacokinetic model. The red lines were indicated the concentration for the MIC of AB1845.



sequencing produced 12,050,346 and 12,191,576 pairs of 150-bp reads for AB1845 and AB2092, respectively. Assembly of the AB1845 and AB2092 genomes resulted in 132 and 131 contigs larger than 500 bp, comprising 4.0 megabases of sequence and representing a median 930-fold coverage. The AB1845 draft assembly has been deposited in GenBank (accession number LVYAO00000000), and raw sequence reads for AB1845 and AB2092 have been submitted to NCBI's Sequence Read Archive under the study accession number SRP072783.

Automated gene prediction detected 3,811 and 3,810 putative coding sequences (CDSs) for AB1845 and AB2092, respectively. A phylogenetic tree was constructed, and the genome similarity was compared (Figure 2). The 3,718 CDSs were homologous (defined as a BLASTN *e*-value $\leq 1e^{-10}$) to the genome of *A. baumannii* MDR-ZJ06, which was a multi-drug-resistant isolate belonging to International clone II and wide spreading in China (Zhou et al., 2011). MDR-ZJ06 was used as a reference genome for annotating genes in AB1845 and AB2092 (Figure 3). The results showed that the two isolates possess 97.6% similarity to MDR-ZJ06, with 921 and 915 SNPs, respectively. Certain genes were missing in both AB1845 and AB2092 compared with MDR-ZJ06. For example, 17 genes had a 15-kb fragment that was missing in the resistant island AbaR22, which includes transposition helper proteins, site-specific tyrosine recombinase, transposase protein A and conserved hypothetical proteins, among others.

The CDS of AB1845 was also mapped to the published plasmid sequence. It showed that certain AB1845 genes mapped to plasmid ABKp1 from the *A. baumannii* 1656-2 plasmid with 97.6% similarity.

A comparison of the CDS of AB1845 and AB2092 revealed only 33 SNPs. All of these SNPs were synonymous, indicating that SNP was not the main reason for the development of resistance (Supplementary Table S1).

β-Lactamase and Gene Amplification

A well-known resistance enzyme for β-lactam antibiotics is β-lactamase. The class A, C, and D classes (*bla*_{OXA-23} and *bla*_{OXA-51}) β-lactamase were identified in both AB1845 and AB2092 (Supplementary Table S2).

Gene amplification was detected by whole-genome sequencing in combination with the read depth method (Cantsilieris et al., 2012). Approximately 99.9% of the genes had a coverage ratio of AB2092 to AB1845 of approximately 1.0, but six genes (out of 3871 genes) had a coverage ratio over 3.0 (Supplementary Table S3). These six genes were AAA ATPase, DEAD/DEAH box helicases, *bla*_{OXA-23}, and three hypothetical genes. Among these genes, AAA ATPase and DEAD/DEAH box helicases are involved in the function of DNA replication, repair, transcription, and two uncharacterized genes (Johnson and McKay, 1999; Iyer et al., 2004). The *bla*_{OXA-23} was gene encoded class D β-lactamase and conferred resistance to carbapenems and cefoperazone (Pascale and Wright, 2010; Bush, 2013). All six genes were located in a 6-kb sequence fragment in AB1845 designated as *Tn2009*. *Tn2009* was flanked by two *ISAbal* elements that contributed to the duplication of *Tn2009* in AB2092 (Liu et al., 2015). The amplified genes were transcribed in the same orientation (Figure 4).

mRNA Level of *bla*_{OXA-23} in AB1845 and AB2092

Quantitative real-time PCR was conducted to measure the mRNA levels of *bla*_{OXA-23} in AB1845 and AB2092. Both isolates were collected at the early log phase, and the qPCR results showed that the mRNA level of *bla*_{OXA-23} was five times higher in AB2092 than in AB1845 (Figure 5). This finding was consistent with the gene amplification results.

DISCUSSION

Acinetobacter baumannii commonly colonizes in respiratory tract of the hospitalized patients (Peleg et al., 2008). It is difficult to characterize *A. baumannii* isolated from sputum belongs to the upper airway colonization or the causative pathogen of pneumonia. In this study, the two isolates were considered causative pathogens, as verified by pneumonia testing and symptoms such as cough, fever, and high white blood cell counts. To examine the mechanism of resistance development during cefoperazone/sulbactam treatment, both PK/PD indices and genomic sequencing were employed to understand the problem from a PK/PD and molecular perspective.

Exposure to a sub-optimal antibiotic concentration is the most important factor in the development of bacterial resistance. To

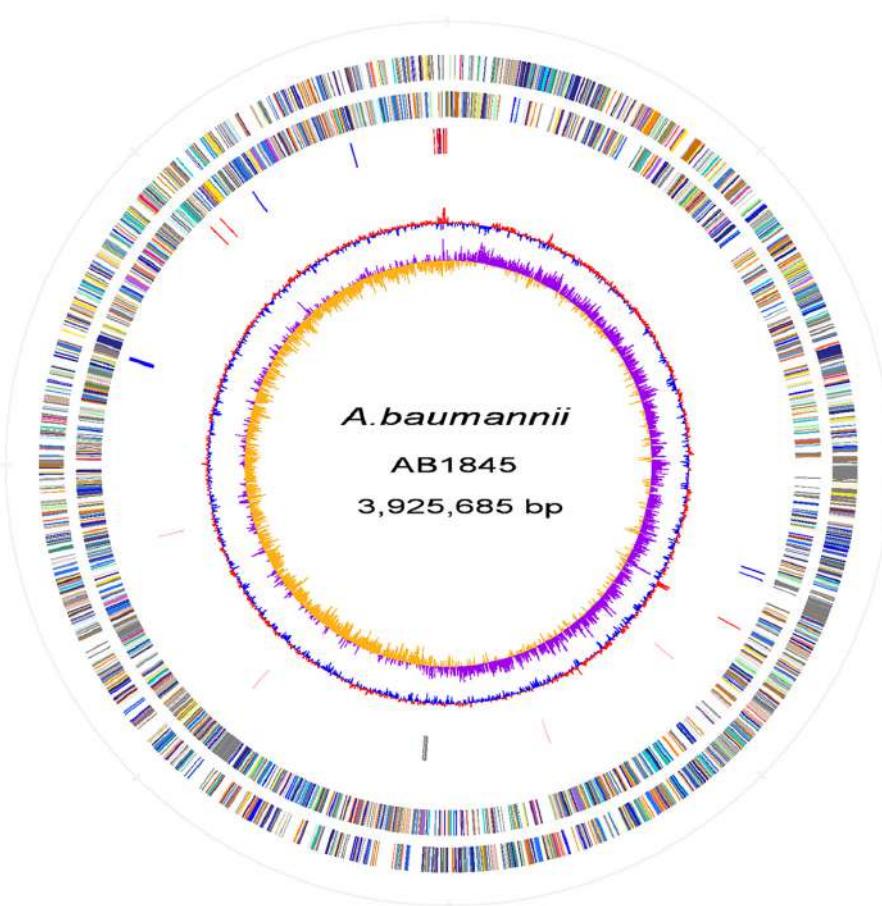


FIGURE 3 | Atlas of the *A. baumannii* AB1845 draft genome. Each concentric circle represents the genomic data for AB1845. The two outer circles illustrate the predicted coding sequences on the plus and minus strands, respectively, colored by functional categories according to the COG classification. The third circle represents the location of the nucleotide substitution between AB1845 and AB2092, with blue representing genes affected by synonymous Single Nucleotide Polymorphisms (SNPs) and red representing SNPs in the intergenic region. The fourth circle displays the loci of the β -lactamase genes (pink) and genes around *blaOXA-23* (gray). The fifth circle shows the GC content, and the 6th circle (innermost) represents GC skew ($G-C/(G+C)$) calculated using a 1-kb window.

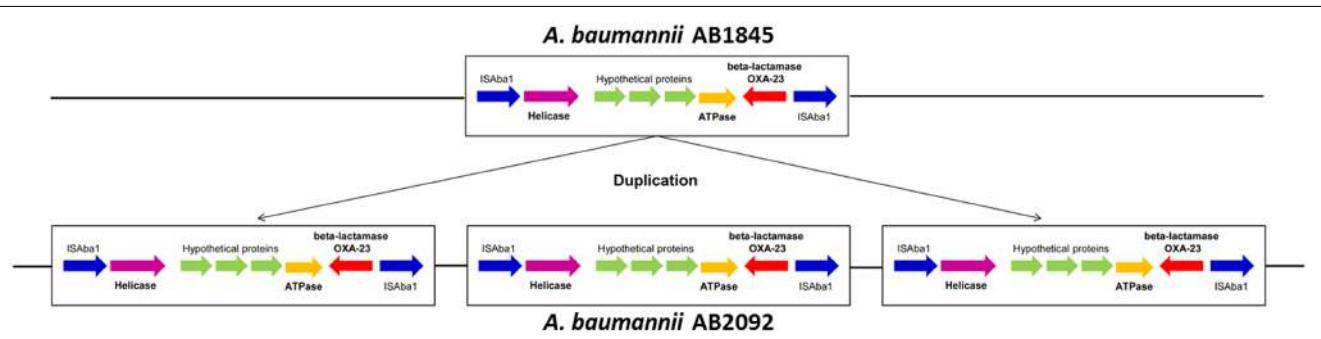


FIGURE 4 | The illustration of the 6-kb DNA fragment of *Tn2009* in AB1845 and AB2092. The fragment was amplified three times in AB2092.

optimize the antibiotic dosage regimen and prevent resistance, the PK/PD which bridges drug exposure and effect, has been widely applied in the clinics (Abdulaziz et al., 2015; Asín-Prieto et al., 2015; Monogue et al., 2015). To date, the PK/PD helps adjust dosage based on the evaluation of steady-state

drug concentrations (Martinez et al., 2012). In this study, blood samples were collected at steady state, and the MIC of the bacteria was determined. As the most predictive PK/PD index, the $\%T_{\geq MIC}$ of cefoperazone and sulbactam was calculated and compared with published PK/PD targets. The published

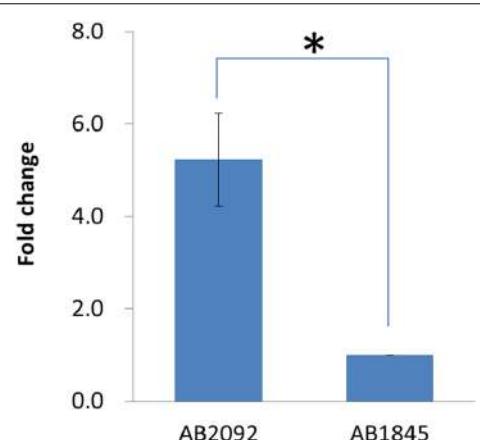


FIGURE 5 | Quantitative real-time PCR results for *bla*_{OXA-23} in AB1845 and AB2092 (*student t-test $p < 0.05$).

target %T_{> MIC} of β -lactam antibiotics was 40–70% (Drusano, 2004); however, there was no published target of sulbactam because of a lack of pharmacodynamic data. In our present dosage regimen (3 g q8h, infusion for 1 h), the target was 100% of cefoperazone and 34.5% of sulbactam for AB1845. Cefoperazone clearly reached its target, whereas there were no criteria for sulbactam. In a study of PK/PD simulation for sulbactam in healthy volunteers, in order for *A. baumannii* with an MIC of 8 mg/L to achieve the target of 40%, the sulbactam had to be administered as a 4-h infusion of 3 g q8h (Jaruratanasirikul et al., 2013). It has even been suggested that the daily dose of sulbactam can be administered up to 6 g for *A. baumannii* infection (Argyres, 2010) to achieve enhanced efficacy. A comparison of these data revealed that sulbactam should be administered at a much higher dose or prolonged infusion time. A suboptimal dose of sulbactam could lead to the development of resistance during treatment. Additional research to identify the PK/PD target of sulbactam specific to *Acinetobacter* is needed.

Whole-genome sequencing was further employed to investigate the mechanism of resistance from a molecular perspective. Comparison of the genome between AB1845 and AB2092 revealed no gene deletions or mutations (only 33 synonymous SNPs). However, the reads depth method showed six amplified genes on *Tn2009*, including *bla*_{OXA-23}. The *bla*_{OXA-23} gene encodes the class D β -lactamase which confers resistance to the last resort carbapenem antibiotics. The OXA-23-producing *A. baumannii* is widely disseminated in multi-drug-resistant *A. baumannii*. Although the *bla*_{OXA-23} gene has been mainly identified on plasmids, its chromosomal location has also been reported (Zhou et al., 2011). The transportable elements, *Tn2006*, *Tn2007*, *Tn2008*, and *Tn2009*, play a key role in the transfer of the *bla*_{OXA-23} gene to different locations in one bacterium or to different isolates (Corvec et al., 2007; Liu et al., 2015). *Tn2008* and *Tn2009* have mostly been associated with the transfer of *bla*_{OXA-23} in China (Adams-Haduch et al., 2008; Zhou et al., 2011; Liu et al.,

2015). IS elements are associated with the genome gain/loss of genes, especially resistant genes. These two transposons share ISAbal upstream from *bla*_{OXA-23}, which belongs to the IS4 family, provides promoter for *bla*_{OXA-23} (Turton et al., 2006; Mugnier et al., 2009), which could explain the gene duplication or amplification in this study. The mRNA level of *bla*_{OXA-23} confirmed the higher expression level of AB2092 and AB1845. There is evidence that gene duplication and amplification in bacteria was directly associated with adaptation to environmental changes, including antibiotic stress (Sandegren and Andersson, 2009). In a study testing the relationships of gene amplification and β -lactams resistance increase showed that 70% colonies of *S. Typhimurium* with increased resistance to cephalosporin had increased *bla*_{TEM} gene copy numbers (Sun et al., 2009). Recent study showed the heteroresistance of *E. Coli* and *S. Typhimurium* was due to the amplification of *pmrD* gene which encoded a protein modifying lipid A. Moreover, the heteroresistance phenotype is associated with different copy numbers of the *pmrD* gene (Hjort et al., 2016). Numerous studies have reported gene duplication and amplification related to bacterial resistance to antibiotics *in vitro* (Bertini et al., 2007). During the macrolide treatment of a *Streptococcus pneumoniae* infection, the bacterium developed resistance due to an 18 bp duplication in the *rplV* gene, leading to the treatment failure. The duplication of *rplV* gene led to the blocking of macrolide binding site on ribosome (Musher et al., 2002). Another example of gene amplification in bacterial isolates from human patients involves the *Streptococcus agalactiae*, a leading cause of neonatal infection. The amplification of a four-fold tandemly amplified 13.5-kbp region and a 92 kbp duplication conferred drug resistance to both sulfonamide and trimethoprim (Brochet et al., 2008).

In this study, the isolates developed resistance to cefoperazone/sulbactam as well as carbapenem antibiotics. The whole-genome sequencing showed the amplified *bla*_{OXA-23} in the isolates is very likely the contribution to the resistance. It is rational to conferred resistance to carbapenem antibiotics by the amplification of *bla*_{OXA-23}. However, this is the first time to report the amplification of *bla*_{OXA-23} results in cefoperazone/sulbactam combination resistance. Further studies will be focused on collecting more clinical isolates pairs of *A. baumannii* to support the mechanism proposed.

AUTHOR CONTRIBUTIONS

XL designed the genomic sequencing experiments. XL and HZ performed the data analysis. XL and ZS conducted the qPCR experiments. YC conducted the pharmacokinetic parameter calculation. LS extracted the genomic DNA for sequencing. MZ conducted the MIC test and the PFGE test. JS and JZ conceived of the study and devised the overall study strategy. XL wrote the manuscript with input from all authors.

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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.2016.01268>

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Functional categorization of carbapenemase-mediated resistance by a combined genotyping and two-tiered Modified Hodge Test approach

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The functional relationship between the detection of carbapenemase activity and phenotypic resistance in Gram-negative bacterial pathogens is often ill-defined. To address this issue, we developed a two-tiered Modified Hodge Test approach for carbapenemase detection and typing, in which the use of *Pseudomonas aeruginosa* strain PAO1 and *Escherichia coli* as indicator strains conferred two levels of sensitivities to carbapenemases. When applied alongside PCR genotyping tests for existence of known carbapenemase genes in 92 carbapenem resistant clinical isolates, this method is extremely useful in elucidating the relative role by which different enzymes contributed to the prevalent carbapenem-resistance phenotypes. With this study approach, we showed that the proportion of *P. aeruginosa* and *Acinetobacter baumannii* strains whose carbapenem resistance phenotypes could at least be partially attributed to carbapenemase were 34 and 89%, respectively. Our data also facilitates detailed functional categorization of carbapenem resistance phenotypes on the basis of the types and activities of detectable carbapenemase produced by the test organism. For example, six *A. baumannii* isolates harboring the *bla*_{OXA-51/23}-like gene without detectable enzymatic activities were identified, suggesting that other resistance mechanisms may be involved. On the other hand, there were seven *P. aeruginosa* strains which produced carbapenemase phenotype without harboring known carbapenemase genes, inferring the existence of some hitherto unknown resistance determinants. Findings in this work therefore provide a comprehensive view on the cellular basis of carbapenem resistance phenotypes in major Gram-negative bacterial species, paving the way for development of novel strategies to reverse the effects of the major resistance mechanisms concerned.

Keywords: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, carbapenem resistance, two-tiered Modified Hodge Test, genotyping

Introduction

Dissemination of carbapenem-resistant organisms continues to cause an increasing number of severe and often untreatable bacterial infections in nosocomial settings (Woodford et al., 2014). Production of active carbapenemase is a major carbapenem resistance mechanism among clinical Gram-negative isolates. There are several major classes of carbapenemase, including Class A serine carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC), Class B metallo- β -lactamase such as New Delhi metallo- β -lactamase (NDM), Verona integron-encoded metallo- β -lactamase (VIM), IMP, and Carbapenem-hydrolyzing Class D β -lactamases (CHDL) such as OXA-48, OXA-23, OXA-24, etc. Nordmann et al. (2011). In addition, OXA-51 has been reported to be intrinsic to *Acinetobacter baumannii* and it normally does not lead to carbapenem resistance unless an Insertion Sequence, ISAbal, is introduced into its upstream region, enhancing the strength of the promoter and causing over-expression of the *oxa-51* gene. Even if this happens, it has been reported that the CHDL OXA-51 produced exhibits only weak carbapenemase activity (Turton et al., 2006). Determination of the presence of carbapenemase activity is pivotal for clinicians to devise a suitable treatment regimen. To date, several enzymatic- and inhibitor-based phenotypic assays have been developed for detection of bacterial carbapenemase activities. Enzymatic assays, including Modified Hodge Test (MHT) and Carba NP test, involve the direct observation of carbapenemase hydrolysing phenotype in the presence of an indicator organism or a pH indicator (Nordmann et al., 2012b). Inhibitor assays which include commercially available E test MBL Strip (Nordmann et al., 2012a) and the Imipenem/EDTA inhibition test, deploy chemicals such as EDTA, a metal chelator, to inhibit enzyme activity, and are mainly used for detecting Metallo- β -lactamase (MBL) activity. Above all, MHT is a recommended method for identifying carbapenemase producers (CLSI, 2013). However, this method is not ideal for detection of CHDL, including OXA-51, OXA-23, and OXA-58 in *Acinetobacter* (Dortet et al., 2014). OXA-51 is intrinsic to *A. baumannii* and normally does not lead to carbapenem resistance, thus it is regarded as a less active carbapenemase, unless it is over-expressed by introduction of a strong promoter in its upstream region as a result of insertion of the insertion sequence ISAbal (Turton et al., 2006). Detection of other CHDLs such as OXA-23 and OXA-58 in *A. baumannii* has always been a challenge due to the low membrane permeability of the organisms (Dortet et al., 2014). Failure to detect carbapenemases produced by clinical strains is a serious concern since false categorization of the corresponding resistant organisms may result in wrong diagnosis and ineffective treatment. Although performance of *Pseudomonas aeruginosa* as an indicator strain in MHT to detect MBLs was found to be unsatisfactory previously due to the low specificity of this method and its high tendency to produce intermediate results (Pasteran et al., 2011), the suitability of this test approach to detect CHDLs has not been evaluated. In this work, replacement of *Escherichia coli* ATCC25922 by *P. aeruginosa* PAO1 as the indicator strain was found to significantly improve sensitivity in detection of less active carbapenemase producers. We showed that combined analysis of

results of carbapenemase detection tests with MHT and PAO1-MHT, which cover detection sensitivity toward CHDLs and other carbapenemases, as well as those of PCR genotyping of carbapenemase genes, allows highly accurate assessment of the type and relative role of the carbapenemase involved in conferring the observable phenotype. This analytical approach shall therefore help better define the molecular basis of clinical carbapenem resistance and establish a profile of carbapenemase-encoding elements commonly harbored by each of the key Gram-negative pathogens. Such data are essential for future development of novel antimicrobial strategies, especially those involved in carbapenemase inhibition.

Materials and Methods

Bacterial Isolates

A total of 102 clinical isolates were collected from patients in The Prince of Wales Hospital, Hong Kong ($n = 27$), and Henan Provincial People's Hospital, Henan, China ($n = 75$). The collection comprised *A. baumannii* ($n = 68$), *P. aeruginosa* ($n = 32$), *Enterobacter cloacae* ($n = 1$) and *E. coli* ($n = 1$). Four *P. otitidis* isolates recovered from food products were also included as control (Table 1). The isolates were identified by the Vitek Bacterial Identification System, followed by 16S rRNA sequencing.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility assay was performed and interpreted according to the CLSI guidelines (CLSI, 2013). Antimicrobial susceptibility testing was carried out by the agar dilution method with meropenem and imipenem as the test agents. Results were interpreted following the CLSI guidelines (CLSI, 2013). *E. coli* strains ATCC 25922, 35218, and *P. aeruginosa* strain ATCC 27853 were used as quality control.

PCR Detection of Carbapenemase Genes

Possession of carbapenemase genes by isolates was investigated by PCR as described previously (Woodford et al., 2006; Poirel et al., 2011; Thaller et al., 2011; Lopes et al., 2012). The PCR assays included detection of *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-51}, *bla*_{OXA-58}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{OXA}, *bla*_{SIM}, *bla*_{KPC}, *bla*_{SPM}, *bla*_{DIM}, *bla*_{GIM}, *bla*_{NDM}, and *bla*_{POM}. Primers used in this study are shown in Table 1. Genomic DNA was obtained by using the Purelink Genomic Mini Kit (Life Technologies). 1 μ l of DNA template was added into PCR reaction mix (1X PCR Buffer, 3 mM MgCl₂, 0.2 mM dNTP, 0.25 μ M primer, 1U Takara rTaq). The assays were validated by including a pET15B vector carrying target constructs as positive control, and amplicons were subjected to sequencing.

Two-Tiered Modified Hodge Test

Modified Hodge Test for meropenem was performed on all isolates as described using the *E. coli* strain ATCC25922 and *P. aeruginosa* strain PAO1 as indicator organisms (designated as MHT and MHT-PAO1, respectively), and a *bla*_{IMP}-producing *E. coli* strain as positive control. Meropenem susceptibility testing disks were obtained from Oxoid, and Mueller-Hinton Agar

TABLE 1 | Primers used in this study.

β -lactamase	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon size (bp)	Reference
<i>bla</i> _{OXA-51}	TAATGCTTGATCGGCCTTG	TGGATTGCACTTCATCTTGG	353	Woodford et al. (2006)
<i>bla</i> _{OXA-23}	GATCGGATTGGAGAACAGA	ATTTCTGACCGCATTTCCAT	501	
<i>bla</i> _{OXA-24}	GGTAGTTGGCCCCCTAA	AGTTGAGCAAAAGGGGATT	246	
<i>bla</i> _{OXA-58}	AAGTATTGGGCTTGTGCTG	CCCCTCTGCGCTCACATAC	599	
<i>bla</i> _{IMP}	GGAATAGTGGCTTAAYTCTC	GGTTTAAAYAAAACAACCACC	232	Poirel et al. (2011)
<i>bla</i> _{VIM}	GATGGTGTGGTCGCATA	CGAATGCGCAGCACCG	390	
<i>bla</i> _{OXA}	GCGTGGTTAAGGATGAACAC	CATCAAGTTCAACCCAACCG	438	
<i>bla</i> _{SIM}	TACAAGGGATTGGCATCG	TAATGGCCTGTTCCCATGTG	570	
<i>bla</i> _{KPC}	CGTCTAGTCTGCTGTCTTG	CTTGTACATCCTGTTAGGCG	798	
<i>bla</i> _{SPM}	AAAATCTGGTACGCAAACG	ACATTATCCGCTGGAACAGG	271	
<i>bla</i> _{DIM}	GCTTGTCTCGCTTGCTAACG	CGTTGGTGGATTGATTG	699	
<i>bla</i> _{GIM}	TCGACACACCTTGGTCTGAA	AACTTCCAACTTGCCATGC	477	
<i>bla</i> _{NDM}	GGTTTGGCGATCTGGTTTC	CGGAATGGCTCATCACGATC	621	
<i>bla</i> _{POM}	ACGTCGCTGATGCTCAG	CCTGCGTCATCAGAGACCTC	1143	Thaller et al. (2011)

(MHA) was obtained from BD. Indicator organisms and test isolates were grown overnight on MHA. Bacterial suspension of indicated organism at 0.5 McFarland Standard was prepared by saline and spread on MHA plates on which meropenem disks (10 μ g, Oxoid) were placed at center. Inoculated plates were allowed to dry for 10 min followed by streaking a line of test strains with a 10 μ l inoculation loop from the drug disk to the edge of agar plate. Results were recorded after 18 h incubation at 37°C. Production of active carbapenemases was regarded as positive upon observation of “clover-leaf” appearance due to enhanced growth of indicator strain toward the meropenem disk alongside the test strain.

Results and Discussion

A total of 106 strains (102 clinical isolates and four food isolates) were first subjected to antibiotic susceptibility tests. Among the 68 *A. baumannii* isolates tested, 52 were resistant to meropenem and imipenem. The rest of the test strains, including 32 *P. aeruginosa* isolates, four *P. Otitis* isolates, one *Enterobacter cloacae* strain, and one *E. coli* strain, were all carbapenem resistant. PCR genotyping experiments showed that known carbapenemase genes were not detectable in as many as 28 carbapenem resistant *P. aeruginosa* strains. The other four strains were found to contain the *bla*_{IMP} (three strains) and *bla*_{KPC} genes (one strain), respectively. MHT tests were then performed to assess the degree of correlation between existence of carbapenemase genes and the detection of carbapenemase activity, with results indicating that the carbapenemase *bla*_{IMP} was detectable by both MHT tests, yet the *bla*_{KPC} enzyme was detectable by MHT-PAO1 only (**Figure 1**). Despite such discrepancy, the four host strains concerned all exhibited high level meropenem resistance ($\geq 32 \mu\text{g}/\text{ml}$), suggesting that active carbapenemase production might not be the sole basis of carbapenem resistance in these organisms. Likewise, 5 of the 28 *P. aeruginosa* strains which did not harbor any known carbapenemase gene were found to

produce a carbapenemase phenotype detectable by MHT-PAO1 only; in addition, another two strains were found to produce an enzyme detectable by both MHT tests (**Figure 1**). Although false positives have been described in MHT to detect carbapenemases in *P. aeruginosa* previously, it can not rule out the possibility that other unknown carbapenem resistance mechanisms are present in these seven strains (Vasoo et al., 2013). **Table 2** summarizes the results of genotyping, MHT tests and antibiotic susceptibility levels of the 106 strains studied in this work. For carbapenem-resistant *P. aeruginosa*, MHT-POA1 can detect 100% (4/4) of known carbapenemase producers and 25% (7/28) of isolates without known carbapenemase genes, while MHT can detect 75% (3/4) of known carbapenemase producers and 7% (2/28) of isolates without known carbapenemase genes.

Of the 68 *A. baumannii* isolates tested, 17 were found to contain the intrinsic *bla*_{OXA-51}-like CHDL-producing gene only, among which were the 14 carbapenem-sensitive strains. Interestingly, the ISAbal element was detectable in the upstream region of a *bla*_{OXA-51}-like gene in the other three of these 17 strains, which were also found to produce a carbapenemase detectable only by MHT-PAO1, corroborating with the fact that the ISAbal element is essential for over-expression of the *bla*_{OXA-51}-like gene. The remaining 51 isolates were found to harbor other carbapenemase-producing genetic elements, with the vast majority (49 strains) containing the *bla*_{OXA-51/23}-like gene. Among these 49 strains, whose meropenem MIC ranged from 8 to $\geq 32 \mu\text{g}/\text{ml}$, 16 were positive in both MHT tests, 27 were positive in the MHT-PAO1 only, and six strains did not produce any detectable carbapenemases (**Figure 1**). These findings appear to suggest that *bla*_{OXA-51/23}-like gene product may not be the sole factor responsible for the development of the carbapenem resistance phenotypes of the organisms concerned.

The other two of the 51 isolates which harbored carbapenemase genes included one highly resistant strain (meropenem MIC $\geq 32 \mu\text{g}/\text{ml}$) which contained a *bla*_{OXA-51/24}-like element. However, this strain was tested positive only in MHT-PAO1, again suggesting the involvement of non-carbapenemase

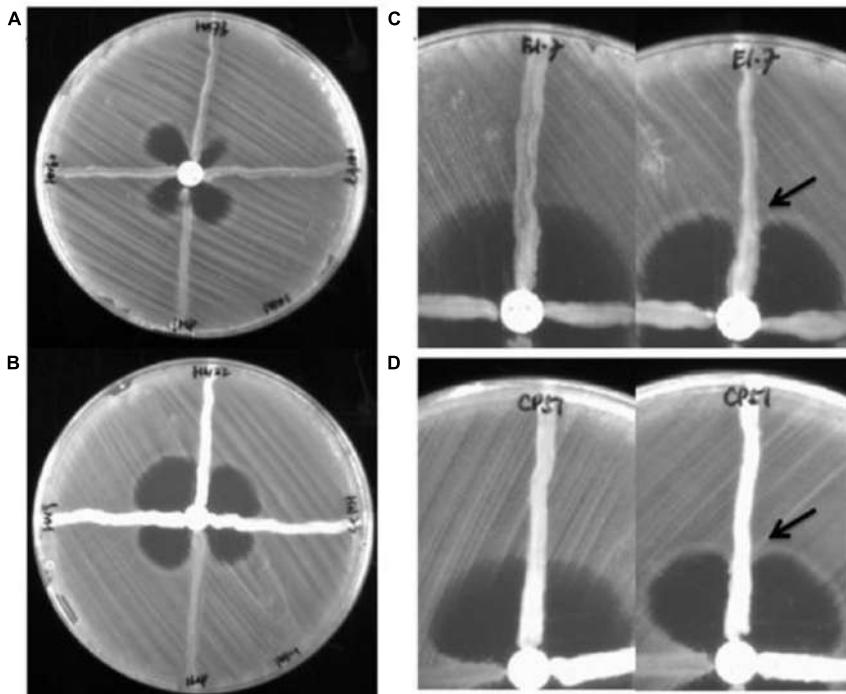


FIGURE 1 | Result of Modified Hodge Test for representative isolates. **(A)** Modified Hodge Test (MHT-PAO1 of three *bla_{IMP}*-borne *Pseudomonas aeruginosa* strains (left, upper, right) with an *Escherichia coli* strain harboring the *bla_{IMP}* gene as positive control (bottom). **(B)** MHT-PAO1 of three *bla_{OXA-23}*-borne *Acinetobacter baumannii* strains

(left, upper, right) with an *E. coli* strain harboring the *bla_{IMP}* gene as positive control (bottom). **(C)** MHT (Left) and MHT-PAO1 (right) of a *bla_{POM}*-borne *P. otitidis* strain (E1-7). **(D)** MHT (Left) and MHT-PAO1 (right) of a *bla_{OXA-23}*-borne *A. baumannii* strain (CP51). Arrows indicate enhanced growth of indicator organism.

mechanisms. Likewise, another strain which contained both the *bla_{OXA-51}*-like and *bla_{NDM}* genes, exhibited a meropenem MIC of $\geq 32 \mu\text{g/ml}$, which is significantly higher than that observed in the three strains containing the *bla_{OXA-51}*-like gene alone, plus an ISAbal element ($8 \mu\text{g/ml}$). Such discrepancy highlighted the additive effects of different enzymes on the strength of phenotypic carbapenem resistance. It should be noted that the phenomenon of high level carbapenem resistance and low level enzyme production was also observable in a meropenem-resistant *E. coli* strain harboring the *bla_{NDM}* gene in this study, as well as several *E. coli* and *K. pneumoniae* isolates described previously (Girlich et al., 2012). This phenomenon suggests the possibility that the level of carbapenem resistance exhibited by a given clinical isolate is more closely related to the type rather than level of carbapenemase produced by the organism. Alternatively, these findings could also indicate that the *bla_{NDM}* enzyme might only confer relatively low level carbapenem resistance, and other factors including altered membrane permeability and expression of efflux may be responsible for the high level carbapenem resistance phenotypes exhibited by the host strains concerned. This possibility is also raised by the finding that the meropenem MIC of an *Enterobacter cloacae* strain, which harbored the *bla_{NDM}* and *bla_{KPC}* genes and exhibited a positive result in both MHT tests, was only $16 \mu\text{g/ml}$. On the other hand, *bla_{POM}*, encoding an intrinsic Metallo- β -Lactamase, was detected in all the four *P. otitidis* isolates (Table 2). This enzyme, which was consistently

detectable by the MHT-PAO1 test only, was associated with a meropenem MIC of $16-\geq 32 \mu\text{g/ml}$. These findings suggest a need to investigate the role of each specific carbapenemase in conferring carbapenem resistance in the host strain. In summary, within 60 isolates of carbapenem-resistant *P. otitidis*, *h. coli*, and *K. pneumoniae*, 90% (54/60) were positive for MHT-PAO1 test, while only 26% (16/60) were positive for MHT testing.

In this work, MHT-PAO1 yielded 63 carbapenemase-positive cases whereas MHT yielded 22 positive cases, with MHT-PAO1 being much more sensitive in detecting CHDL in *A. baumannii*, as well as the *bla_{NDM}* enzyme. Detection of CHDLs produced by *Acinetobacter* by MHT has always been a challenge due to the relative weak activity of enzymes and low membrane permeability of the organism. Unsatisfactory CHDL detection by MHT, including those produced by *bla_{OXA-23}*, *bla_{OXA-24}*, and *bla_{OXA-58}*, has been documented (Bonniin et al., 2012). Previous studies have also shown that false negative results of MHT in detection of *bla_{NDM}* in *Acinetobacter* and *Enterobacteriaceae* were not uncommon, which may be due to the variation in zinc content in MHA produced by different suppliers (Pasteran et al., 2011; Girlich et al., 2012). Although performance of *P. aeruginosa* ATCC27853 as indicator strain in MHT to detect certain *P. aeruginosa* MBLs has been attempted by another group which reported a low sensitivity and specificity (Pasteran et al., 2011), our data confirmed that the use of MHT-PAO1 can successfully identify the enzymes encoded by *bla_{IMP}*, *bla_{NDM}*, and

TABLE 2 | Summary of genotypic and phenotypic characteristics of 106 Gram-negative bacterial isolates tested in this study.

Bacterial species tested (Carbapenemases)	Meropenem MIC(µg/ml)	No. of isolates	No. of positive Modified Hodge Test (MHT)	
			<i>Pseudomonas aeruginosa</i> PAO1	<i>Escherichia coli</i> ATCC25922
<i>P. aeruginosa</i>				
blaIMP	≥32	3	3	3
blaKPC	≥32	1	1	0
Not Detected	16 – ≥32	28	7	2†
<i>P. aeruginosa</i>				
blaPOM	16 – ≥32	4	4	0
<i>Acinetobacter baumannii</i>				
blaOXA-51like	<0.5	14*	0	0
ISAbA1 + blaOXA-51like	8	3	3	0
blaOXA-51/23like	8 – ≥32	49	43	16†
blaOXA-51/24like	≥32	1	1	0
blaOXA-51like + blaNDM	≥32	1	1	0
<i>Enterobacter cloacae</i>				
blaNDM + blaKPC	16	1	1	1
<i>E. coli</i>				
blaNDM	16	1	1	0
Total		106	63	22

*Carbapenem-susceptible. †Also positive in MHT-PAO1 test.

TABLE 3 | Categorization of carbapenem susceptibility phenotypes.

Category	Carbapenem susceptibility phenotypes	MHT (<i>E. coli</i>)	MHT- PAO1	PCR- genotyping	Implication	Examples
I	S	–	–	+	The carbapenemase gene detectable by genotyping does not produce carbapenemase	14 carbapenem sensitive <i>A. baumannii</i> strains harboring the blaOXA-51 like gene
II	R	–	–	–	Carbapenem resistance not due to carbapenemase	21 carbapenem resistant <i>P. aeruginosa</i> strains
III	R	+/-	+	–	Carbapenem resistance due to a combination of resistance mechanisms including an unknown carbapenemase or overexpression of AmpC	Seven <i>P. aeruginosa</i> strains
IV	R	+	+	+	Carbapenem resistance due to carbapenemase encoded by the gene detectable by genotyping	Various <i>P. aeruginosa</i> and <i>A. baumannii</i> strains
V	R	–	+	+	Carbapenem resistance due to a combination of resistance mechanisms including a weakly detected carbapenemase	<i>P. aeruginosa</i> and <i>A. baumannii</i> strains with a meropenem MIC ≥32 µg/ml
VI	I	–	+	+	Carbapenem resistance due to a weakly detected carbapenemase encoded by the gene detectable by genotyping	Three <i>A. baumannii</i> strains harboring the ISAbA1+ blaOXA-51like elements

Categorization according to results of the two-tiered MHT tests and genotyping of carbapenemase genes. S, carbapenem sensitive; R, carbapenem resistant; I, intermediate or low level carbapenem resistance.

blaPOM in *P. aeruginosa*, *E. coli*, and *P. aeruginosa*, respectively. Currently, testing carbapenemase activity by MHT or other assays has been widely used to determine the carbapenem resistance potential of clinical Gram-negative bacterial pathogens to guide the treatment decision. False negative detection of CHDLs by traditional MHT may result in wrong diagnosis and ineffective treatment. The improved two-tiered MHT can achieve

much less false negative, around 9% (6/64) in isolates carrying known carbapenemase genes compared to 71% (45/64) by MHT test, suggesting the high reliability of the two-tiered MHT in the clinical screening of carbapenem-resistant Gram-negative pathogens in particular CHDL producers. If the discrepancy in results of conventional MHT and MHT-PAO1 observable in this study is indeed due to the low enzyme levels as discussed

above, future research efforts should be focused on investigating the underlying basis of high level carbapenem resistance in organisms producing less active carbapenemase.

Findings of this study allow us to systematically categorize the test strains according to their carbapenem susceptibility phenotypes and results of the MHT and genotyping tests. As shown in **Table 3** which lists the scenarios which give rise to at least six possible combinations of the phenotypic and genotypic test results, we can not only better define the relative role of each carbapenemase in the production of a resistance phenotype on the basis of the test results, but also identify strains whose resistance phenotypes were also due to other resistance mechanisms. For example, if a highly resistant strain with positive genotyping result is found to produce a weakly detected carbapenamase (positive in MHT-PAO1 test), it is likely that the resistance phenotype is due to multiple mechanisms including production of active carbapenemase. Here we showed that 34% of the clinical carbapenem resistant *P. aeruginosa* strains tested were at least partially due to carbapenemase, yet the corresponding rate for *A. baumannii* was as high as 89%. Detailed delineation of the molecular basis of carbapenem resistance in these Gram-negative pathogens, which were previously not possible to perform on the basis of a single phenotypic or molecular test, shall greatly facilitate development

of novel strategies to treat bacterial infections, especially those aimed at devising a carbapenemase inhibition approach.

Conclusion

A two-tiered MHT approach which conferred two levels of sensitivities for carbapenemase detection can be applied alongside PCR genotyping of known carbapenemase genes to determine the degree of contribution of specific carbapenemase in expression of phenotypic carbapenem resistance in Gram-negative bacterial pathogens. This analytical approach therefore facilitates categorization of carbapenem resistance phenotypes and identification of the major mechanisms concerned in bacterial species in which clinical carbapenem resistance is common.

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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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Antibiotic Resistance Patterns of *Pseudomonas* spp. Isolated from the River Danube

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Spread and persistence of antibiotic resistance pose a severe threat to human health, yet there is still lack of knowledge about reservoirs of antibiotic resistant bacteria in the environment. We took the opportunity of the Joint Danube Survey 3 (JDS3), the world's biggest river research expedition of its kind in 2013, to analyse samples originating from different sampling points along the whole length of the river. Due to its high clinical relevance, we concentrated on the characterization of *Pseudomonas* spp. and evaluated the resistance profiles of *Pseudomonas* spp. which were isolated from eight sampling points. In total, 520 *Pseudomonas* isolates were found, 344 (66.0%) isolates were identified as *Pseudomonas putida*, and 141 (27.1%) as *Pseudomonas fluorescens*, all other *Pseudomonas* species were represented by less than five isolates, among those two *P. aeruginosa* isolates. Thirty seven percent (37%) of all isolated *Pseudomonas* species showed resistance to at least one out of 10 tested antibiotics. The most common resistance was against meropenem (30.4%/158 isolates) piperacillin/tazobactam (10.6%/55 isolates) and ceftazidime (4.2%/22 isolates). 16 isolates (3.1%/16 isolates) were multi-resistant. For each tested antibiotic at least one resistant isolate could be detected. Sampling points from the upper stretch of the River Danube showed more resistant isolates than downriver. Our results suggest that antibiotic resistance can be acquired by and persists even in *Pseudomonas* species that are normally not in direct contact with humans. A possible scenario is that these bacteria provide a reservoir of antibiotic resistance genes that can spread to related human pathogens by horizontal gene transfer.

Keywords: JDS3, *Pseudomonas*, antibiotic resistance, water, Danube

INTRODUCTION

Multiresistant bacteria are present in many surface waters (Girlich et al., 2011; Czekalski et al., 2012; Tissera and Lee, 2013; Blaak et al., 2015; Maravic et al., 2015). Typically, evidence is provided through fecal indicators, and mostly relates to short river sections or sampling at individual points. Thus, investigations of whole water systems are rare, especially if the river

passes through 10 riparian countries, like the River Danube. Water samples from the third Joint Danube Survey (JDS3), the world's biggest river research expedition of its kind, offered a chance for evaluating resistance of bacteria over a whole river system. Based on these samples, a resistance profile of *Pseudomonas* spp. over the course of the multinational River Danube was created in our study.

Pseudomonas species can be naturally found in all surface waters, lakes and rivers, but they are rarely found in drinking water. *Pseudomonas* spp. can survive in both low and high nutrition environments (Mena and Gerba, 2009) or even in double distilled water and, in addition, can help *Salmonellae* survive in this environment (Warburton et al., 1994). The whole group of non-fermenting Gram negative bacilli is suspected of establishing the basis for multiresistance in Gram negative bacteria, as the members of this group carry multiple intrinsic resistances and have the ability to acquire and evolve additional resistances (Farinas and Martinez-Martinez, 2013). *Pseudomonas* species are known to harbor multiple intrinsic and acquired resistance genes, host several mobile genetic elements, and also exchange them with other families of Gram negative bacilli like *Enterobacteriaceae* (Juan Nicolau and Oliver, 2010; Pfeifer et al., 2010). Hence *Pseudomonas* are known starting points of several important carbapenemases families (Pfeifer et al., 2010). The occurrence and spread of carbapenemases have become a substantial global health problem, as they inactivate a substantial antibiotic class.

The most common pathogen in this genus is *Pseudomonas aeruginosa*. It causes a variety of different infections, from easy-to-cure ear infections, serious infections of burn patients, to severe lung infections which lead to major complications in cystic fibrosis patients (Barbier and Wolff, 2010; Azzopardi et al., 2014). Besides *Pseudomonas aeruginosa*, other species e.g., *Pseudomonas putida* or *Pseudomonas fluorescens* are also a cause for infections in clinical settings (Gilarranz et al., 2013; Erol et al., 2014; Bhattacharya et al., 2015; Mazurier et al., 2015).

The aim of the study was to evaluate the resistance profiles of *Pseudomonas* spp. isolated at selected sites along the whole course of the River Danube. *Pseudomonas* spp. were chosen for various reasons: they belong to the native bacterial community in surface waters, they are clinically relevant, and changes in their natural resistance profiles indicate anthropogenic influence. This study, therefore, aims at monitoring the presence of resistances of *Pseudomonas* spp. to clinically important antibiotics along the river course. Doing so, changes in the resistance profiles were to be detected, if possible.

MATERIALS AND METHODS

Sample Collection

Samples were collected during the JDS 3, which was organized by the International Commission for the Protection of the Danube River (ICPDR). The ICPDR is a transnational body, which has been established to implement the Danube River Protection Convention. All Danube countries are member states of the ICPDR on the base of the "Convention on Cooperation for the

Protection and Sustainable use of the Danube River" (Danube River Protection Convention).

Between Aug. 12 and Sep. 26, 2013, surface water samples for microbiological investigations were collected from 68 sampling sites along the river Danube (JDS 3, 2015).

For each sampling site, water samples were taken at three sampling points, on the left, in the middle and on the right side of the River Danube. Samples were collected in sterile 1-L glass flasks, from 30 cm below the river surface (Figure 1, pink and violet dots, high resolution map is added as Supplementary Figure S1). Duplicate volumes of the samples (45 ml) were filled into sterile non-toxic 50 ml plastic vials (Techno Plastic Products AG, TPP, Switzerland), containing 5 ml glycerol (final conc. 10% v/v). The vials were shaken and turned around to homogenize glycerol and water and after that immediately stored at -20°C on board of the JDS3 research ship. After transfer to the home laboratory (beginning of October 2013) the samples were stored at -80°C. Out of the 68 sampling sites the four sites directly downstream from the cities Vienna, Budapest, Novi Sad, and Bucharest were chosen for investigation. In addition four non-city related sampling sites were chosen (including the delta and near the starting point of the JDS3; Table 1).

Isolation of Bacteria

The frozen samples were thawed and 15 ml (left, middle, right 5 ml each) were plated in 0.5 ml portions on different selective agars: Endo Agar, Xylose Lysine Deoxycholate Agar (XLD agar), and Chromocult Coliform Agar (CCA), (all Merck, Austria). Growth conditions were 37 ± 1°C for 18–24 h. Identification of *Pseudomonas* by MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) was performed as described previously (Jamal et al., 2014). A single bacterial colony was deposited on the target slide, followed by the addition of the matrix (VITEK MS-CHCA) and air drying. Samples were processed in the MALDI-TOF-MS spectrometer VITEK® MS (Biomerieux, Austria). Microbial identification was achieved by obtaining the spectra using MALDI-TOF technology and analyzing the spectra with the VITEK MS database. The peaks from these spectra were compared with the characteristic pattern for the species, genus or family of the microorganism, leading to identification of the organism.

Antibiotic Susceptibility Testing

For all identified *Pseudomonas* spp., antibiotic susceptibility testing was performed as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) including recommended controls. Inhibition zone diameters were interpreted according to EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/) (EUCAST, 2013; Matuschek et al., 2014). Classification of multiresistance of *Pseudomonas* spp. was evaluated according to the Robert Koch Institut (RKI, Germany, http://www.rki.de/EN/Home/homepage_node.html). The suspension for inoculation was prepared from an over-night pure culture on a blood agar (non-selective medium). Colonies were picked with



FIGURE 1 | Overview of the JDS3 sampling points along the river Danube. The map was taken with kind permission of the ICPDR. (<http://www.danubesurvey.org/results>).

TABLE 1 | Investigated sampling sites names and numbers according to JDS3, country and detailed location (ds = downstream, us = upstream).

Site	Name	Country	Location	E. coli [MPN/100ml]			
				rkm	Left	Middle	Right
JDS03	Geisling	Germany	ds Regensburg	2354	1728	1739	1304
JDS10	Wildungsmauer	Austria	ds Vienna	1895	1044	917	1739
JDS22	DS Budapest	Hungary	ds Budapest	1632	4320	6792	6310
JDS28	US Drava	Croatia/Serbia	us tributary Drava	1384	2445	1998	2880
JDS36	DS Tisa, Serbia	Serbia	ds Novi Sad	1200	11060	9900	4960
JDS59	DS Arges	Romania/Bulgaria	ds tributary Arges*	429	288000	720	1983
JDS63	Siret	Romania	Galati	154	7565	11851	7120
JDS68	St. Gheorge arm	Romania	river delta	104	4880	2424	2431

Basic microbiological parameters. MPN, most probable numbers; rkm, river kilometer. E. coli concentrations were determined according to ISO 9308-2 (JDS 3, 2015).

*Arges is the river flowing through Bucharest.

a sterile loop and suspended in sterile saline (0.85% NaCl w/v in water) to the density of a McFarland 0.5 standard (DensiCheck, Biomerieux, Austria). The suspension was then plated on Mueller-Hinton Agar by using an automatic plate rotator (Retro

C80, Biomerieux, Austria). Antibiotic test disks were applied firmly on the agar surface within 15 min of inoculation of the plates. Plates were incubated at 36°C for 16–20 h. After incubation, inhibition zones were measured.

TABLE 2 | EUCAST Clinical Breakpoints for *Pseudomonas* spp.

Antibiotic	Antibiotic concentration on test disk (μg)	Susceptible inhibition zone (mm)	Resistant (including intermediate) inhibition zone (mm)
Piperacillin/tazobactam	30/6	≥ 18	<18
Ceftazidime	10	≥ 16	<16
Cefepime	30	≥ 19	<19
Meropenem	10	≥ 18	<18
Imipenem	10	≥ 20	<20
Amikacin	30	≥ 24	<24
Gentamicin	10	≥ 15	<15
Tobramycin	10	≥ 16	<16
Ciprofloxacin	5	≥ 25	<25
Levofloxacin	5	≥ 20	<20

The following antibiotics were tested:

Piperacillin/tazobactam (TZP), ceftazidime (CAZ), cefepime (FEP), meropenem (MEM), imipenem (IPM), amikacin (AN), gentamicin (GM), tobramycin (NN), ciprofloxacin (CIP), levofloxacin (LEV), and sulfamethoxazole/trimethoprim (SXT) (all Becton Dickinson, Schwebach, Austria; **Table 2**).

SXT was evaluated because sulfamethoxazole was part of the chemical analysis of the River Danube water. There are no sulfamethoxazole/trimethoprim breakpoints according to EUCAST for *Pseudomonas* spp. To include the sensitivity of *Pseudomonas* spp. to sulfamethoxazole/trimethoprim, diameters of inhibition areas were evaluated and compared (SXT test discs specification: sulfamethoxazole/trimethoprim: 1.25/23.75 μg).

Modified Hodge Test

To estimate the presence of carbapenemases, a modified Hodge test was performed with all isolates resistant to at least one carbapenem. In brief: after plating of a carbapenem sensitive *Klebsiella pneumoniae* (ATCC 700603) a 10 μg imipenem or meropenem disc was placed in the center, and each test isolate was streaked from the disk to the edge of the plate. After incubation ($37 \pm 1^\circ\text{C}$ for 18–24 h) the plates were checked for showing a “cloverleaf shaped” inhibition zone. Isolates that produced carbapenemases enabled growth of the sensitive *Klebsiella* closer to the antibiotic disk (Bennett et al., 2009).

Data Analyses

Statistical analyses were calculated with GraphPadPrism™ 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS

Species Composition of Isolates

In total, 520 *Pseudomonas* spp. were isolated, the fewest isolates were obtained from JDS68 (32 isolates), and the highest number could be isolated from JDS28 sample with 117 isolates (**Table 3**). The most abundant *Pseudomonas* species were *Pseudomonas putida* (66.0%/344 isolates) and *Pseudomonas*

fluorescens (27.1%/141 isolates). Each of the other detected species represented less than 1% of all isolates (five or fewer isolates). Only two *Pseudomonas aeruginosa* were isolated, both from JDS28.

Antibiotic Resistances

Wild type *Pseudomonas* species are susceptible to all tested antibiotics except SXT (EUCAST, 2013). The highest number of resistances was the one against meropenem with 158 resistant isolates (30.4%), 55 isolates (10.6%) were resistant to piperacillin/tazobactam and 22 isolates (4.2%) resistant to ceftazidime, 11 (2.1%) to imipenem and four (0.8%) to cefepime (**Table 3**). In the fluoroquinolone group only six (1.2%) of the isolates showed levofloxacin resistance, whereas 18 (3.4%) isolates were resistant against ciprofloxacin. Resistance to ciprofloxacin turned out to be the most frequent resistance of all tested non beta-lactam antibiotics in this study. One isolate which was resistant to levofloxacin was still sensitive to ciprofloxacin. Resistance to aminoglycosides was very rare, two isolates were resistant to amikacin and gentamicin respectively and only one isolate showed no susceptibility to tobramycin (**Table 3**). The 11 isolates resistant to imipenem were also resistant to meropenem and were positive in the modified Hodge test, indicating carbapenemase activity. 327 (62.9%) isolates were susceptible to all tested antibiotics with EUCAST breakpoints (clinical resistance wild type), 128 (24.6%) isolates showed resistance to one, and 49 (9.4%) isolates were resistant to two tested antibiotics. The most common combination of resistances was to meropenem and piperacillin/tazobactam. Sixteen isolates revealed resistance to 3 or more antibiotics, including 12 (2.3%) isolates with three resistances, three (0.6%) isolates with four and one (0.2%) with five. Eight of them were classified as multi-resistant as they were resistant to three or four different antibiotic classes (**Table 4**).

The 12 isolates with three antibiotic resistances split in eight different resistance patterns, the isolates with four resistances all displayed the same pattern. The two *Pseudomonas aeruginosa* were susceptible to all tested antibiotics. Out of all other isolated species in this study at least one showed the EUCAST defined antibiotic susceptibility wild type, susceptible to all tested antibiotics (except SXT; **Supplementary Table S1**).

Four sampling points had less than 50% isolates with clinical resistance wild type, JDS03, JDS10, JDS22, and JDS59. Three sampling points revealed no isolate that was non-susceptible to three or more tested antibiotics, JDS10, JDS63 and JDS68 (**Table 4**).

Surprisingly, the upstream sampling points (JDS03, JDS10, JDS22, and JDS28) revealed higher proportions of resistant bacteria than the downstream ones (**Figure 2**). Sampling point JDS03 revealed the highest proportions of resistance to ceftazidime (28.6% of JDS03 isolates) and ciprofloxacin (25.7% of JDS03 isolates). Sampling point JDS22 showed high rates for piperacillin/tazobactam (32.6% of the JDS22 isolates), meropenem (69.6% of the JDS22 isolates), and imipenem (13.0% of the JDS22 isolates).

SXT was chosen for testing as sulfamethoxazole was measured directly during JDS3. Chemical analysis revealed a sampling

TABLE 3 | Number of isolated *Pseudomonas* spp. at the investigated sampling points (SP) and the number of resistant isolates to the testes antibiotic.

SP	TZP	CAZ	FEP	MEM	IPM	AN	GM	NN	CIP	LEV	No. Isolates
JDS03	3	10	1	15	0	0	1	0	9	1	35
JDS10	2	2	0	16	2	0	0	0	1	0	33
JDS22	15	5	0	32	6	1	0	0	2	0	46
JDS28	19	4	2	35	3	0	0	0	2	3	117
JDS36	6	0	0	10	0	1	1	1	1	1	109
JDS59	6	1	1	21	0	0	0	0	0	0	46
JDS63	3	0	0	21	0	0	0	0	2	1	102
JDS68	1	0	0	8	0	0	0	0	1	0	32
Sum	55	22	4	158	11	2	2	1	18	6	520

Tested antibiotics and their abbreviation: (TZP), piperacillin/tazobactam; (CAZ), ceftazidime; (FEP), cefepime; (MEM), meropenem; (IPM), imipenem; (AN), amikacin; (GM), gentamicin; (NN), tobramycin; (CIP), ciprofloxacin; (LEV), levofloxacin.

TABLE 4 | Resistance pattern of isolates showing resistances to three or more of the tested antibiotics.

Isolate	Species	Resistance Pattern	Multidrug Resistance (MDR)
JDS03PS007	<i>Pseudomonas fluorescens</i>	CAZ, MEM, CIP	MDR 3
JDS03PS016	<i>Pseudomonas fluorescens</i>	TZP, CAZ, GM, MEM, CIP	MDR 4
JDS03PS019	<i>Pseudomonas putida</i>	TZP, MEM, CIP	MDR 3
JDS03PS020	<i>Pseudomonas fluorescens</i>	CAZ, MEM, CIP	MDR 3
JDS03PS032	<i>Pseudomonas fluorescens</i>	CAZ, CIP, LEV	
JDS22PS016	<i>Pseudomonas putida</i>	TZP, CAZ, MEM	MDR 3
JDS22PS018	<i>Pseudomonas fluorescens</i>	CAZ, IMP, MEM	
JDS22PS032	<i>Pseudomonas putida</i>	TZP, IMP, MEM	
JDS22PS035	<i>Pseudomonas putida</i>	TZP, IMP, MEM	
JDS22PS043	<i>Pseudomonas putida</i>	TZP, MEM, CIP	MDR 3
JDS28PS083	<i>Pseudomonas fluorescens</i>	CAZ, FEP, IMP, MEM	
JDS28PS113	<i>Pseudomonas fluorescens</i>	CAZ, IMP, MEM	
JDS28PS115	<i>Pseudomonas putida</i>	TZP, MEM, CIP, LEV	MDR 3
JDS28PS117	<i>Pseudomonas putida</i>	TZP, MEM, LEV	MDR 3
JDS36PS036	<i>Pseudomonas putida</i>	TZP, AN, GM, NN	
JDS59PS020	<i>Pseudomonas fluorescens</i>	TZP, CAZ, FEP	

Multidrug resistance was assigned to an isolate if it revealed a resistance to three (MDR 3) or four (MDR 4) antibiotic classes. Antibiotic classes: acylureidopenicillins: (TZP) piperacillin/tazobactam; cephalosporins: (CAZ) ceftazidime, (FEP) cefepime; carbapenems: (MEM) meropenem, (IPM) imipenem; aminoglycosides: (AN) amikacin, (GM) gentamicin, (NN) tobramycin; fluoroquinolones: (CIP) ciprofloxacin, (LEV) levofloxacin.

site (JDS58) with elevated levels for sulfamethoxazole (Arges, tributary) (JDS 3, 2015). The subsequent River Danube sampling site JDS59 did not show significantly reduced diameters ($p = 0.68$) (Figure 3). JDS03 and JDS68 showed both elevated diameters (6.59 mm JDS03, 8.13 mm JDS68) but only JDS68 differed significantly from the other sampling points ($p < 0.05$).

DISCUSSION

The spread of antibiotic resistant bacteria, their distribution, and their reservoirs in the environment are important issues. Within the last years many different possible sources have been intensively investigated to shed light on the spread of antibiotic resistant bacteria. Farming and the spread of liquid manure are known to contribute to the spread of multiresistant bacteria (Sengupta et al., 2011; Friese et al., 2013). Recently, the focus

has been put on waste water, as bacteria of all kind and with all possible genetic features are mixed up there. And a very critical feature in waste water is the possibility that bacteria harboring resistance exchange their resistance determinants with other bacteria (Korzeniewska and Harnisz, 2013; Reinthaler et al., 2013; Amador et al., 2015). Microorganisms from these and other sources can be relatively easily flushed into surface waters (Czekalski et al., 2012; Zurfluh et al., 2013; Hess and Gallert, 2014), but except for a few studies on relatively small rivers that deal with this topic, the fate of deposited bacteria is quite unclear.

The distribution of susceptible and resistant *Pseudomonas* spp. at the investigated JDS3 sampling points showed site-specific differences. At the upstream sampling points, there was a trend to more resistant bacteria (JDS03, JDS10, JDS22, JDS28), and multiresistant *Pseudomonas* spp. could only be detected in this part of the river. One reason for this finding

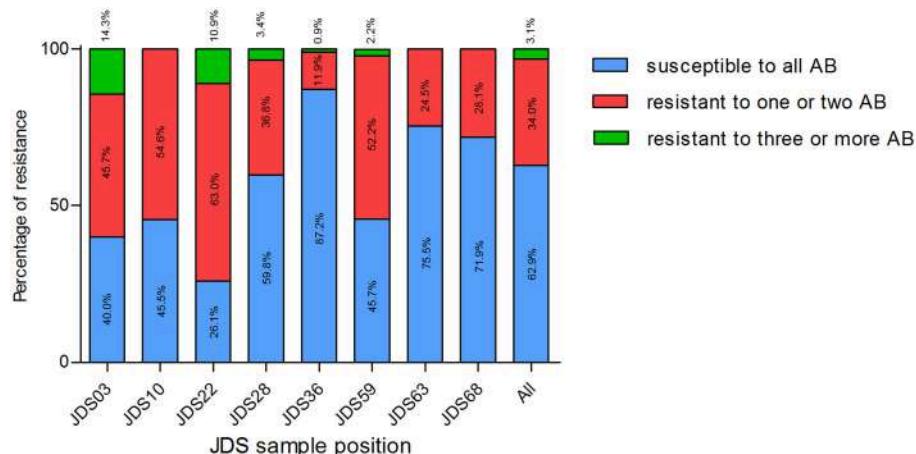


FIGURE 2 | Proportion of isolates susceptible to all tested antibiotics (blue bars), resistant to one or two tested antibiotics (red bars) and resistant to three or more tested antibiotics (green bars), at different sampling points. JDS03, JDS28, JDS63, and JDS68 are non-urban sampling sites, whereas JDS10 is ds Vienna, JDS22 ds Budapest, JDS36 ds Novi Sad and JDS59 is ds Bucharest. Kruskal-Wallis test revealed a non-Gaussian distribution with a $p < 0.0001$.

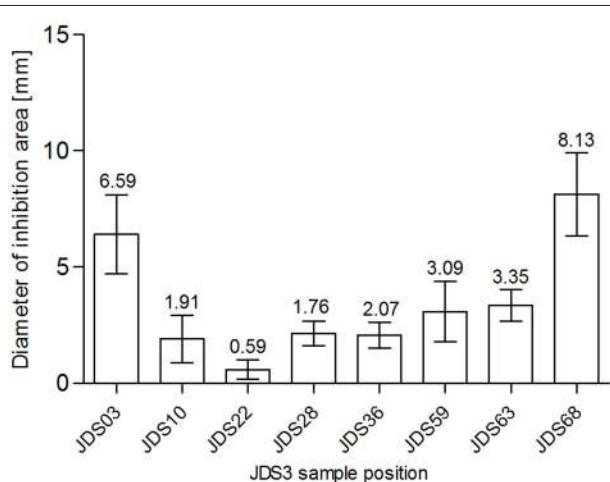


FIGURE 3 | Zone of inhibition diameters (mm) for SXT at the investigated sampling sites. Bars show mean values (values given on top of the bars, mm) and standard deviation errors bars (SD error bars) for each sampling site.

could be the lower river water volume in the upstream parts of the River Danube, which might result in less dilution of the resistant microorganisms. Downstream from the cities of Vienna, Budapest, and Bucharest (JDS10, JDS22, and JDS59 via Arges; Figure 3), the occurrence of resistant bacterial isolates was also elevated, with anthropogenic influence very likely being the reason for that.

Under similar non-selective isolation conditions Suzuki et al. found no resistance to meropenem, gentamicin, amikacin, or ciprofloxacin (Suzuki et al., 2013). A presence of only 8 (1.5%) multiresistant *Pseudomonas* spp. present in three of the eight sampling points seems to be a low number. But still, if we extrapolate the number of 8 multiresistant *Pseudomonaceae* in a

collected volume of 75 ml at the 5 sampling points (5×15 ml) to 1 liter, we might estimate over 100 multiresistant bacteria of the *Pseudomonas* group in one liter of Danube water.

The isolates all over the course of the river Danube showed high resistance rates against meropenem (9.2–69.6%). The resistance against carbapenems in *Pseudomonas* spp. is mostly mediated via efflux pumps (intrinsic resistance), especially in water environment (Tacao et al., 2015). However, carbapenem resistance poses a challenge for therapy, regardless of the underlying mechanism. For example, *Pseudomonas putida* (66% of all isolates in this study) is increasingly involved in hospital infections (Kim et al., 2012; Molina et al., 2014). These infections come up with severe complications and high mortality rates (up to 40%). In most of these cases multiresistant *Pseudomonas putida* was the reason for the infection or the nosocomial outbreak (Kim et al., 2012).

This study is the first study investigating bacterial resistance in a transnational river survey (2500 rkm). Although it was limited by a small sample volume and a fixed time course, the results of this study substantiate the occurrence of waterborne *Pseudomonas* spp. with non-wild type resistance pattern in the whole River Danube. Their presence and their distribution suggest human influence.

AUTHOR CONTRIBUTIONS

CK study design, laboratory work, manuscript preparation, data analysis, ML laboratory work, data conservation. RB laboratory work. BF laboratory work. GK data analysis, manuscript preparartion. DT laboratory work, data analysis. AL laboratory work. AG manuscript preparation. AF manuscript preparation. AK sample collection, manuscript preparation. GZ study design, laboratory work, data analysis, manuscript preparation.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure S1 | Figure of the JDS3 River Danube Survey (high resolution.pdf).

Supplementary Table S1 | Table of all *Pseudomonas* spp. isolates with resistance pattern. (TZP), Piperacilin/tazobactam; (CAZ), ceftazidime; (FEP), cefepime; (MEM), meropenem; (IPM), imipenem; (AN), amikacin; (GM), gentamicin; (NN), tobramycin; (CIP), ciprofloxacin; (LEV), levofloxacin; and (SXT), sulfamethoxazole/trimethoprim.

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Vancomycin modifies the expression of the *agr* system in multidrug-resistant *Staphylococcus aureus* clinical isolates

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Staphylococcus aureus is an opportunistic pathogen that colonizes human hosts and causes a wide variety of diseases. Two interacting regulatory systems called *agr* (accessory gene regulator) and *sar* (staphylococcal accessory regulator) are involved in the regulation of virulence factors. The aim of this study was to evaluate the effect of vancomycin on *hld* and *spa* gene expression during the exponential and post-exponential growth phases in multidrug-resistant (MDR) *S. aureus*.

Methods: Antibiotic susceptibility was evaluated by the standard microdilution method. The phylogenetic profile was obtained by pulsed-field gel electrophoresis (PFGE). Polymorphisms of *agr* and *SCCmec* (staphylococcal cassette chromosome *mec*) were analyzed by multiplex polymerase chain reaction (PCR). The expression levels of *hld* and *spa* were analyzed by reverse transcription-PCR. An enzyme-linked immunosorbent assay (ELISA) was performed to detect protein A, and biofilm formation was analyzed via crystal violet staining.

Results: In total, 60.60% (20/33) of *S. aureus* clinical isolates were MDR. Half (10/20) of the MDR *S. aureus* isolates were distributed in subcluster 10, with >90% similarity among them. In the isolates of this subcluster, a high prevalence (100%) for the *agrII* and the cassette *SCCmec II* polymorphisms was found. Our data showed significant increases in *hld* expression during the post-exponential phase in the presence and absence of vancomycin. Significant increases in *spa* expression, protein A production and biofilm formation were observed during the post-exponential phase when the MDR *S. aureus* isolates were challenged with vancomycin.

Conclusion: The polymorphism *agrII*, which is associated with nosocomial isolates, was the most prevalent polymorphism in MDR *S. aureus*. Additionally, under our study conditions, vancomycin modified *hld* and *spa* expression in these clinical isolates. Therefore, vancomycin may regulate alternative systems that jointly participate in the regulation of these virulence factors.

Keywords: *Staphylococcus aureus*, multidrug-resistant, vancomycin, *agr* system, antibiotics

Introduction

Staphylococcus aureus is an opportunistic pathogen capable of causing a wide variety of diseases in humans, ranging from localized infections of the skin and soft tissues to life-threatening systemic infections (Archer, 1998; Shopsin and Kreiswirth, 2001; David and Daum, 2010; Sowash and Uhlemann, 2014). An infection is initiated when *S. aureus* has access to subcutaneous tissues and is disseminated by the circulatory system, and it infects nearly every organ, leading to severe osteomyelitis, sepsis, abscesses, endocarditis, pneumonia, and toxic shock syndrome (Archer, 1998; Shopsin and Kreiswirth, 2001; David and Daum, 2010; Sowash and Uhlemann, 2014). *S. aureus* can produce a remarkable array of wall surface and secreted virulence factors that contribute to the establishment and maintenance of infection (Novick et al., 1993). These cell surface virulence factors include microbial surface components that recognize extracellular matrix proteins, such as fibrinogen, laminin, plasminogen, vitronectin, fibronectin, thrombospondin, and bone sialoprotein (Falord et al., 2011; Yamamoto et al., 2013). The secreted virulence factors are generally produced during the post-exponential or stationary phase, and they include several extracellular toxins (i.e., alpha-, beta-, gamma-, and delta-hemolysin, enterotoxins, exfoliative toxins A and B, and toxic shock syndrome toxin-1) and exoenzymes (i.e., lipase, nucleases, proteases, hyaluronate lyase, and metalloproteases; Chan and Foster, 1998; Kolar et al., 2013).

The expression of virulence genes in *S. aureus* is regulated under the partial control of the two-component quorum-sensing system encoded by genes at the *agr* locus (Bronner et al., 2004). The P2 transcript (RNAII) encodes the quorum-sensing system, which consists of the following four proteins: AgrB (the secreted protein responsible for the export and processing of AgrD to its active form), AgrD (a signaling peptide), and AgrC-ArgA (a two-component system in which AgrC is the transmembrane receptor histidine kinase and AgrA is the DNA-binding response regulator; Novick et al., 1995; Gilot et al., 2002; Novick, 2003; Gilot and van Leeuwen, 2004). A high cell population density causes the activation of AgrA, which induces the transcription of the P3 promoter. Next, P3 drives the transcription of RNAIII, a regulatory RNA that is both a positive and negative regulator of virulence factor production. The activation of RNAIII transcription in response to an increase in cell population density induces a transition in gene expression correlated with metabolic changes and stress adaptations. Toxin- and extracellular enzyme-encoding genes are positively regulated by the *agr-hld* (δ -lysin gene) system, and the genes coding for protein A (*spa*) and coagulase are negatively regulated (Morfeldt et al., 1988). Protein A has a molecular weight of 42 kDa and is covalently anchored to the peptidoglycan of *S. aureus* (Palmqvist et al., 2002). Ninety percent of the molecule is localized in the cell wall, and 10% is in the bacterial cytoplasm. Protein A is an important virulence factor of *S. aureus* based on its ability to bind to a variety of ligands, including the Fc region of IgG, the von Willebrand factor, tumor necrosis factor receptor-1 (TNFR-1), the Fab-heavy chains of the Vh3 subclass, and the epidermal growth factor receptor (EGFR; Cedergren et al., 1993;

Viau and Zouali, 2005; Gómez et al., 2006; O'Seaghda et al., 2006). An increase in protein A during the post-exponential phase has been observed in *agr*-deleted *S. aureus* strains (Novick, 2003).

In addition, AgrA activation leads to increased transcription of the δ -lysin gene (*hld*), which is located immediately upstream of the *agr* operon (Janzon and Arvidson, 1990). δ -lysin is a small polypeptide of only 26 amino acids. It is secreted without a signal peptide, and it makes cation-selective channels in the phospholipid bilayers (Lee and Birkbeck, 1984). δ -lysin is a virulence factor with lytic activity in a wide range of cells, such as neutrophils, macrophages, mammalian erythrocytes, and bacterial protoplasts, as well as in cellular organelles (Julander et al., 1983).

The aim of this study was to assess *agr* system expression by quantifying *hld* and *spa* expression in multidrug-resistant (MDR) *S. aureus* clinical isolates cultured from the exponential to post-exponential growth phases in the presence of vancomycin. In addition, the *agr* group I–IV polymorphisms were evaluated as a factor that predisposes the permanence and survival of MDR *S. aureus* clinical isolates during nosocomial or hospital-acquired infections in the Hospital Infantil de México Federico Gómez (HIMFG).

Materials and Methods

Bacterial Isolates

Thirty-three *S. aureus* from different infections were obtained from a clinical isolates collection at the Central Laboratory of the HIMFG. The *S. aureus* isolates were collected from January 2006 to June 2007. They were cultured on 5% sheep blood agar plates (Becton Dickinson, East Rutherford, NJ, USA) at 37°C under 5% CO₂ for 24 h and kept at -70°C in skim milk (Becton Dickinson, East Rutherford, NJ, USA).

Diagnostic Tests for Identifying *S. aureus*

The bacteria were grown on blood agar, and identification was performed using conventional bacteriological techniques, such as colony morphology examination, catalase assays, coagulase assays, Gram staining (Sigma-Aldrich, St. Louis, MO, USA), mannitol fermentation, and Brain Heart Infusion (BHI) broth growth assays (Becton Dickinson, East Rutherford, NJ, USA) with 15% NaCl (MacFadin, 1996).

Antimicrobial Susceptibility

The antibiotic susceptibility profiles of the *S. aureus* isolates were determined by the Minimum Inhibitory Concentration (MIC) technique with the microdilution method in Mueller-Hinton broth (MH; Becton Dickinson, East Rutherford, NJ, USA), as recommended by the Clinical and Laboratory Standards Institute (2014). The MIC tests were conducted with vancomycin, ciprofloxacin, erythromycin (MP Biomedicals, Solon, OH, USA), clarithromycin (Grünenthal GmbH, Aachen, Germany), oxacillin, clindamycin, linezolid (Sigma-Aldrich, St. Louis, MO, USA), meropenem (AstraZeneca Pharmaceuticals LP, Wilmington, DE, USA), trimethoprim, sulfamethoxazole (Roche, Basel, Switzerland), and gentamicin (Schering-Plough Pharmaceuticals,

Kenilworth, NJ, USA). To identify methicillin-resistant *S. aureus* clinical isolates, the bacteria were tested for oxacillin resistance by the oxacillin-salt screening method. Oxacillin is a more stable antibiotic than methicillin, although they are chemically identical. *S. aureus* strain ATCC 29213 (American Type Culture Collection, Manassas, VA, USA) was used as a positive control.

Molecular Genotyping Assays

Pulsed-field gel electrophoresis (PFGE) was performed using a previously described protocol (Pereira et al., 2014). The chromosomal DNA from MDR and sensitive *S. aureus* isolates was digested with the Sma I restriction enzyme (Thermo Fisher Scientific Inc., Life Technologies, Grand Island, NY, USA) and subjected to electrophoresis on 1% agarose gels (Promega; Madison, WI, USA) using the following parameters: 200 V (6 v/cm) at 14°C for 21.5 h, with an initial change of 5 s and a final change of 40 s. The gels were stained with 0.5 µg/mL ethidium bromide solution (Sigma-Aldrich, St. Louis, MO, USA) and visualized using a gel imaging system (ChemiDoc™ MP System, Biorad, Hercules, CA, USA). The DNA fragment patterns generated by PFGE were analyzed with NTSY-pc software (version 2.0, Applied Biostatistics, Inc., Port Jefferson, NY, USA; Ramazanzadeh et al., 2013) using the Sørensen-Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) clustering approach (Dice, 1945).

Multiplex Polymerase Chain Reaction (PCR) Conditions

The *S. aureus* clinical isolates were recovered from frozen stock onto BHI agar plates and incubated at 37°C for 18–24 h. Genomic DNA extraction was performed with a Wizard Genomic DNA

Purification Kit (Promega, Madison, WI, USA) from a bacterial culture grown in BHI broth. Briefly, the bacterial culture pellet was mixed with TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0), lysozyme (0.25 mg/mL; Sigma-Aldrich, St. Louis, MO, USA), proteinase K (0.0125 mg/mL; Sigma-Aldrich, St. Louis, MO, USA), and lysostaphin (0.062 mg/mL; Sigma-Aldrich, St. Louis, MO, USA). Multiplex polymerase chain reaction (PCR) assays for detecting *agr* polymorphisms (*agrI*, *agrII*, *agrIII*, and *agrIV*; Table 1) were prepared according to the protocol for Go Taq Green Master Mix (Promega, Madison, WI, USA). Multiplex PCR reactions were prepared in a final volume of 25 µL as follows: 12.5 µL of Go Taq Green Master Mix 2x (Promega, Madison, WI, USA), 2 µL of bacterial DNA (100 ng/µL), 5 µL of *agr* primers (*agrI*, *agrII*, *agrIII*, and *agrIV*) at 10 pg/µL, and 5.5 µL of nuclease-free water. The DNA amplification was performed in a Veriti 96-Well Thermal Cycler-Life Technologies (Applied Biosystems, Foster City, CA, USA) with the following parameters: an initial denaturation at 94°C for 5 min followed by 26 amplification cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s), ending with a final extension at 72°C for 7 min. An external positive control [DNA extracted from *S. aureus* strains USA300 (*agrI*), 1749 (*agrII*), and ATCC 25923 (*agrIII*)] and an external negative control (DNase/RNase-free distilled water) were included with each run. PCR amplicons (10 µL) were loaded into a 1.5% (wt/v) agarose gel (Promega, Madison, WI, USA) using a 100 bp DNA ladder (Promega, Madison, WI, USA), and electrophoresis was performed in 1x TAE buffer at 100 V for 1 h. The bands were visualized using a gel imaging system (ChemiDoc™ MP System, Biorad, Hercules, CA, USA).

The SCCmec genes (I, II, III, and IVa) were characterized by multiplex PCR according to Cázares-Domínguez et al. (2015).

TABLE 1 | Primers used in *agr* and SCCmec typing by multiplex PCR.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
<i>agrI</i>	<i>agr</i> I-F	ATGCACATGGTGACATGC	441	Gilot et al. (2002)
	<i>agr</i> I-R	GTCACAAGTACTATAAGCTGCGAT		
<i>agrII</i>	<i>agr</i> II-F	ATGCACATGGTGACATGC	575	Gilot et al. (2002)
	<i>agr</i> II-R	TATTACTAATTGAAAAGTGGCCATAGC		
<i>agrIII</i>	<i>agr</i> III-F	ATGCACATGGTGACATGC	323	Gilot et al. (2002)
	<i>agr</i> III-R	GTAATGTAATACCTTGTAAAAAGTGGCCATAGC		
<i>agrIV</i>	<i>agr</i> IV-F	ATGCACATGGTGACATGC	659	Gilot et al. (2002)
	<i>agr</i> IV-R	CGATAATGCCGTATAACCCG		
SCCmec I	<i>mec</i> I-F	GCTTTAAAGAGTGTGTTACAGG	613	Zhang et al. (2005)
	<i>mec</i> I-R	GTTCTCTCATAGTATGACGTCC		
SCCmec II	<i>mec</i> II-F	CGTTGAAGATGATGAAGCG	398	Zhang et al. (2005)
	<i>mec</i> II-R	CGAAATCAATGGTTAATGGACC		
SCCmec III	<i>mec</i> III-F	CCATATTGTGTACGGATGCG	280	Zhang et al. (2005)
	<i>mec</i> III-R	CCTTAGTTGTCGTAACAGATCG		
SCCmec IVa	<i>mec</i> IVa-F	GCCTTATTGCAAGAAACCG	776	Zhang et al. (2005)
	<i>mec</i> IVa-R	CTACTCTCTGAAAAGCGTCG		
SCCmec V	<i>mec</i> V-F	GAACATTGTTACTTAAATGAGCG	325	Zhang et al. (2005)
	<i>mec</i> V-R	TGAAAGTTGACCTTGACACC		
<i>mecA</i>	<i>mec</i> 147-F	GTGAAGATACCAAGTGATT	147	Zhang et al. (2005)
	<i>mec</i> 147-R	ATGCGCTATAGATTGAAAGGAT		

F, forward; R, reverse.

RNA Extraction from *S. aureus* Clinical Isolates

Staphylococcus aureus isolates that were grown overnight were adjusted to an optical density of 0.05 at 600 nm and incubated in BHI with 1 µg/mL vancomycin. The bacterial cultures were grown for 4 h until reaching the exponential phase (OD_{600} of 0.6–0.8) and for 11 h just in the post-exponential phase (OD_{600} of 1.2–1.4). Aliquots of bacterial cultures were harvested by centrifugation at $10,000 \times g$ for 3 min at 4°C. Each pellet was washed in an equal volume of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) three times and lysed with the same buffer supplemented with 0.25 mg/mL lysozyme, 0.0125 mg/mL proteinase K, and 0.062 mg/mL lysostaphin. Total bacterial RNA was isolated (TRIzol, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's directions. After purification, contaminating DNA was removed with RNase-free DNase I (2 U/10 µg of total bacterial RNA at 37°C for 30 min). The RNA was then re-purified with RNeasy Minicolumns (Qiagen Incorporated, Lñ Valencia, CA, USA). The amount of recovered RNA was determined spectrophotometrically, and the samples were then stored at –80°C.

Transcriptional Expression Analyses of the spa and hld Genes

The relative expression levels of the *spa* and *hld* genes were determined by cDNA-PCR. The purified RNA of all *S. aureus* clinical isolates was employed for reverse transcription (RT)-PCR assays with the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA, USA), using specific primers for the *spa* (encoding protein A) and *hld* (encoding a delta toxin) genes (Table 2). A GeneAmp RNA PCR Kit was used with 0.2 µg of total RNA per reaction as a template for PCR amplification. Reactions containing *S. aureus* cells alone, only RNA, or no reverse transcriptase were used as negative controls. Specific primers were used for the amplification of 16S RNA, which was used as an internal control (Table 2).

The expression levels of *spa* and *hld* transcripts from *S. aureus* clinical isolates were quantified by densitometric analysis with Bio-Rad image software (Bio-Rad chemi-doc, Quantity one 4.4.1). The data are expressed as the mean ± standard error of the means. A *p*-value of less than 0.05 was considered significant. All experiments were repeated at least three times, and a representative result is shown for each experiment.

Quantitative Measurements of Protein A

A qualitative screening test for the production of protein A was conducted by an enzyme linked immunosorbent assay (ELISA). Briefly, 96-well plates containing 200 µL of BHI were inoculated with 10 µL (1.5×10^8 bacteria/mL) of bacterial suspensions and incubated at 37°C in the presence or absence of vancomycin to the exponential phase (4 h) and post-exponential phase (11 h). Cell wall-associated protein A was identified using anti-protein A, followed by mouse anti-IgG antibodies, *o*-phenylenediamine (OPD) compounds, and ELISA as previously described (Ohkura et al., 1995).

Quantitative Determination by Biofilm Assays

Biofilm formation was quantitatively analyzed according to the protocol described by Erdem et al. (2008). MDR and sensitive *S. aureus* clinical isolates were grown in BHI broth overnight at 37°C. Then, 96-well plates containing 200 µL of BHI were inoculated with 10 µL (1.5×10^8 bacteria/mL) of bacterial suspensions and grown at 37°C in the presence or absence of vancomycin in the exponential phase (4 h) and post-exponential phase (11 h). The biofilms that developed on the surfaces of the wells were gently washed three times with 1x phosphate-buffered saline (PBS; pH 7.4) and fixed with 2% formaldehyde at 4°C overnight. Wells with fixed biofilms were decanted, washed three times with PBS and stained with 200 µL of 1% crystal violet for 30 min. The excess crystal violet was removed, and the plates were washed twice with water. Crystal violet was subsequently solubilized in 70% methanol, and the absorbance was determined at 620 nm. Assays were performed in triplicate and repeated three consecutive times.

Results

The Antimicrobial Susceptibility Testing of *S. aureus* Clinical Isolates

Thirty-three *S. aureus* clinical isolates were tested for antimicrobial susceptibility. In total, 60.60% (20/33) of *S. aureus* clinical isolates were MDR, 100% (33/33) were sensitive to four antibiotics (vancomycin, trimethoprim/sulfamethoxazole, gentamicin, and linezolid), and 39.39% (13/33) were sensitive to all antimicrobials (Table 3).

Molecular Typing Analysis of the MDR and Sensitive *S. aureus* Isolates by PFGE

A total of 19 DNA pulsotypes grouped in four clusters (I–IV) were identified, revealing patterns that consisted of 11–18 DNA fragments ranging in size from 48.5 to 339.5 Kb (Figure 1). In total, 3.03% (1/33) of the *S. aureus* clinical isolates were identified as pulsotype F, and cluster I showed 38% genetic similarity when compared with other pulsotypes. Four pulsotypes (E, I, K, and M) belonging to cluster II included 21.21% (7/33) of the *S. aureus* clinical isolates with 64% genetic similarity. In addition, the nine pulsotypes (H, J, L, N, O, P, Q, R, and S) organized in cluster III included 54.55% (18/33) of the *S. aureus* clinical isolates with

TABLE 2 | Primers used in *hld* and *spa* expression analysis by RT-PCR.

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
<i>spa</i>	TATCTGGTGGCGTAACACCTG GATGAAGCCGTTACGTTGTC	322	Goerke et al. (2000)
<i>hld</i>	GAAGGAGTGTTCAATGG TAAGAAAATACATAGCACTGAG	260	Goerke et al. (2000)
16S	TCCGGAATTATTGGGCGTAA CCACTTCCCTCTCTGCACCTCA	121	Goerke et al. (2000)

TABLE 3 | MIC analysis for the MDR and sensitive *S. aureus* clinical isolates.

Clinical isolates	Antibiotics ($\mu\text{g/mL}$)										
	CEC	CEP	VAN	CLA	CIP	STX	OXA	GEN	ERI	MEM	LZN
488H, 428H, 10H, 714H	≥ 128	≥ 128	1	≥ 128	64	0.5	16	1	≥ 128	64	2
242H, 143H	≥ 128	≥ 128	1	≥ 128	2	≥ 128	0.5	≥ 128	32	4	
246H, 175H	≥ 128	≥ 128	1	≥ 128	1	≥ 128	0.5	≥ 128	16	4	
882HR	≥ 128	≥ 128	1	≥ 128	64	1	≥ 128	1	≥ 128	32	2
425LCR	≥ 128	≥ 128	1	≥ 128	64	0.5	16	0.5	≥ 128	64	2
330H	≥ 128	≥ 128	1	≥ 128	2	≥ 128	1	≥ 128	32	4	
783H	≥ 128	≥ 128	1	≥ 128	2	≥ 128	1	≥ 128	32	4	
902H	≥ 128	≥ 128	1	≥ 128	64	1	≥ 128	2	≥ 128	32	4
54H	≥ 128	≥ 128	0.5	≥ 128	64	0.5	16	0.5	≥ 128	32	2
A-32	≥ 128	≥ 128	0.5	≥ 128	2	≥ 128	0.5	≥ 128	16	2	
828H	8	≥ 128	1	≥ 128	64	0.5	16	0.5	≥ 128	64	2
931H	8	≥ 128	1	≥ 128	64	0.5	16	1	≥ 128	64	2
260H	0.125	64	0.5	32	0.25	0.5	64	0.5	≥ 128	8	0.5
622H	0.125	≥ 128	1	0.06	0.25	1	32	0.5	0.25	4	4
299H	0.060	≥ 128	1	≥ 128	32	0.5	8	0.5	≥ 128	0.12	0.5
679H, 318LCR, 573H, 770H, 633H, 779H, 291H, 336H, 18H, 108H, 780H, 493H, 198H	≤ 0.25	≤ 8	≤ 1	≤ 0.25	≤ 2	≤ 1	≤ 1	≤ 1	≤ 1	≤ 0.125	≤ 4
CVR*	≥ 32	≥ 32	≥ 16	≥ 8	≥ 4	≥ 4	≥ 4	≥ 16	≥ 8	≥ 16	≥ 8

CEC, cefaclor; CEP, cephalothin; VAN, vancomycin; CLA, clarithromycin; CIP, ciprofloxacin; STX, trimethoprim/sulfametoxazole; OXA, oxacillin; GEN, gentamicin; ERI, erythromycin; MEM, meropenem; LZN, linezolid. The MDR *S. aureus* clinical isolates are marked in gray. *Cut-off values for resistance to MIC ($\mu\text{g/mL}$; CVR).

56% genetic similarity. In this cluster, the *S. aureus* clinical isolates with >90% genetic similarity were grouped in subcluster 10 and distributed in the following order: two isolates in pulsotype Q, one isolate in pulsotype R, and eight isolates in pulsotype J (**Figure 1**). In addition, 21.21% (7/33) of the *S. aureus* clinical isolates were classified as cluster IV with 62% genetic similarity and distributed as pulsotypes A, B, C, D, and G (**Figure 1**).

Only one *S. aureus* clinical isolate sensitive to all antibiotics was identified (pulsotype F of cluster I; **Figure 1**). Four MDR and three sensitive *S. aureus* clinical isolates were distributed in the four pulsotypes (E, I, K, and M) of cluster II (**Figure 1**). Fourteen MDR and four sensitive *S. aureus* clinical isolates were distributed into the nine pulsotypes (H, J, L, N, O, P, Q, R, and S) of cluster III. Interestingly, seven MDR and one sensitive *S. aureus* clinical isolates that were distributed in pulsotype J were organized in subcluster 10. In addition, two MDR and five sensitive *S. aureus* clinical isolates were distributed over the five pulsotypes (A, B, C, D, and G) of cluster IV (**Figure 1**).

Distributing *agr*, *SCCmec*, *hld*, and *spa* Genes by Multiplex PCR Endpoint Analysis

Multiplex PCR amplification assays were performed for *agr*-specific group identification in the selected MDR and sensitive *S. aureus* clinical isolates. Our results showed three different *agr* specificity groups (**Figures 2A,B**), which were identified according to the expected product sizes (**Table 1**). Briefly, the sensitive *S. aureus* clinical isolates were 30.76% (4/13) *agrI*, 30.76% (4/13) *agrII*, and 38.46% (5/13) *agrIII* (**Figures 2B,C**). The MDR *S. aureus* clinical isolates were 70% (14/20) *agrII*, 10% (2/20) *agrI*, and 10% (2/20) *agrIII* (**Figures 2A,C**). Furthermore,

both the *agrI/II* and *agrII/III* polymorphism groups were identified in a single (1/20; 5%) clinical isolate of MDR *S. aureus* (**Figures 2A,C**). The expression of the *agrIV* was not identified in either the MDR or sensitive *S. aureus* clinical isolates.

A 398 bp product corresponding to the *SCCmec* group II polymorphism was present in 60.60% (20/33) of the MDR *S. aureus* clinical isolates that were distributed in the fourth cluster as determined by PFGE. A *SCCmec* II polymorphism was observed in 12.12% (4/33) of the isolates in cluster II, 42.42% (14/33) of the isolates in cluster III, and 6.06% (2/33) of the isolates in cluster IV. However, the *SCCmec* II polymorphism was not identified in cluster I (**Figure 1**). Moreover, *SCCmec* polymorphism types I, III, and IV were not identified in the MDR *S. aureus* clinical isolates (**Figure 1**). It is important to emphasize that the presence of the *SCCmec* gene explains methicillin (oxacillin) resistance; therefore, it was not identified in sensitive *S. aureus* clinical isolates (**Figure 1**).

The frequencies of the *hld* and *spa* genes were determined using specific primers to be 100% (33/33) in MDR and sensitive *S. aureus* clinical isolates. Our results showed two bands, which corresponded to *hld* at 260 bp and *spa* at 322 bp (data not shown).

hld and *spa* Gene Expression as Determined by RT-PCR

The specific transcripts of the *spa* and *hld* genes produced by the ten MDR *S. aureus* isolates that were distributed in subcluster 10 of cluster III were quantified by RT-PCR-densitometry. These MDR *S. aureus* isolates were treated with and without vancomycin during two different stages of the growth phase

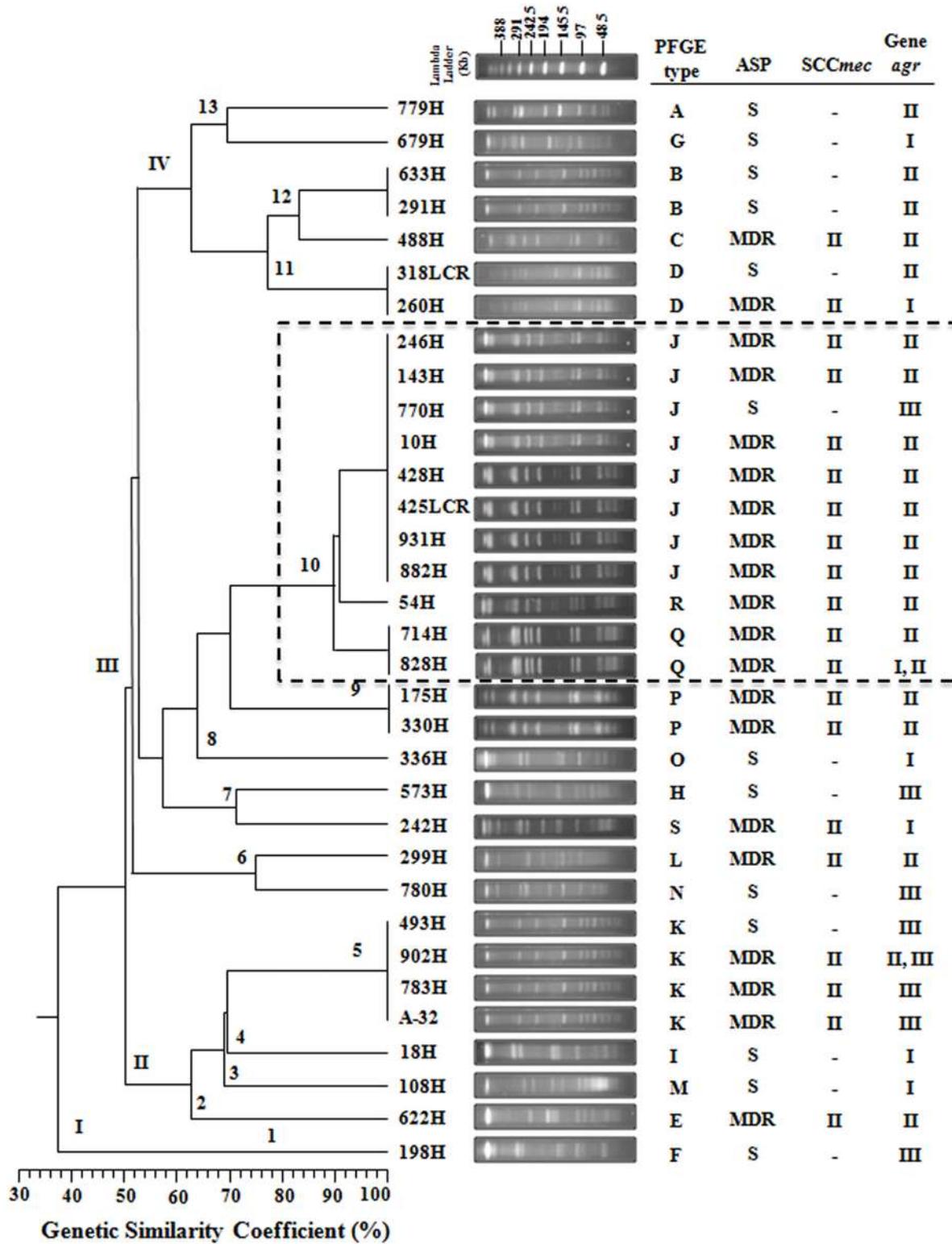


FIGURE 1 | Dendrogram analysis of PFGE results showing the genetic relationships among the PFGE profiles and the presence of *agr* and *SCCmec* type among the 33 *S. aureus* clinical isolates. A phylogenetic analysis was conducted using the Sørensen–Dice similarity coefficient in association with the UPGMA algorithm as the grouping method. The

dendrogram was evaluated by obtaining the cophenetic correlation coefficient with the Mantel test, which yielded an *r*-value of 0.9077. Antimicrobial susceptibility profile (ASP), multidrug-resistant (MDR), sensitive (S). The dotted line indicates that the clinical isolates that belong to subcluster 10 with >90% genetic similarity.

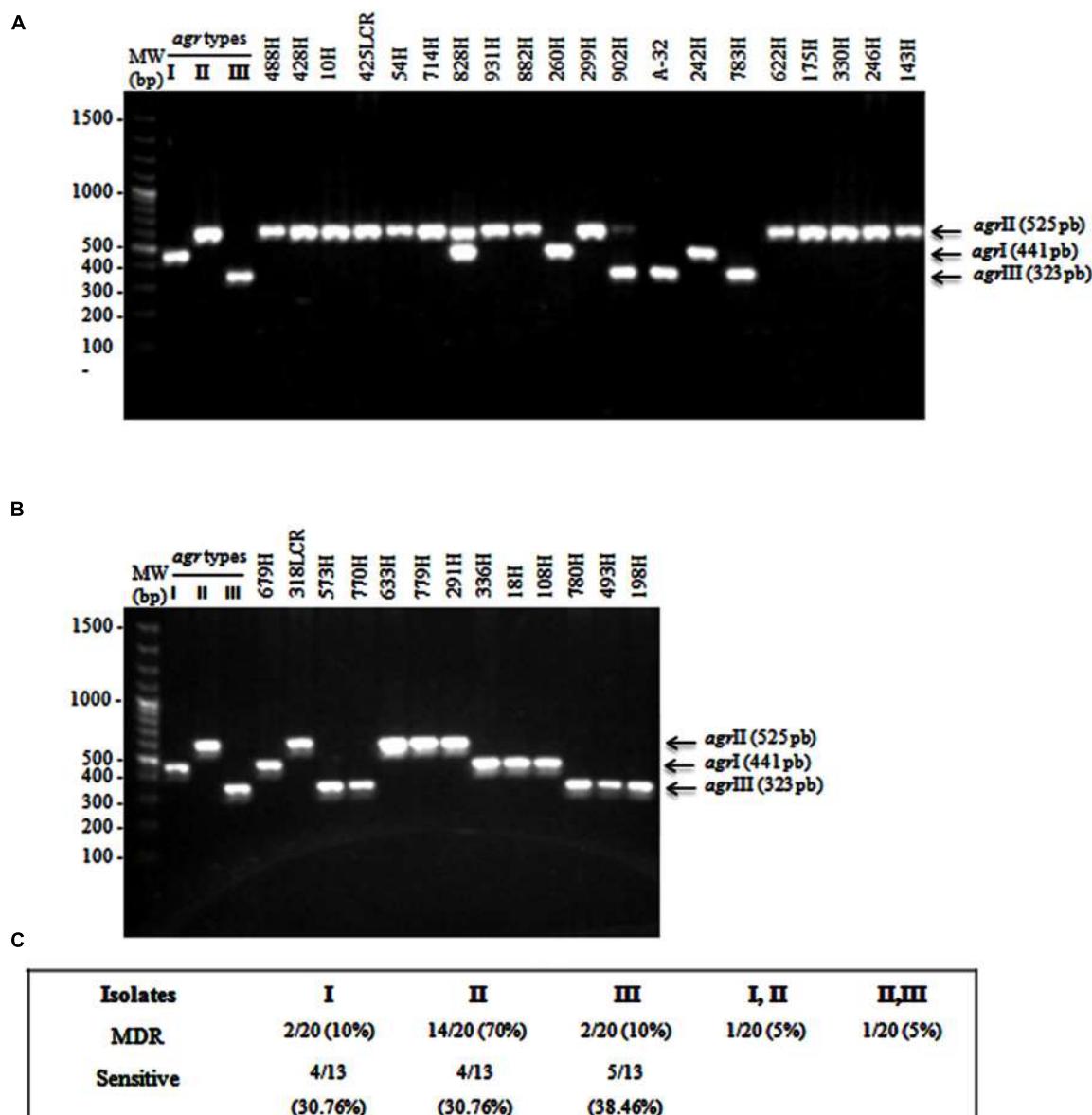


FIGURE 2 | Multiplex PCR assays for identifying *agr* types. (A) MDR *S. aureus* clinical isolates showing the *agrI* (441 bp), *agrII* (575 bp), and *agrIII* (323 bp) types. **(B)** Sensitive *S. aureus* clinical isolates. **(C)** The percentages of

agr types that were identified in MDR and sensible *S. aureus* clinical isolates. *S. aureus* strain USA300 (*agrI*), 1749 (*agrII*), and ATCC 25923 (*agrIII*) were used as reference strains. MW, molecular weight (bp).

(exponential and post-exponential). The *hld* expression in MDR *S. aureus* clinical isolates showed a significant increase (1.68-fold; $p = 0.0001$) when cultured from the exponential to the post-exponential growth phase in the absence of vancomycin as well as a significant increase (2.04-fold; $p = 0.0001$) when grown in the presence of vancomycin (Figure 3A). Interestingly, under the same test conditions, a significant increase (2.07-fold; $p = 0.0001$) in *hld* expression was observed in MDR *S. aureus* clinical isolates in the exponential growth phase treated with vancomycin compared to those treated without vancomycin (Figure 3A). In addition, a significant increase (2.53-fold; $p = 0.0001$) in *hld* expression was observed in the MDR *S. aureus* clinical isolates

cultured to the post-exponential growth phase when challenged with 1 μ g/mL vancomycin compared to without vancomycin challenge (Figure 3A). The *hld* expression in sensitive *S. aureus* isolates showed increases from the exponential to the post-exponential growth phase in both the presence and absence of vancomycin (data not shown).

The *spa* expression level showed a significant reduction (4.01-fold; $p = 0.0001$) when the MDR *S. aureus* clinical isolates were cultured from the exponential to post-exponential growth phases in the absence of vancomycin (Figure 3B). Interestingly, a remarkable increase (3.67-fold; $p = 0.0001$) in *spa* expression was observed from the exponential to the post-exponential

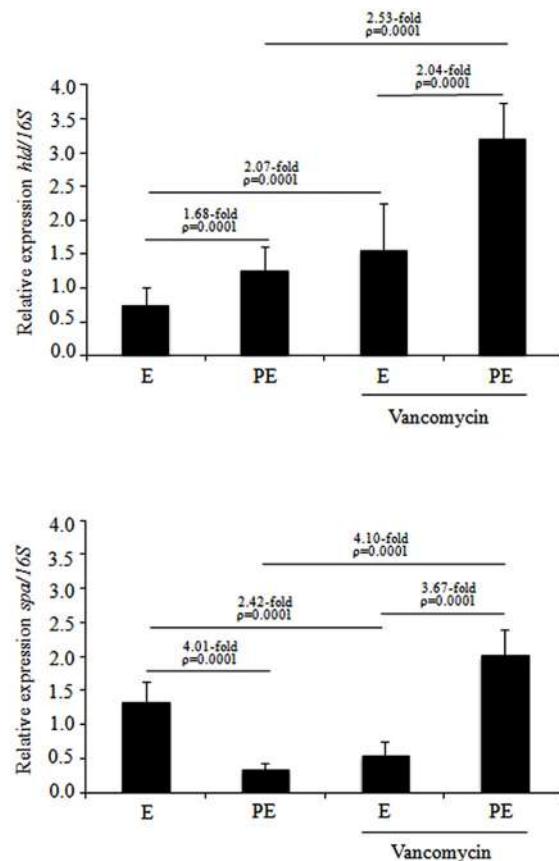


FIGURE 3 | Expression levels of the *hld* and *spa* genes determined by RT-PCR in MDR *S. aureus* clinical isolates cultured from the exponential to post-exponential growth phases in the presence of vancomycin. (A) *hld* expression; (B) *spa* expression. The densitometric results of the transcripts were quantified using the Quantity One program and analyzed by paired Student's *t*-tests. E, exponential; PE, post-exponential.

phases in MDR *S. aureus* clinical isolates cultured in the presence of vancomycin (Figure 3B). During the exponential phase, a significant reduction in *spa* expression (2.42-fold; $p = 0.0001$) was observed when the MDR *S. aureus* clinical isolates were challenged with vancomycin compared to those grown in the absence of vancomycin; the MDR *S. aureus* clinical isolates in the post-exponential growth phase showed a significant increase (4.10-fold; $p = 0.0001$) in *spa* expression when challenged with vancomycin compared to those grown in the absence of vancomycin (Figure 3B). The *spa* expression level did not show significant changes (reduction and/or increase) when the sensitive *S. aureus* clinical isolates were cultured in the exponential and post-exponential growth phases in the presence or absence of vancomycin (data not shown).

Protein A Immunodetection by ELISA

Protein A immunodetection assays were performed for the MDR *S. aureus* clinical isolates distributed in subcluster 10 of cluster III. Quantitative analysis showed a significant reduction (2.39-fold; $p = 0.0001$) in protein A expression in the

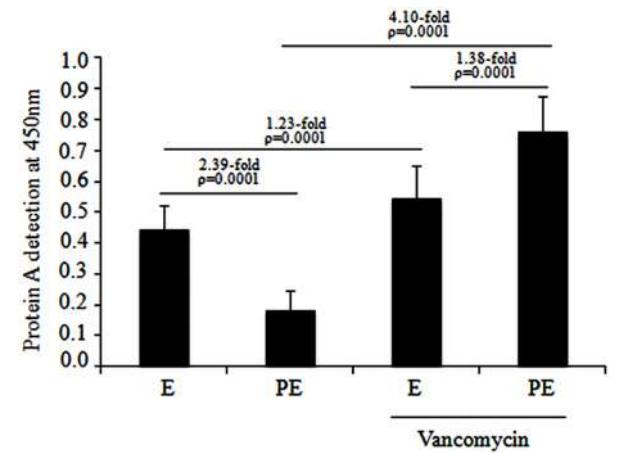


FIGURE 4 | Protein A production in the MDR *S. aureus* clinical isolates cultured from the exponential to post-exponential growth phases in the presence of vancomycin. A significant reduction of 2.39-fold ($p = 0.0001$) was observed in the MDR *S. aureus* clinical isolates when they were cultured from the exponential to post-exponential growth phases in the absence of vancomycin and there was a significant increase of 1.38-fold ($p = 0.0001$) when cultured from the exponential to post-exponential growth phase when challenged with 1 μ g/mL vancomycin.

post-exponential phase compared to the exponential phase in the absence of vancomycin (Figure 4). Furthermore, a significant increase (1.38-fold; $p = 0.0001$) in protein A expression was observed from the exponential to the post-exponential growth phase in the MDR *S. aureus* clinical isolates cultured in the presence of 1 μ g/mL vancomycin. Interestingly, a significant increase (4.10-fold; $p = 0.0001$) in protein A expression was observed in these clinical isolates cultured to the post-exponential growth phase in the presence of vancomycin compared to in the absence of vancomycin (Figure 4). Likewise, a significant increase (1.23-fold; $p = 0.0001$) in protein A expression was observed in the MDR *S. aureus* clinical isolates that were cultured to the exponential growth phase in the presence of vancomycin compared to in the absence of vancomycin.

Clinical Strains of MDR and Sensitive *S. aureus* Isolates Produce Biofilms

A crystal-violet biofilm assay was performed for the MDR *S. aureus* clinical isolates that were distributed in subcluster 10 of cluster III. Quantitative analysis showed no difference in biofilm formation when the MDR *S. aureus* clinical isolates were cultured to the exponential and post-exponential growth phases in the absence of vancomycin. In the presence of vancomycin, a significant increase (1.42-fold; $p = 0.0001$) was observed in the biofilm formation of the MDR *S. aureus* clinical isolates cultured to the exponential growth phase and a significant increase (1.85-fold; $p = 0.0001$) was observed in the isolates cultured to the post-exponential growth phase. Furthermore, a significant increase (1.35-fold; $p = 0.0001$) in the biofilm formation of these isolates was observed from the exponential to

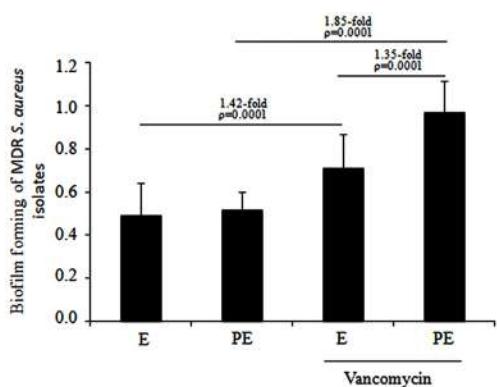


FIGURE 5 | Biofilm assays of the MDR *S. aureus* clinical isolates when cultured from the exponential to post-exponential growth phases in the presence of vancomycin. Interestingly, a significant increase (1.35-fold; $p = 0.0001$) in biofilm formation was observed in the MDR *S. aureus* clinical isolates cultured from the exponential to post-exponential growth phases in the presence of the vancomycin.

post-exponential growth phases in the presence of vancomycin (Figure 5).

Discussion

In this study, 100% of *S. aureus* clinical isolates showed sensitivity to vancomycin, trimethoprim/sulfamethoxazole, gentamicin, and linezolid. Low rates of resistance to tetracycline, gentamicin, and trimethoprim/sulfamethoxazole in clinical methicillin-resistant staphylococcus aureus (MRSA) isolates have been described in other studies (Bordon et al., 2010; Davis et al., 2013). However, the impressive ability of *S. aureus* to develop resistance against various antibiotics through point mutations, transposons, plasmids, and resistance cassettes is the most likely reason for the rapid increase in antimicrobial resistance worldwide in recent years (Sakoulas and Moellering, 2008). High levels of erythromycin resistance and increasing ciprofloxacin resistance in MRSA clinical isolates have been observed (Davis et al., 2013). The clinical condition, the administration route, and the resistance pattern of the organism are also risk factors that are considered in treating MRSA infections when drugs such as vancomycin, linezolid, daptomycin, clindamycin, and mupirocin are administered (Liu et al., 2011). The increase of vancomycin-resistant *S. aureus* (VRSA) isolates in the United States could be explained by the selective pressure originating from the excessive use of vancomycin to treat MRSA infections (David and Daum, 2010). The high frequency of resistance to the antibiotics tested in our study may reflect the fact that the public health service in Mexico prescribes all of these antibiotics for treating pediatric patients; these drugs are considered to be essential in this health sector.

According to the PFGE analysis, 33.33% (11/33) of the *S. aureus* clinical isolates distributed in subcluster 10 shared 90% similarity. In particular, 50% of the MDR *S. aureus* clinical isolates were distributed in subcluster 10 and showed closely

related pulsotypes with three clones that were assigned as J, Q, and R according to the criteria of Tenover et al. (1995). These results suggest that MDR *S. aureus* isolates are associated with patients with infections acquired during their current hospital stay. MRSA isolates with a highly related PFGE type have been associated with an MDR profile to β -lactams, gentamicin, ciprofloxacin, clindamycin, and erythromycin (Velazquez-Meza et al., 2004). In total, 90.90% (10/11) of the *S. aureus* clinical isolates belonging to subcluster 10 were resistant to six antibiotics, namely cephalothin, clarithromycin, ciprofloxacin, oxacillin, erythromycin, and meropenem. Furthermore, 72.72% (8/11) of these clinical isolates were resistant to cefaclor.

Our data showed a high prevalence of the *agr* group II polymorphism, with a PCR-amplified product of 575 kb, in the MDR *S. aureus* clinical isolates belonging to subcluster 10 by PFGE analysis. Several studies performed in Japan and the USA described the *agr* group II polymorphism as the *agr* type that was most frequent in MDR *S. aureus* clinical isolates, and it has been associated with nosocomial infections from pediatric patients (Sakoulas et al., 2003). Similar findings indicated that all *S. aureus* clinical isolates from diverse geographic origins and those recovered from patients undergoing intubation showed the *agr* group II polymorphism (Sakoulas et al., 2002; Goerke et al., 2003; Moise et al., 2004). Furthermore, the *agr* group II polymorphism in MRSA predicts the failure of vancomycin therapy (Moise et al., 2007).

We further evaluated *agr* expression through indirect mechanisms by quantification of *hld* and *spa* expression in MDR *S. aureus* clinical isolates during the exponential and post-exponential growth phases and upon vancomycin challenge. Our RT-PCR analysis showed that *hld* activation occurs during the post-exponential growth phase, resulting in an increase of 1.68-fold without vancomycin and 2.04-fold with vancomycin compared with the exponential phase. The increased *hld* expression in the MDR *S. aureus* clinical isolates of subcluster 10 suggested that the activation of this gene is influenced by the growth phase and vancomycin challenge. Other studies have shown significant increases in *hld* expression at the end of the exponential growth phase by an *agr*-dependent mechanism that is involved in the regulation of virulence genes. *agrA* codes for a protein that can activate *hld* transcription as a response to the growth phase (Janzon and Arvidson, 1990). Our data indicated that the MDR *S. aureus* isolates were stimulated by vancomycin at subinhibitory concentrations, as indicated by increases in *hld* expression. These results indicate that the antibiotic stimulates the *agr* system. In a previous study, the over-expression of the *hld* transcript in vancomycin-resistant *S. aureus* strains associated with activation of the sigma factor was observed when these isolates were exposed to subinhibitory antibiotic concentrations (Chen et al., 2011). Recently, community-associated (CA) MRSA was challenged with subinhibitory concentrations of tetracycline and clindamycin, which had a strong stimulatory effect on the activity of the *agr* operon (Joo et al., 2010). These results, together with the data obtained in our studies, suggest that vancomycin also exerts a strong stimulatory effect on the activity of the *agr* operon. It is important to note that *agr* controls many virulence factors of

S. aureus and that vancomycin is frequently used in Mexico for treating MRSA infections in pediatric patients.

Interestingly, a reduction in *spa* expression was observed during the exponential to post-exponential growth phases when MDR *S. aureus* clinical isolates were cultured without vancomycin. In contrast, a significant increase (3.67-fold) in *spa* expression was observed when MDR *S. aureus* clinical isolates were only cultured with vancomycin during the post-exponential growth phase. These data suggest that *spa* activation or repression is regulated by the growth phase and that expression of *spa* mRNA is modified by drugs such as vancomycin. In previous studies, CA-MRSA isolates treated with daptomycin and vancomycin showed no change in *spa* mRNA and SpA protein levels (Subrt et al., 2011; Otto et al., 2013). However, in a different study, the expression levels of different virulence factors in the CA-MRSA isolates were suppressed in the presence of clindamycin and linezolid (Otto et al., 2013).

The expression levels of the *spa* gene are directly correlated with protein A production because most of the gene transcript is translated as protein A. The localization of protein A in the cell wall could contribute to biofilm formation in MRSA clinical isolates during the post-exponential phase and when challenged with vancomycin. The XdrA regulator has almost as strong an activating effect on *spa* as SarS, and it acts on the same *spa* operator regions (identified potential *cis*-acting regulatory regions) as SarS or on closely overlapping regions (McCallum et al., 2010). The current evidence suggests that XdrA directly regulates *spa* transcripts independently of other well-characterized regulators. The over-expression of *spa* transcripts and protein A in the MDR *S. aureus* clinical isolates challenged with vancomycin could also be related to the action of the regulator XdrA, which should be examined in future studies. In addition, the variation in the levels of *spa* transcripts during different growth phases is likely due to the influence of other regulators and/or to the characteristics of clinical isolates.

Conclusion

Staphylococcus aureus clinical strains are influenced by inter-cellular signaling through the *agr* system, which modulates the activation and/or repression of many outer membrane proteins associated with biofilms (Cafiso et al., 2012). Vancomycin is the antibiotic of choice for treating nosocomial infections of MDR *S. aureus*.

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aureus clinical isolates in pediatric patients at the HIMFG. These data provide a direct evidence for evaluating the role of virulence genes (*hld* and *spa*) associated with *agr* regulation and suggest the participation of other regulatory elements; which are include an operon that activates many virulence factors that could be important during infection by this nosocomial pathogen. In our study, the polymorphism *agrII* was associated with nosocomial isolates and was the most prevalent polymorphism in MDR *S. aureus*. Our finding showed that vancomycin modified the *hld* and *spa* expression in the MDR *S. aureus* clinical isolates; suggesting that, vancomycin may regulate alternative systems that jointly participate in the regulation of virulence factors involved in bacteria pathogenesis, which allows spread and adaptation into a hospital environmental.

Author Contributions

Designed and conceived the experiments: VCD, SAO, ACC, NVG, and JXC. Performed the experiments: VCD, GE, and SAO. Analyzed the data: VCD, SAO, ALO, and GER. Contributed reagents/materials/analysis tools: VCD, SAO, ACC, JJOT, and JXC. Wrote and reviewed the manuscript: VCD, SAO, NVG, and JXC.

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Comparative Genomic Analysis of the ICESa2603 Family ICEs and Spread of *erm(B)*- and *tet(O)*-Carrying Transferable 89K-Subtype ICEs in Swine and Bovine Isolates in China

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Integrative and conjugative elements (ICEs) of the ICESa2603 family have been isolated from several species of *Streptococcus* spp.; however, the comparative genomic and evolutionary analyses of these particular ICEs are currently only at their initial stages. By investigating 13 ICEs of the ICESa2603 family and two ICESa2603 family-like ICEs derived from diverse hosts and locations, we have determined that ICEs comprised a backbone of 30 identical syntenic core genes and accessory genes that were restricted to the intergenic sites or the 3'-end of the non-conserved domain of core genes to maintain its function. ICESa2603 family integrase $\text{Int}_{\text{ICESa2603}}$ specifically recognized a 15-bp *att* sequence (TTATTTAAGAGTAAC) at the 3'-end of *rplL*, which was highly conserved in genus *Streptococcus*. Phylogenetic analyses suggest that extensive recombination/insertion and the occurrence of a hybrid/mosaic in the ICESa2603 family were responsible for the significant increase in ICE diversity, thereby broadening its host range. Approximately 42.5 and 38.1% of the tested *Streptococcus suis* and *Streptococcus agalactiae* clinical isolates respectively contained ICESa2603 family Type IV secretion system (T4SS) genes, and 80.5 and 62.5% of which also respectively carried $\text{int}_{\text{ICESa2603}}$, indicating that ICESa2603 family is widely distributed across these bacteria. Sequencing and conjugation transfer of a novel sequence type ST303 clinical *S. suis* isolate HB1011 demonstrated that the 89K-subtype ICESsuHB1011 retained its transferable function, thereby conferring tetracycline and macrolide resistance.

Keywords: integrative and conjugative elements, antimicrobial resistance, horizontal gene transfer, ICESa2603 family, *erm(B)*, *tet(O)*, *Streptococcus suis*, *Streptococcus agalactiae*

INTRODUCTION

Integrative and conjugative elements (ICEs) are self-transmissible mobile genetic elements (MGEs) that primarily reside in the host cell's chromosome, yet have the ability to be transferred between cells by conjugation (Burrus et al., 2002; Burrus and Waldor, 2004). ICEs are considered as mosaic elements with both phage- and plasmid-like features that can integrate into and replicate

with the host cell's chromosomes similar to those observed in bacteriophages, as well as transfers, via conjugation in plasmids. The number of identified ICEs continues to increase with the exponential expansion of sequenced complete genomes (Te Poele et al., 2008; Wozniak and Waldor, 2010). Guglielmini et al (Guglielmini et al., 2011) previously described the prevalence and diversity of ICEs by conducting bioinformatics analysis of clustered conjugative apparatus modules in various chromosomal locations. Based on this definition, 18% of sequenced prokaryotic genomes contain at least one ICE, implying that the role of ICEs in horizontal gene transfer is more important than previously conceived.

An ICE typically comprises three distinct modular structures that mediate its integration and excision, conjugation, and regulation. In addition, ICEs contain genes that confer specific phenotypes that are related to its existence in its hosts such as resistance to antibiotics and heavy metals. Therefore, ICEs are important vectors for the horizontal transfer of genetic information, thereby facilitating rapid bacterial evolution (Bi et al., 2011; Rodriguez-Blanco et al., 2012). The SXT/R391 family is the most extensively studied ICE family that was initially identified in *Vibrio cholerae* O139 (Waldor et al., 1996). To date, more than 40 members of this family have been identified in different clinical and environmental *Vibrio* species, as well as related gammaproteobacteria (Mata et al., 2011; Rodriguez-Blanco et al., 2012). The SXT/R391 family shares a core structure containing 52 conserved genes and a common chromosomal integration site at the 5'-end of the *prfC* gene, with other parts all integrating into 3'-ends of distinct element-specific tRNA gene loci (Wozniak et al., 2009; Wozniak and Waldor, 2010). All known elements contain variable DNAs that are inserted into specific positions of the backbone (hot spots) and encoding various beneficial traits such as antibiotic resistant determinants including but not limited to *floR*, *strBA*, *sul2*, *dfrA1*, *dfr18*, *teta'*, *kanR*, and an emerging extended-spectrum cephalosporin resistance gene *blaCMY-2* (Harada et al., 2010). SetR, a homolog of the phage λ repressor CI, derepresses the master transcriptional activators required for SXT transfer, including the *int* and *tra* operons (Daccord et al., 2012). Beaber et al. (2004) have shown that DNA-damaging agents mitomycin C and quinolones which induce the host SOS response, increase the transfer of SXT by several hundred times. The horizontal dissemination of antibiotic resistance genes through ICEs under selective pressure poses new challenges to the use of antimicrobial agents. Extensive knowledge of SXT/R391 family ICEs prompted us to explore the genetic features of other family ICEs.

Comparative analysis of the genomes of different isolates of *Streptococcus agalactiae* has revealed that nearly 2/3 of its regions of diversity in the isolates consist of ICEs or ICE-like elements (Brochet et al., 2008), which include two of the largest families, namely, Tn916 and ICESa2603. The Tn916 family is extensively distributed across various organisms, including important human pathogens such as *Enterococcus faecalis*, *Clostridium difficile*, *Staphylococcus aureus*, and *Streptococcus* spp. (Roberts and Mullany, 2011). Furthermore, its transfer mechanism has been well studied (Roberts and Mullany, 2009).

In contrast, the ICESa2603 family, although identified in various *Streptococcus* species, including *S. agalactiae* and *Streptococcus suis*, has not been comprehensively investigated. In the current study, we first comparatively analyzed 13 ICEs of the ICESa2603 family and two ICESa2603 family-like ICEs to perform an in-depth genetic characterization of the core genes that are present in all members of this ICE family. Next, we investigated the potential accessory functions encoded by the variable DNA harbored by these mobile elements as well as the conserved *att* site for integration and the *oriT* site for origination transfer. Phylogenetic analyses of the 30 core genes allowed us to classify the 15 ICEs into four subgroups. Furthermore, the distribution of these ICEs was determined in *S. suis* and *S. agalactiae*. Finally, an experimental transfer of a swine origin 89K-like subgroup transferable ICE, ICESsuHB1011, was introduced. It is worth noting that isolates with *erm(B)*- and *tet(O)*-carrying transferable 89K-subtype ICEs were detected in different swine and bovine farms of China, thereby disseminating tetracycline and macrolide resistance genes and contributing to human pathogenesis (Tang et al., 2006).

MATERIALS AND METHODS

Bacterial Strains and Genomic DNA Extraction

A total of 73 *S. suis* isolates from swine (including one *S. suis* strain from ATCC) and 21 *S. agalactiae* isolates from bovine (including one *S. agalactiae* strain from ATCC) were used in the present study (Table S1). Isolates were routinely grown overnight on tryptic soya broth or agar plates supplemented with 5% calf serum at 37°C. The bacterial culture was centrifuged (10,000 g for 5 min at room temperature), and the pellets were harvested and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with lysozyme (5 mg/mL) and incubated for 30 min at 37°C. Genomic DNA was prepared using a Bacteria DNA kit (Omega, Norcross, GA, USA), following the manufacturer's instructions. The extracted DNA was used as template for PCR and sequencing.

In silico identification of the ICESa2603 Family

To identify and compare the ICESa2603 family ICEs, the reference genomes of ICESa2603 family were obtained from NCBI (Table 1). ICESa2603, ICESde3396, ICESdy12394-1, ICESpa43144-1, ICESthJIM8232-1, ICESsu32457, ICESsuBM407-2, and ICESsuSC84 were previously classified as members of the ICESa2603 family (Bi et al., 2011; <http://db-mmj.sjtu.edu.cn/ICEberg/index.php>). ICESa09mas018883, ICESsu05ZYH33-1 (also designated as 89K according to its size), ICESsu98HAH33-1, ICESsuD9, ICESsuSS12, and ICESsuHB1011 were grouped into this family because these encode the integrase gene that is closely related to *int*_{ICESa2603} and share significant sequence alignment and syntonic core structure. ICESsuT15 and ICESluvan were identified as ICESa2603 family-like ICEs because these encoded a serine recombinase (SR) family integrase gene instead of the tyrosine family integrase gene, *int*_{ICESa2603}.

TABLE 1 | Properties of the ICESa2603 family ICEs.

ICE name	Host strain	Accession	Size (bp)	% GC	Origin	References
ICESa2603	<i>S. agalactiae</i> 2603V/R	AE009948.1	54349	38	United States	Tettelin et al., 2002
ICESde3396	<i>S. dysgalactiae</i> subsp. <i>equisimilis</i> NS3396	EU142041.1	63668	31	Australia, 2001	Davies et al., 2005, 2009
ICESdy12394-1	<i>S. dysgalactiae</i> subsp. <i>equisimilis</i> ATCC12394	CP002215.1	50568	39	United States, 1939	Suzuki et al., 2011
ICESpa43144-1	<i>S. pasteurianus</i> ATCC43144	AP012054.1	58476	37	United States	Lin et al., 2011
ICESthJIM8232-1	<i>S. thermophilus</i> JIM8232	FR875178.1	50501	37	France, 2002	Delorme et al., 2011
ICESa09mas018883	<i>S. agalactiae</i> 09mas018883	HF952104.1	46903	37	–	Zubair et al., 2013
ICESsu32457	<i>S. suis</i> 32457	FR823304.2	54879	50.5	Italy, 2007	Palmieri et al., 2012
ICESsu05ZYH33-1	<i>S. suis</i> 05ZYH33	CP000407.1	88851	37	China, 2005	Chen et al., 2007
ICESsu98HAH33-1	<i>S. suis</i> 98HAH33	CP000408.1	89154	37	China, 1998	Chen et al., 2007
ICESsuSC84	<i>S. suis</i> SC84	FM252031.1	89165	37	China, 2005	Holden et al., 2009
ICESsuBM407-2 ^a	<i>S. suis</i> BM407	FM252032.1	80320	36	Vietnam, 2004	Holden et al., 2009
ICESsuD9	<i>S. suis</i> D9	CP002641.1	55989	39.8	China	Zhang et al., 2011
ICESsuSS12	<i>S. suis</i> SS12	CP002640.1	64284	38.2	China	Zhang et al., 2011
ICESsuT15 ^{a,b}	<i>S. suis</i> T15	CP006246.1	71412	36.3	–	–
ICESluvan ^b	<i>S. lutetiensis</i> 5-F9	HE963029.1	94189	44	Netherlands	Bjorkeng et al., 2013
ICESsuHB1011 ^c	<i>S. suis</i> HB1011	–	–	–	China, 2010	This study

^aThe SNF2 family protein was absent (ICESsuBM407-2) or partially absent (ICESsuT15).

^bICESsuT15 and ICESluvan with a serine recombinase (SR) family integrase were referred as ICESa2603 family-like ICEs.

^cICESsuHB1011 sequence was not complete.

Phylogenetic Analyses

The ICESa2603 family and family-like ICEs were aligned using ClustalW (Larkin et al., 2007) with default settings. Nucleotide and amino acid conservations were assessed using the appropriate BLAST algorithms. MAUVE 2.4 (Darling et al., 2004) and ACT software (Carver et al., 2005) were used to identify core genes. The core genes of each ICE were further aligned with the corresponding genes of ICESa2603 to calculate nucleic acid percentage identity (**Figure 1A**).

To obtain an overview of the relationship of the ICESa2603 family and family-like ICEs and its possible evolution, phylogenetic trees were generated from the alignment of whole core genes or each core gene using the neighbor-joining method with bootstrapping ($N = 1000$) parameters by the software, MEGA6 (Tamura et al., 2013) and FigTree v1.4.2.

Evaluation of the *att* and *oriT* Sites

To identify the putative *att* sites, genomic sequences of the ICE-carrying strains were analyzed for the presence of directly repeated sequences flanking the ICEs. The ICESa2603 family-like ICESsuT15, where there are no conserved directly repeated sequences presented, the putative *att* site was predicted by comparing the junction sequence with *S. suis* P1/7.

The predicted *oriT* site of ICESsu05ZYH33-1 was first identified by Li et al. (2011). We determined that the *oriT* site is conserved in all other ICESa2603 family members. MEGA 6 (Tamura et al., 2013) and BioEdit v7 were used to align and map the *oriT* site the members of the ICESa2603 family.

PCR and Sequencing

To determine the presence of ICESa2603 family ICEs in *S. suis* and *S. agalactiae*, primers for the ICESa2603 core genes were

designed and used in PCR analysis (Table S2). The integrated form and the extrachromosomal circular form of the ICEs of *S. suis* and *S. agalactiae* were detected by using combination primers, P1–P4 (**Figures 1B–D** and Table S2). PCR was also performed to detect the presence of ICE fragments in *S. suis* and *S. agalactiae* by PCR tiling assay as described previously (Sitkiewicz et al., 2011) using specific oligonucleotide primers (**Figure 1E** and Table S2). The products of a representative *S. suis* strain, HB1011, with the ICEs backbone as determined by PCR tiling were sequenced by Genewiz, Inc. (Suzhou, Jiangsu, China).

Antimicrobials and Susceptibility Tests

Tetracycline, erythromycin, streptomycin, and rifampin (Sigma, St. Louis, MO, USA) were used in the present study. MICs were determined according to the guidelines described by the Clinical and Laboratory Standards Institute (CLSI, 2010).

Conjugation Experiments

S. suis BAA-853 and *S. agalactiae* G9, which were susceptible to tetracycline and erythromycin, were used to generate a rifampin- and streptomycin-resistant phenotype by using the stepwise-induced method with sub-MICs of rifampin and streptomycin (Haenni et al., 2010). In the mating experiments, the above two induced strains (tetracycline- and erythromycin-susceptible but rifampin- and streptomycin-resistant) were used as recipients and *S. suis* HB1011 (tetracycline- and erythromycin-resistant but rifampin- and streptomycin-susceptible) with the ICESsuHB1011 was utilized as donor. Filter mating assays were performed as previously described (Li et al., 2011). The transconjugant was further confirmed by PCR, sequencing, and MLST typing.

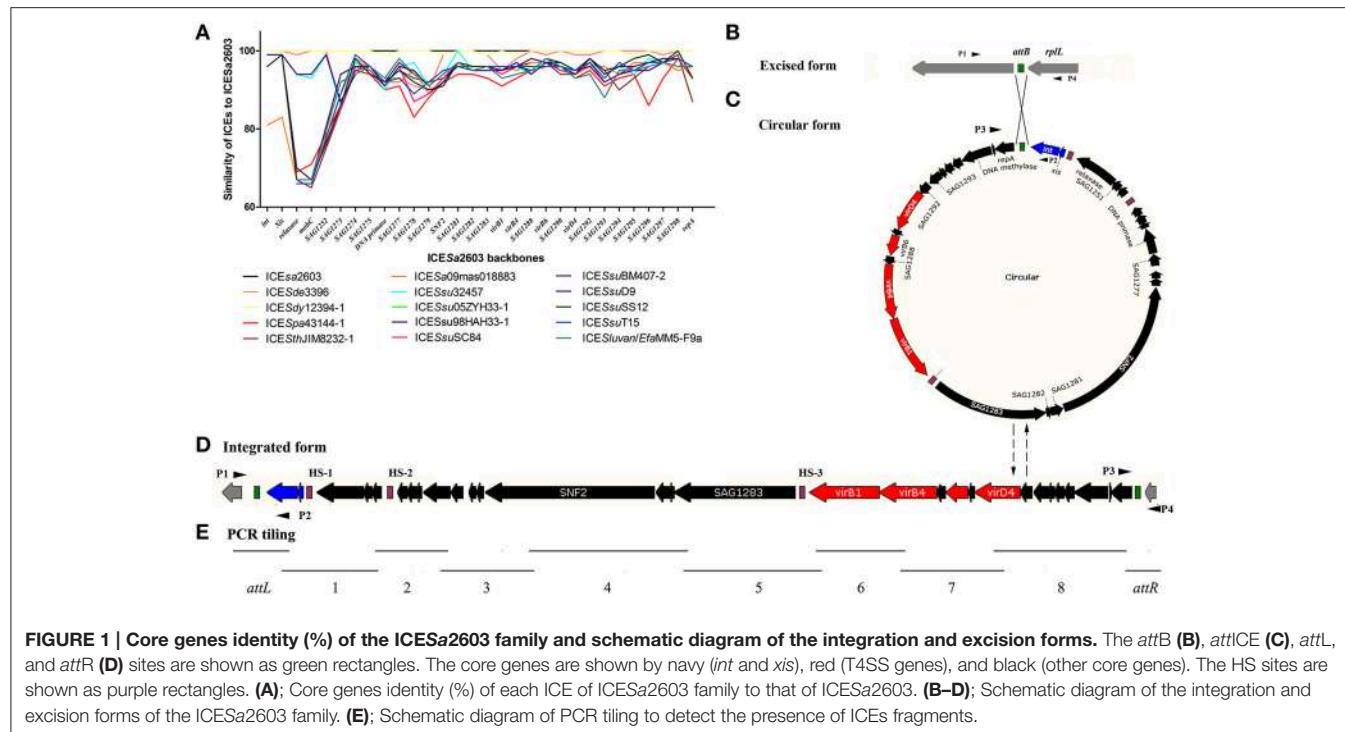


FIGURE 1 | Core genes identity (%) of the ICESa2603 family and schematic diagram of the integration and excision forms. The *attB* (**B**), *attICE* (**C**), *attL*, and *attR* (**D**) sites are shown as green rectangles. The core genes are shown by navy (*int* and *xis*), red (T4SS genes), and black (other core genes). The HS sites are shown as purple rectangles. (**A**): Core genes identity (%) of each ICE of ICESa2603 family to that of ICESa2603. (**B–D**): Schematic diagram of the integration and excision forms of the ICESa2603 family. (**E**): Schematic diagram of PCR tiling to detect the presence of ICEs fragments.

RESULTS

General Features of the ICESa2603 Family

A list of 13 ICEs of the ICESa2603 family and two ICESa2603 family-like ICEs from the NCBI complete genome database were analyzed and compared in this study (Table 1). An ICE that encodes an integrase gene closely related to *int*_{ICESa2603}, defined as having >60% gene or protein homology, and has significant sequence alignment (60% nucleic acid identity of core genes) and syntenic core structure were classified as a member of ICESa2603 family (Bi et al., 2011; Figure 2A) (<http://db-mml.sjtu.edu.cn/ICEberg/index.php>). Although ICESsuT15 and ICESluvan contained the backbone sequences of the ICESa2603 family, its integrase genes belonged to the SR family instead of the tyrosine family site-specific integrase of *int*_{ICESa2603}, and these were referred to as ICESa2603 family-like ICEs (Figure 2B). The strains, which were originally isolated from different countries around the world, belonged to six species of the *Streptococcus* spp. (Table 1).

The ICEs of the ICESa2603 family were first compared by using the MAUVE 2.4 (Darling et al., 2004) and ACT software (Carver et al., 2005) to visualize conserved and variable regions. All ICEs, with sizes ranging from 46,903 bp (ICESa09mas018883) to 89,165 bp (ICESsuSC84), shared a common conserved structure and had variable regions (Table 1 and Figure 2A). The syntenic core structure of the ICESa2603 family backbone (33 kb) contained 30 conserved core genes (Figures 1A, 2). There were three sites within the conserved ICESa2603 family structure for inserting variable DNAs in all of the ICEs (Figure 2). The three sites were named as HS-1, HS-2, and HS-3 (i.e., hotspots) for ICE acquisition of new genes of resistance to

antibiotics and heavy metals. The three hotspots were located in the intergenic region of the ICE backbone, suggesting that the acquisition of these variable DNA regions did not interrupt core ICE gene. Additional DNA was inserted into the 3'-end non-conserved region of gene SAG1277 (Gene ID: 1014084), SAG1278 (Gene ID: 1014085), SAG1279 (Gene ID: 1014086), or SNF2 (Gene ID: 1014087) of some ICEs (Figure 2). The four inserted sites were designated as I-1, I-2, I-3, and I-4, respectively. The function of SAG1277, SAG1278, and SAG1279 remains unclear.

The ICE Core Genes

The present study has identified 30 core genes in 13 ICEs, which comprised the recombination module, the conjugation module, and various other genes of unknown functions. Integrases were members of the tyrosine recombinase family that contained a signature R.. H.. RH.. Y active site residue within its C-terminal catalytic domain. ICESa2603 family integrase *Int*_{ICESa2603} mediates site-specific recombination between identical *attICE* and *attB* sites and integration into the 3'-end of 50S ribosomal subunit protein L7/L12 gene (*rplL*) (Haenni et al., 2010), which was determined to be conserved in all *Streptococcus* spp. (Table S3).

The conjugative transfer module of the ICESa2603 family was grouped into two parts clustered. The first cluster comprised 3 genes (*SAG1250*, *SAG1251*, and *SAG1252* in *S. agalactiae* 2603V/R), which were annotated as *Tn5252 orf4 relaxase*, *Tn5252 orf9 mobC*, and *Tn5252 orf10*, respectively. Protein SAG1250 showed similarities to various prokaryotic DNA relaxases that covalently bind to and nick the DNA at the origin of transfer (*oriT*) with the help of the auxiliary MobC to initiate DNA

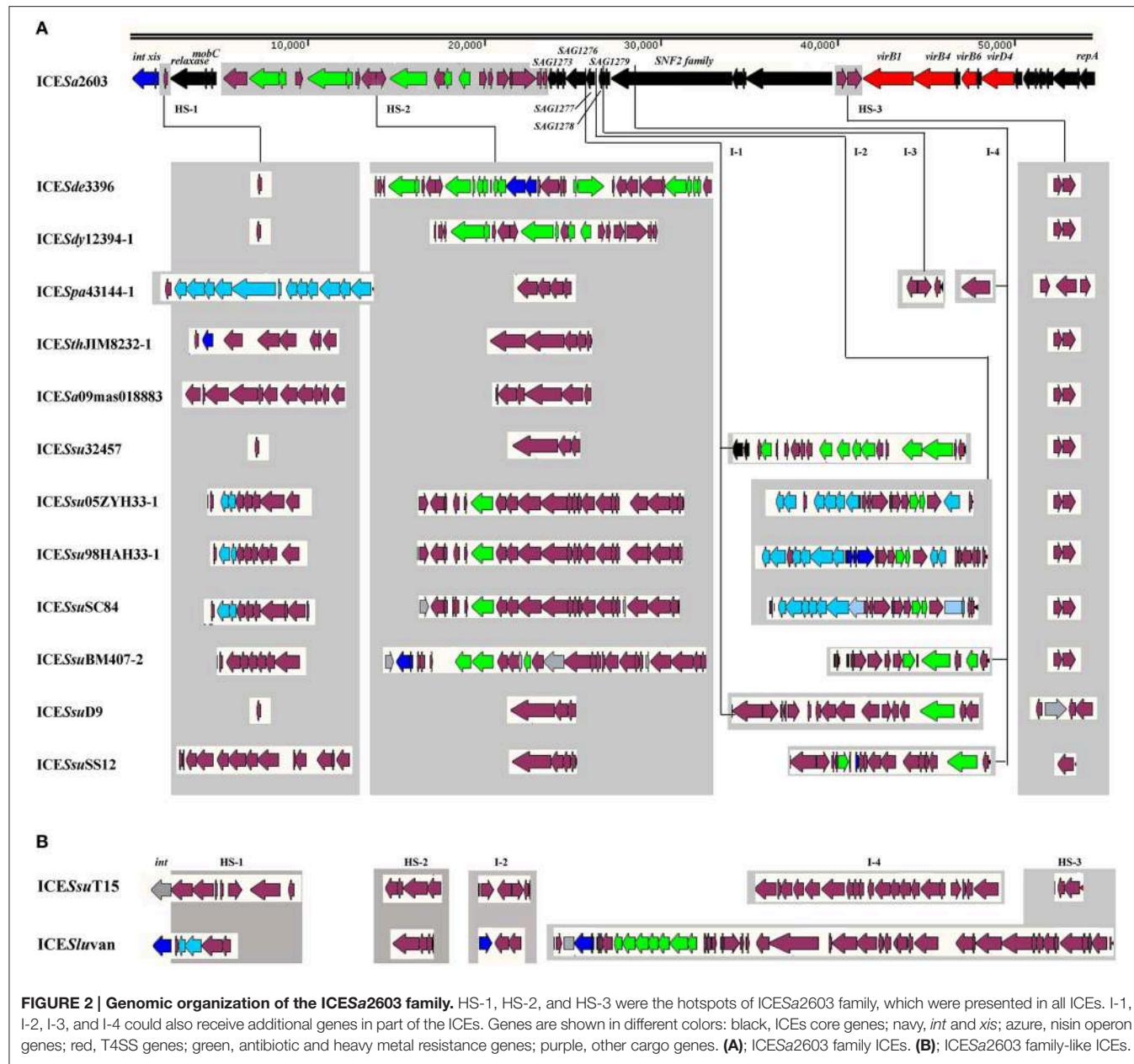


FIGURE 2 | Genomic organization of the ICESa2603 family. HS-1, HS-2, and HS-3 were the hotspots of ICESa2603 family, which were presented in all ICEs. I-1, I-2, I-3, and I-4 could also receive additional genes in part of the ICEs. Genes are shown in different colors: black, ICEs core genes; navy, *int* and *xis*; azure, nisin operon genes; red, T4SS genes; green, antibiotic and heavy metal resistance genes; purple, other cargo genes. **(A)**: ICESa2603 family ICEs. **(B)**: ICESa2603 family-like ICEs.

processing (Lanka and Wilkins, 1995), which include ORF4 relaxase (AAC98434.1) of conjugative transposon *Tn5252* (52% identity and 72% similarity) and Moba (AAQ55244.1) of *S. aureus* plasmid pC223 (22% identity and 41% similarity). Protein SAG1251, which is located adjacent to relaxase, shared 57% identity and 79% similarity to *Tn5252* ORF9 MobC (AAC98435.1) and 27% identity and 50% similarity to MobC (AAQ55243.1) of *S. aureus* plasmid pC223. The other part of the conjugation module belonged to a type IV secretion system (T4SS). The VirD4 coupling protein (40% identity to *Agrobacterium tumefaciens* VirD4), which functions as a substrate receptor, binds to the ICE DNA-relaxase complex (relaxosome) and presents it to the mating pair formation

(Mpf) channels. Compared to *Agrobacterium tumefaciens* Vir T4SS (Christie, 1997), which are assembled from virB1–VirB11, only VirB1 (transglycosylase, 18% identity), VirB4 (ATPase, 41% identity), and VirB6 (inner-membrane protein, 12% identity) have been identified among the Mpf channels of the ICESa2603 family (Figure 2).

Additional conserved genes were located adjacent to the T4SS genes. Upstream of the *virD4* gene were genes coding for cytosine DNA methylase (*SAG1297*), replication initiation factors (*SAG1299*), and other unknown conserved genes, whereas downstream of the cluster were genes of the SNF2 family (*SAG1280*), DNA primase (*SAG1276*), and other unknown conserved genes.

Variable DNA Regions

In addition to conserved core genes in all ICEs analyzed, variable DNA regions were also detected in the ICE backbone sequences (**Figure 2**). Most of the variable DNA sequences were inserted at three intergenic hotspots (HS), namely, HS-1, HS-2, and HS-3. The DNA content of various ICEs in HS was highly identical. For example, ICESa2603, ICESde3396, ICESdy12394-1, ICESsu32457, and ICESsuD9 had identical contents in HS-1. The DNA contents in HS-2 were more variable (only ICESsu32457 and ICESsuD9 shared identical genes), whereas in HS-3, these were more conserved (only ICESpa43144-1, ICESsuD9, and ICESsuSS12 varied from the other ICEs). There were other exogenous DNA insertion sites (I), namely, I-1, I-2, I-3, and I-4 in at the 3'-end of the non-conserved region of genes SAG1277, SAG1278, SAG1279, and SNF2 in some ICEs, respectively. For example, ICESsu32457 and ICESsuD9 had exogenous DNAs in I-1, whereas ICESsu05ZYH33-1, ICESsu98HAH33-1, ICESsuSC84, ICESsuT15, and ICESluvan had variable DNAs in I-2. Additional DNA inserts were identified at the I-3 site in ICESpa43144-1 and at the I-4 site in ICESpa43144-1, ICESsuBM407-2, ICESsuSS12, and ICESsuT15.

Other notable accessory genes in this family were identified as resistance genes for antibiotics and heavy metals, intact or remnant lantibiotic biosynthesis operon, and other MGEs such as IS elements and Tn916-like elements (**Figure 2**). The antibiotic and heavy metal resistance genes in ICEs are presented in **Table 2**. ICESa2603, ICESde3396, and ICESdy12394-1 carry heavy metal resistance genes, whereas ICEs from *S. suis* mostly contain

macrolide resistance gene *erm*(B) and/or tetracycline resistance gene *tet*(M) or *tet*(O). ICESpa43144-1, ICESsu05ZYH33-1, ICESsu98HAH33-1, ICESsuSC84, and ICESluvan had remnant nisin ORFs, in which a two-component signal transduction system nisK/nisR, is essential for full virulence of highly invasive *S. suis* serotype 2 (Li et al., 2008; Wang et al., 2014). In the first four ICEs, remnant nisin ORFs were presented in two variable regions at the insertion sites of HS-1 and I-2 (**Figure 2A**).

MGEs were also found to be inserted in HS or I sites. For example, IS116 and IS4 family IS elements were inserted in the I-4 region of ICESpa43144-1 and ICESsuD9, respectively (**Figure 2A**). Moreover, a Tn916-like element was inserted in HS-2 of ICESsu05ZYH33-1, ICESsu98HAH33-1, ICESsuSC84, and ICESsuBM407-2 (**Figure 2A**). A Tn1549-like element was inserted in I-4 of ICESluvan, thereby forming a mosaic ICE ((Bjorkeng et al., 2013); **Figure 2B**). The integrase of ICESluvan, which belongs to the SR family instead of the tyrosine recombinase family of Int_{ICESa2603}, resulted in the alteration of the insertion site, thus generating a new ICE (ICESa2603 family-like ICE).

The *att* Site and *oriT* Site

ICEs of the ICESa2603 family were found to specially integrate at the 3'-end of the *rplL* gene of *Streptococcus* spp., by recognizing the *att* sequence, TTATTTAAGAGTAAC (**Table 2**). Although different identities of *rplL* gene were observed, the *att* sequence (15 bp from the 3'-end of the *rplL* gene) appeared more conserved in *Streptococcus* spp. (**Table 2** and Table S3), thus

TABLE 2 | Attachment site analysis and resistance profile of the ICESa2603 family.

ICEs name	% Identity to <i>Int</i> ₂₆₀₃	% Identity to <i>rplL</i> ₂₆₀₃	<i>attB</i> (3)	Putative <i>att</i> sites	Resistance profile
ICESa2603	100	100	<i>rplL</i>	TTATTTAAGAGTAAC	Hg, Cd, Cu
ICESde3396	100	—	—	—	Cd, Cu, As
ICESdy12394-1	100	84	<i>rplL</i>	TTATTTAAGAGGTGAT	Cd, Cu
ICESpa43144-1	96	84	hypothetical protein	TTATTTAAGAGTAAC	
ICES <i>thJIM</i> 8232-1	96	88	<i>rplL</i>	TTATTTAAGAGTAAC	
ICESa09mas018883	81	99	<i>rplL</i>	TTATTTAAGAGTAAC	
ICESsu32457	99	— ^a	<i>rplL</i>	TTATTTAAGAGTAAC	
ICESsu05ZYH33-1	96	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>erm</i> (B), <i>aadE</i> , <i>aphA</i> , <i>tet</i> (40), <i>tet</i> (O/W/32/O)
ICESsu98HAH33-1	96	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (M), <i>aadE</i>
ICESsuSC84	96	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (M), <i>aadE</i>
ICESsuBM407-2	96	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (M), <i>tet</i> (L), <i>tet</i> (O), <i>erm</i> (B), <i>aadE</i> , <i>cat</i>
ICESsuD9	99	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (O), <i>erm</i> (B)
ICESsuSS12	96	84	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (O), <i>erm</i> (B)
ICESsuT15 ^b	—	84	SSU0877 in <i>S. suis</i> P1/7	CCTCTTATGTCAAGTACTG (<i>attL</i>) CATTATTATGACACAATCCC (<i>attR</i>)	
ICESluvan ^b	—	— ^a	<i>rumA</i>	CACGTGGAGTGCCTAGTGTT (<i>attL</i>) TTCTCAAGGACCAGACAACAA (<i>attR</i>)	<i>van</i> resistance operon
ICESsuHB1011 ^c	98	—	<i>rplL</i>	TTATTTAAGAGTAAC	<i>tet</i> (O), <i>erm</i> (B)

^aThe *rplL* sequences were not indicated.

^bThe ICESsuT15 and ICESluvan's *Int* belonging to serine recombinase (SR) family shared low homology with the ICESa2603 tyrosine family site-specific integrase. ICESluvan was inserted in *rum*, and the *att* site was experimentally confirmed by Bjorkeng et al. (2013). ICESsuT15 was inserted in the 344 sites of SSU0877 in *S. suis* P1/7, the putative *att* site was predicted, and the *Int* of ICESsuT15 and ICESluvan may recognize CA dinucleotide in certain region of chromosome sites.

^cICESsuHB1011 sequence was not complete and the *rplL* sequences were not known.

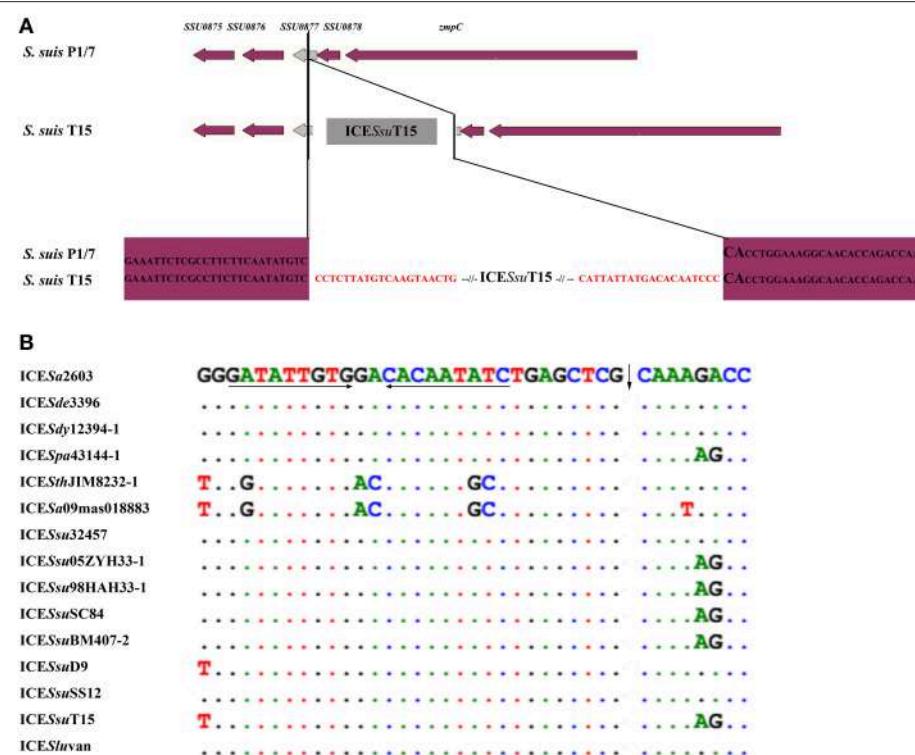


FIGURE 3 | att site of ICESSuT15 (A) and oriT site of the ICESA2603 family (B). (A): ORFs in purple represent the chromosome genes while in gray shows the integraing site of ICESSuT15, the putative *att* sequence was indicated in red. (B): the *oriT* site of the ICESA2603 were listed in the top, the bases different from ICESA2603 were indicated. Arrows under the sequences represent the locations of inverted repeats, and vertical arrows show the nick sites.

providing the sequence basis for its distribution among members of the family. ICESsuT15 and ICESluvan with Int and belonging to SR instead of tyrosine family integrase of ICESa2603 family were assumed to have a different *att* site. As shown in Figure 3A, there were no consensus insertion sequences have been identified in ICESsuT15.

A ~200 bp highly conserved region (92–100% identity) highlighted in the visual comparison with Mauve 2.4 (Darling et al., 2004) is considered to be the putative the *oriT* region. The left adjacent region was SAG1252 (*Tn5252 orf10*, 65–100% identity) gene that showed relatively low identity and the right adjacent region was a variable DNA hotspot site. In all ICEs except for ICES*th*JIM8232-1 and ICES*a*09mas018883, the *oriT* site contained a 9-nt inverted repeat sequence, GATATTGTG, which served as a binding site for relaxase, and the precise nickase cleavage site was predicted to reside at position 5'-CTCG/CAAA (Figure 3B).

Phylogenetic Analyses of the ICE Core Genomes

To analyze the evolution of the ICE core genomes, we first compared the identity of each ICE core genes to the corresponding ICESa2603 genes (**Figure 1A**). Most ICE core genes exhibited a >90% identity at the nucleotide level. On the other hand, the less conserved genes were located at the junction between core genes and variable region. For example, the

DNA processing genes (*SAG1250*–*SAG1252*) of the conjugative transfer module between HS-1 and HS-2 were much less conserved (65–72%) than other core genes, whereas the *oriT* site remained highly conserved.

Next, we generated phylogenetic trees based on all the 30 core genes (**Figure 4A**) or individual representative genes (**Figure 4B**). The ICEs were then further divided into four subgroups: ICESa2603 family groups I-III (ICESa2603-like, 89K-like, and ICESthJIM8232-1-like) and ICESa2603 family-like group IV. Interestingly, these subgroups also showed variable features: The ICEs of group IV could be distinguished from that of other groups based on the *int*, only one regulatory gene (*SAG1249*) was found in HS-1 region of group I ICEs (ICESa2603-like); and a transposon Tn916 was inserted into HS-2 region of group II ICEs, thereby generating mosaic ICEs (**Figures 2, 4A**).

To gain more insights into the evolution of individual core genes, we created phylogenetic trees for each core gene. However, most core genes were determined to be highly conserved, thus making it difficult to generate a reliable tree. Hence, we concentrated on the less conserved genes and selected *int*, *relaxase*, and *mobC* for phylogenetic analyses. **Figure 4B** shows the two subgroups of *int*: the tyrosine recombinase family *int* genes, which were highly conserved and present in all ICESa2603 families (88–100% identity), and the SR family *int* genes that were present in ICESa2603 family-like ICEs. According to the tree of

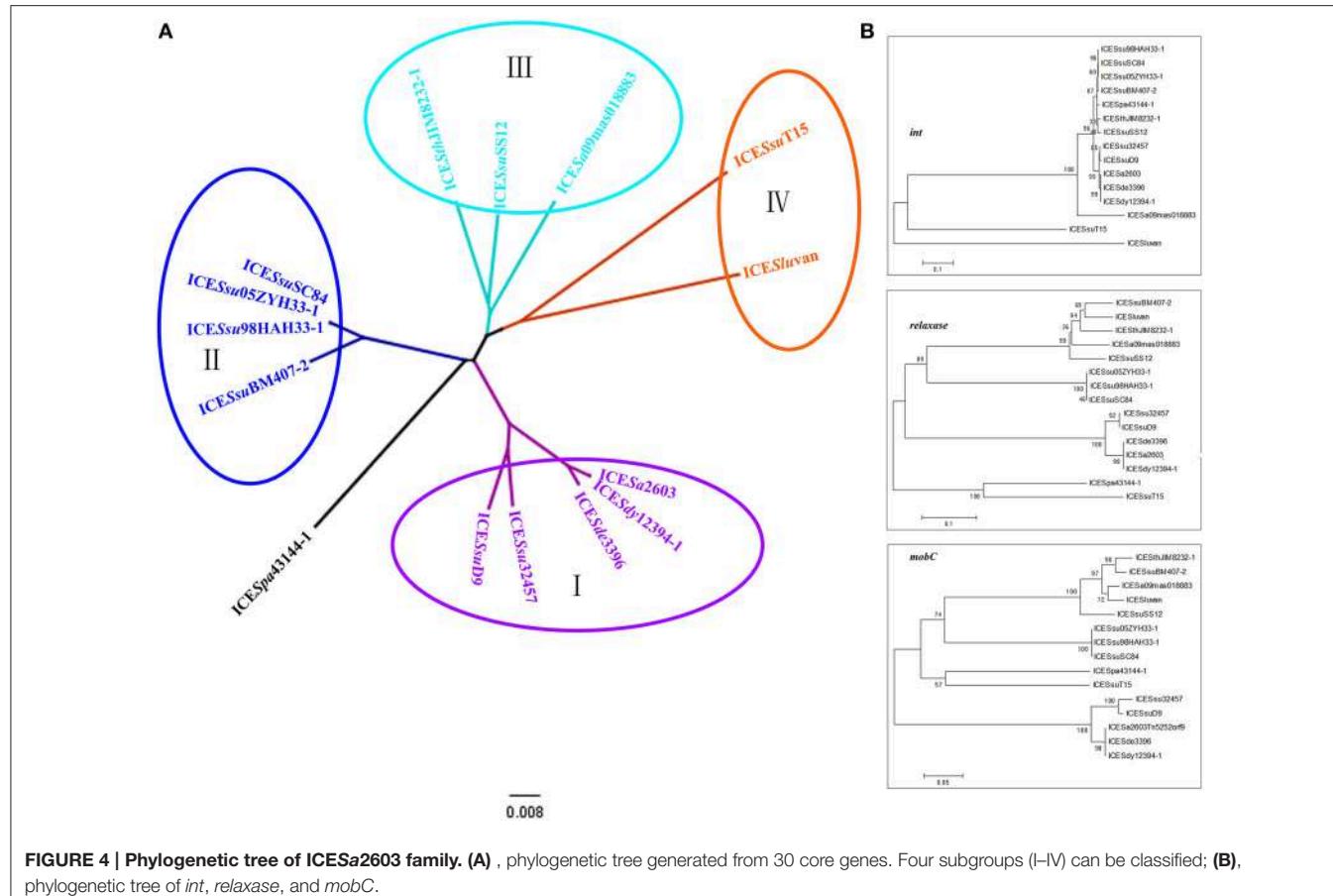


TABLE 3 | Distribution of ICESa2603 family core genes in *S. suis* and *S. agalactiae*.

Strains	<i>int</i> _{ICESa2603}	DNA primase	T4SS genes				<i>repA</i>	ICE at <i>rplL</i> site confirmed by PCR ^a
			<i>virB1</i>	<i>virB4</i>	<i>virB6</i>	<i>virD4</i>		
<i>S. suis</i> (<i>n</i> = 73) ^b	25	65	32	32	32	31	30	22
<i>S. agalactiae</i> (<i>n</i> = 21) ^c	5	19	8	8	8	8	8	5

^aThe extra chromosomal form and integrating form of ICEs can be seen in Figures 1B–D.

^b*S. suis* were isolated from swine in China (Table S1).

^c*S. agalactiae* were isolated from bovine in China (Table S1).

relaxase and *mobC*, all ICEs in group I were aligned together and shared ~94% similarity, whereas other ICEs generated distinct branching patterns (with <80% similarity).

Distribution of Members of the ICESa2603 Family in *S. suis* and *S. agalactiae*

To evaluate the presence of ICEs in *S. suis* isolates from pigs and *S. agalactiae* isolates from cows in China, several core ICEs genes were detected (Table 3 and Table S1). Of the 73 *S. suis* isolates screened, 31 were determined to contain ICEs based on the presence of T4SS genes (*virB1*, *virB4*, *virB6*, and *virD4*), in which 80.5% (25/31) also carried *int*_{ICESa2603}. Similarly, 38.1% of the tested *S. agalactiae* isolates harbored T4SS genes, amongst which 62.5% (5/8) were *int*_{ICESa2603}-positive.

Different primer pairs P1–P4 were employed and identified the integration/excision form of ICESa2603 family in the present study (Table 3). Table 3 shows that 88% (22/25) of the ICESa2603 family strains were excised from the chromosomes, thereby forming circular intermediates prior to its conjugative transfer into recipient cells.

To determine whether the ICEs were intact, PCR tiling was performed. Ten overlapping fragments were amplified using the long-PCR method (Figure 1E and Table S2). Table 4 and Table S1 showed that almost all fragments of ICE core regions (fragments 4, 6, 7, and 8) could be amplified in macrolide- and tetracycline-resistant *S. suis*, whereas other fragments (1, 2, 3, 5) linking the variable regions (HS-1–HS-3 and I-1–I-4) were more variable. PCR tiling also detected various ICE

TABLE 4 | Analyses of ICESa2603-family fragments in part of macrolide and tetracycline resistant *S. suis* by PCR tiling assay.

Fragments ^a	attL	1	2	3 ^b	4	5	6	7	8	attR
HB1004	+	+	-	-	+	+	+	+	+	+
HB1006	-	-	-	-	+	+	+	+	+	+
HB1011 ^c	+	+	+	-	+	+	+	+	+	+
HB1012	+	+	-	-	+	+	+	+	+	+
HB1013	-	-	-	-	+	+	+	-	-	-
NJ4	+	-	-	-	+	+	+	-	-	-
JS07002	+	+	-	-	+	-	+	+	+	+
JS07015	+	+	-	-	+	+	+	+	+	+
SC05017	-	-	-	-	+	+	+	+	+	-
SS2-XY	+	-	-	-	+	+	+	+	+	+
YY060816	-	-	-	-	+	+	+	+	+	-

^aThe PCR tiling assay and fragments can be seen in **Figure 1E**.

^bFailure of amplifying this fragment, probably due to too long additional DNA segments inserted in the 4 regions (I-1 to I-4)

^cThe fragment 7 in HB1011 was ~2 kb larger than ICESa2603 core region.

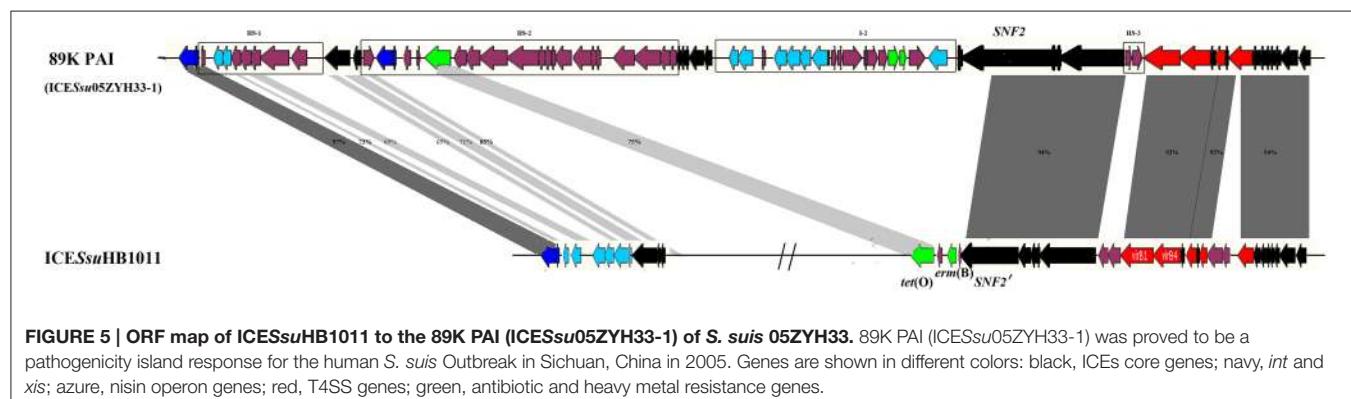


FIGURE 5 | ORF map of ICESsuHB1011 to the 89K PAI (ICESsu05ZYH33-1) of *S. suis* 05ZYH33. 89K PAI (ICESsu05ZYH33-1) was proved to be a pathogenicity island response for the human *S. suis* Outbreak in Sichuan, China in 2005. Genes are shown in different colors: black, ICEs core genes; navy, *int* and *xis*; blue, aztreonam operon genes; red, T4SS genes; green, antibiotic and heavy metal resistance genes.

subgroups. For example, the strain with fragments 2–8, but without fragments *attL*, 1, and *attR*, belonged to subgroup IV (ICESa2603 family-like); Furthermore, subgroup I could be distinguished from other subgroups based on the size of fragment 1.

ICESsuHB1011, a Swine Origin 89K-Like Subgroup Transferable ICEs

Because ICEs play an important role in the horizontal transmission of antibiotic resistance, we selected a swine-derived clinical macrolide- and tetracycline-resistant strain HB1011 (ST303, a new ST type) for subsequent conjugation experiments. The transfer of macrolide and tetracycline resistance from HB1011 to *S. suis* BAA-853 (ST1) was confirmed by sequencing of the PCR products (Figures S1, S2) and MLST typing. The MICs of erythromycin and tetracycline and PCR products of *erm(B)* and *tet(O)* in conjugant JH-1 confirmed that erythromycin and tetracycline resistance genes *erm(B)* and *tet(O)* were indeed transferred (Table S4). We further sequenced the tiling fragments of ICESsuHB1011, which is an 89K-like ICE (**Figure 5**). The results showed that *erm(B)* and *tet(O)* genes were inserted into the SNF2 gene (I-4, **Figure 1**).

DISCUSSION

ICEs are self-transmissible MGEs that encode the machinery for conjugation, as well as intricate regulatory systems to control excision from chromosomes and its conjugative transfer (Wozniak and Waldor, 2010). ICEs can be considered as mosaic elements that present the combined features of plasmids and bacteriophages. Similar to plasmids, ICEs transfer via conjugation and in parallel to phages, ICEs integrate into and replicate with its host chromosome (Burrus and Waldor, 2004). ICEs often carry accessory genes that encode for antibiotic resistance, virulence factors and various other functions. These are therefore important vectors for the horizontal dissemination of genetic information, thereby facilitating rapid bacterial evolution.

ICESa2603 is a ~54 kb ICE that was first discovered in *S. agalactiae* 2603V/R (Tettelin et al., 2002). It has the capacity to retain its features of transferability to *S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. uberis* (Haenni et al., 2010). Subsequently, ICESa2603-like ICEs have been detected in clinical isolates of *Streptococcus* spp. from both human and animal species from Europe and Asia (**Table 1**). Therefore, it is likely that the members of the ICESa2603 family have

been distributed and have the ability to undergo horizontal transmission in *Streptococcus* spp. The number of ICESa2603 family ICEs and its host range may therefore further expand as additional research studies are currently being conducted. However, comparative genomics of ICESa2603 family ICEs and its evolution have not been reported. Here, we first comparatively analyzed 13 ICEs of the ICESa2603 family. The results showed that the ICESa2603 family has a backbone of 30 core genes that encode the core functions of integration/excision, conjugation and regulation.

The integration/excision modules of ICESa2603 consisted of Int_{ICESa2603} (SAG1247) and Xis_{ICESa2603} (SAG1248). Int_{ICESa2603} belongs to the tyrosine based site-specific recombinases. This family of integrase originated from bacterial phages and conjugate transposons. One member is the integrase from *Bacillus subtilis* conjugative transposon ICEBs1, which mediates integration into the 3'-ends of tRNA-Leu loci (Lee et al., 2007). Int_{ICESa2603} shares 23% identity with Int_{ICEBs1}, but mediate site-specific recombination between identical attICE and attB sites and the integration into the 3'-end of the 50S ribosomal subunit protein L7/L12 gene (Haenni et al., 2010). The attB sites (TTATTTAAGAGTAAC) of ICESa2603 family is conserved in *Streptococcus* spp. and even *Enterococcus faecium* and *Enterococcus faecalis*, which provided the sequence basis for the horizontal transfer of this family. To date, members of the ICESa2603 family has been identified in six species of *Streptococcus* spp. (**Table 2**). The ICESa2603 family-like ICEs, share 28 of 30 core genes with ICESa2603 family ICEs, which contain the SR family integrase, although a low level of identity was observed between ICESluvan and ICESSuT15 (**Figures 1A, 4B**). A study on another SR Int_{Tn5397} has shown that the insertion site contains a central GA dinucleotide; however, no other consensus insertion sequences have been identified (Wang and Mullany, 2000). Based on the experimental transfer of ICESluvan (Bjorkeng et al., 2013) and the analysis of ICESSuT15 (**Figure 3A**), we believe that Int of ICESluvan and ICESSuT15 recognize the CA dinucleotide in certain regions of chromosomal sites. The ICESluvan is capable of transmission, which provides an illustration that acquisition of a new integration/excision module (most likely via recombination) generates new types of ICEs (here we termed as ICESa2603 family-like ICEs). Xis, the adjacent recombination directionality factor (RDF), both facilitates excision and inhibits integration. It binds to the ends of the integrated element adjacent to Int binding sites. Deletion of the xis gene of ICESSu05ZYH33-1 demonstrated that xis stimulates, but is not essential for ICESSu05ZYH33-1 excision from chromosomes (Li et al., 2011).

The conjugation module of ICESa2603 family is similar with that of plasmids. When ICESa2603 transfers, a relaxase (SAG1250) first binds to and excises circular ICE DNA at oriT sites (Li et al., 2011). Thereafter, with the help of a coupling protein, MobC (SAG1251), the relaxase-DNA complex is presented to type IV coupling protein (T4CP) and the Mpf complex. Finally, the T4CPMpf complex transfers the ICE DNA to the receipt cell. The T4SS complex includes genes encoding for proteins similar to *A. tumefaciens* VirB1, VirB4, VirB6, and VirD4. Zhang et al. reported that these T4SS gene clusters were

conserved both in composition and order in the genomes of various *Streptococcal* species and thus collectively described these as a Type-IVC secretion system (Zhang et al., 2012). Knockout of two key components genes (i.e., virD4 and virB4) of the Type-IVC secretion system not only abolished its transferability but also eliminated the lethality of the highly virulent strain and impaired its ability to trigger a host immune response in infected mouse models (Li et al., 2011; Zhao et al., 2011). These results suggest that T4SS has retained its ancestral function as a conjugation module while its capacity to translocate effector protein(s) to the cell surface or into eukaryotic target cells undergoes evolution.

Our understanding of the mechanism underlying the regulation module of the ICESa2603 family is limited. Comparative analysis of the 30 core genes of ICESa2603 family ICEs has shown that no common regulators were conserved in all ICEs of this family. Instead, a gene (SAG1249) encoding a Cro/CI family transcriptional regulator was detected in all experimentally proven transferable ICEs, despite not being classified as core genes in the present study. This regulator possibly regulates ICE gene expression and ICE transfer (Beaber and Waldor, 2004). Recently, Xu et al. described a two-component system, NisK/NisR, in subgroup II (89K-subgroup, **Figure 4A**) ICEs that contributes to the virulence of *S. suis* serotype 2 (Xu et al., 2014). It is tempting to know whether this two-component system is involved in the regulation of transfer. A bacteriophage abortive infection (Abi) system, AbiE, which assists bacteria from being killed by bacteriophages, has been recently identified as a type IV toxin-antitoxin system in ICESa2603 (SAG1284 and SAG1285) (Chopin et al., 2005; Dy et al., 2014). Dy et al. (2014) demonstrated that the AbiE system enables plasmid maintenance when these were introduced into pUC19. It is possible that the abiE operon and native promoter enable the maintenance of the circular form of ICESa2603, thereby increasing the frequency for ICE transfer.

Phylogenetic and comparative analyses have shown that most of the core genes of the ICESa2603 family exhibited 91–100% identity at the nucleotide level (**Figure 1A**). No detectable difference in the degree of conservation of most core genes was observed, which suggests equal selective pressures on core genes of the ICEs. However, some genes (i.e., Int, xis, relaxase, mobC, and SAG1278) exhibited lower degrees of conservation. The difference in the identity of the Int and xis genes, as earlier described, was probably generated by recombination. The relaxase and mobC genes can be divided into four subgroups (**Figure 4B**). The relaxase and mobC genes exhibited a high level of identity within subgroups (>80%), but a low identity between subgroups (65–80%). These results suggest that the different subgroups of relaxase and mobC genes (DNA processing module) might have also been generated by recombination of the entire DNA processing module. The relative low level of conservation of SAG1278, in which of the 3'-end is often the insertion site (I-2), might have been ascribed to the insertion of accessory genes, as indicated by the lower level of identity of this gene in each ICE. These results also suggest that individual core genes are exposed to different evolutionary pressures.

BLAST analysis of the core conjugation and integration module proteins of the ICESa2603 family showed that ICESa2603 shared some similarity with the *Streptococcus pneumoniae* conjugative transposon Tn5252 (Alarcon-Chaidez et al., 1997). Based on limited knowledge, we propose that ICEs were disseminated across a diverse range of hosts, thereby resulting in exposure to different selective stress. To survive stress, cargo genes encoding antibiotic resistance, nitrogen fixation, virulence factors, and various other functions were introduced into the HS and I sites. In the case of the ICESa2603 family, insertion of variable DNAs might have disrupted the core regulatory module gene (*SAG1249*), but another regulatory module gene could be functioned to compensate and restore stability in the chromosome (Li et al., 2008, 2011). Another benefit is that recombination of the core module increases the adaptability and diversity of ICEs or MGEs. Examples include the ICESa2603 family-like ICEs, ICESluvan, in which only the conjugation module *int_{Tyr-xis}* was replaced by SR family *int_{SR}* yet retained its transferability (Bjorkeng et al., 2013). A very recent study (Marini et al., 2015) has shown that mating between *S. suis* 32457 (donor) and *S. agalactiae* 2603V/R (recipient) yields a hybrid ICE, ICESa2603/ICESsu32457, and interestingly, could be transferred across various *Streptococcus pyogenes* strains. These findings strongly support the concept that recombination plays an important role in horizontal gene transfer and generates novel ICEs or even new types of MGEs. These findings and the above mentioned recombination/insertion/hybrid events further broaden range of ICE diversity. The mosaic patterns of the ICESa2603 family consisted of genes from groups A, B, and G *Streptococcus* organisms, *Faecalibacterium*, enterococci, and possibly *Listeria innocua* and *Enterococci faecalis*, indicating that recombination and mobilization events are key factors in the assembly of these families. The exchange of integration modules among ICEs or between ICEs and other integrating MGEs (e.g., phages) could potentially create new ICEs with altered insertion site specificities or modified host ranges.

Figure 4A shows that all four subgroups of the ICESa2603 family were detected in *S. suis*, which is indicative of its important role in the transmission of these types of ICEs of *S. suis*. Screening of ICESa2603 core genes showed that 42.5% of swine-derived *S. suis* isolates contained ICE T4SS genes and 80.5% of these belong to ICESa2603 family. These results suggest that *S. suis* isolates harbor ICESa2603 family ICEs that are extensively distributed in swine in China, which has become a challenge to the hog industry and even a threat to human health.

89K-like subgroup ICEs (ICESsu98HAH33-1 and ICESsu05ZYH33-1) were detected in two *S. suis* isolates and have caused two extensive human outbreaks of streptococcal toxic shock-like syndrome (STSLs) in China in 1998 and 2005 (Tang et al., 2006). Further studies (Li et al., 2008; Zhao et al., 2011; Zhong et al., 2014) have demonstrated that 89K is a pathogenicity island (PAI). Li et al. (2008) earlier showed that a two-component signal transduction system (SalK/SalR) is essential for full virulence of highly invasive *S. suis* serotype 2. SalK/SalR, which is also the regulatory module of the nisin

lantibiotic operon, was inserted into the HS-1, and possibly acts as a regulator, together with SAG1249. Furthermore, knockout of three key components (*virB1*, *virB4*, and *virD4*) of T4SS significantly reduces but does not abolish the virulence of *S. suis* in a mouse model (Zhao et al., 2011; Zhong et al., 2014). These findings indicate that T4SS harbored by 89K PAI contributes to the development of STSLs and mediates the horizontal transfer of 89K. However, those types of ICEs did not contain genes that encode resistance to macrolides and tetracycline. In the present study, we observed that almost all of the macrolide- and tetracycline-resistant *S. suis* also contained T4SS genes, indicating that ICEs play an important role in spreading macrolide and tetracycline resistance in *S. suis*. The isolated strain HB1011, which was determined to harbor ICESsuHB1011 that carried *erm(B)* and *tet(O)*, which was indicative of its multiple functions, including horizontal transfer resistance and pathogenicity. In addition, other strains of *S. agalactiae* and *S. dysgalactiae* subsp. *equisimilis* whose ICEs harbored T4SS that was similar to 89K did not cause STSLs. Therefore, it is of great importance to identify factors and mechanisms by which the T4SS of 89K mediates pathogenicity. It has been proposed that ICESa2603 family T4SS might have retained its ancestral function as a mobile DNA element, but have also evolved its capacity to translocate effector protein(s) to the cell surface or into eukaryotic target cells (Bhatti et al., 2013).

CONCLUSIONS

In summary, the structures of the ICESa2603 family ICEs in isolates of different *streptococcal* species are clearly illustrated in this study by comparative analyzing 13 ICEs of the ICESa2603 family and two ICESa2603 family-like ICEs. Screening of ICESa2603 core genes shows that ICESa2603 family ICEs are distributed widely in *S. suis* and *S. agalactiae* in China and mainly belong to the 89K-subtype family ICEs. Furthermore, the spread of the ICEs containing resistance genes *erm(B)* and *tet(O)* among *S. suis* strains by conjugation had a profound effect on the horizontal transmission of antibiotic resistance to macrolides and tetracyclines. Further research is needed to characterize the mechanisms involved in triggering ICEs transfer intra- and inter-species.

AUTHOR CONTRIBUTIONS

JH, DG, and LW developed the concept and designed experiments. JH, YL, KS, and LG performed the experiments and collected the data. JH, YL, and JK conducted all bioinformatics analyses. JH and LW prepared the manuscript. All authors have contributed to, seen and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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- blaCMY-2 in *Proteus mirabilis*. *J. Antimicrob. Chemother.* 66, 2266–2270. doi: 10.1093/jac/dkr286
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Bacillus cereus efflux protein BC3310 – a multidrug transporter of the unknown major facilitator family, UMF-2

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Phylogenetic classification divides the major facilitator superfamily (MFS) into 82 families, including 25 families that are comprised of transporters with no characterized functions. This study describes functional data for BC3310 from *Bacillus cereus* ATCC 14579, a member of the “unknown major facilitator family-2” (UMF-2). BC3310 was shown to be a multidrug efflux pump conferring resistance to ethidium bromide, SDS and silver nitrate when heterologously expressed in *Escherichia coli* DH5α ΔacrAB. A conserved aspartate residue (D105) in putative transmembrane helix 4 was identified, which was essential for the energy dependent ethidium bromide efflux by BC3310. Transport proteins of the MFS comprise specific sequence motifs. Sequence analysis of UMF-2 proteins revealed that they carry a variant of the MFS motif A, which may be used as a marker to distinguish easily between this family and other MFS proteins. Genes orthologous to *bc3310* are highly conserved within the *B. cereus* group of organisms and thus belong to the core genome, suggesting an important conserved functional role in the normal physiology of these bacteria.

Keywords: MFS, drug resistance, efflux protein, *Bacillus cereus*, UMF-2

Introduction

Bacillus cereus sensu stricto (*B. cereus*) is a Gram-positive, endospore forming organism known to cause foodborne illness in humans. It is a member of the *B. cereus* group of bacteria (*Bacillus cereus sensu lato*) that, in addition to *B. cereus* encompasses the species *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. cytotoxicus* (Kolsto et al., 2009; Guinebretiere et al., 2013). The *B. cereus* group members are genetically closely related with high level of syntheny (conserved gene order). The high similarity results in an intertwinement of the *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* branches in the phylogenetic tree (Ash et al., 1991). However, the *B. cereus* group organisms exhibit different phenotypes, inhabit diverse ecological niches and are pathogenic against different hosts. The three species *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* are regarded as non-pathogenic. *B. anthracis* is the causative agent of anthrax in humans and animals (Mock and Fouet, 2001). *B. thuringiensis* is

an insect pathogen that is commercially used as a biopesticide (Melo et al., 2014). *B. cytotoxicus* causes enteritis in humans and is thermotolerant and highly cytotoxic (Guinebretiere et al., 2013). In the natural environment *B. cereus* is found as a saprophyte in soil, associated with the rhizosphere of plants and in the gut of invertebrates (Jensen et al., 2003; Berg et al., 2005). Even though *B. cereus* is most frequently associated with food-borne enteric infections in humans, it is able to cause other local or systemic infections such as endophthalmitis, cutaneous infections, endocarditis, central nervous system infection, or bacteremia (Steen et al., 1992; Callegan et al., 1999; Centers for Disease Control and Prevention, 2005; Callegan et al., 2006; Martinez et al., 2007; Kim et al., 2010; Sasahara et al., 2011; Stevens et al., 2012). Clinically serious infections of *B. cereus* are treated with antibiotics such as carbapenems, clindamycin, ciprofloxacin, and vancomycin (Kerrick et al., 1990; Bottone, 2010; Uchino et al., 2012; Matsuda et al., 2014). However, resistance against carbapenem and clindamycin has been reported, which eventually led to failed treatments including cases with fatal outcomes (Kerrick et al., 1990; Kiyomizu et al., 2008; Savini et al., 2009; Uchino et al., 2012).

According to the transportdb database, the *B. cereus* group strains constitute between 390 and 455 transporters per strain (Ren et al., 2007; Ren and Paulsen, 2007). The unusually high number of transporters per *B. cereus* group strain may reflect the different lifestyles of these bacteria. Importantly, each group member contains approximately 100 transporters, predicted to efflux drugs.

Drug efflux systems are part of the resistance machinery to counteract antibiotics (Sun et al., 2014). They are divided into six different transporter superfamilies: (i) MFS (major facilitator superfamily); (ii) ABC (ATP binding cassette) transporter superfamily; (iii) MATE (multidrug and toxic compound extrusion) family; (iv) RND (resistance nodulation division) family; (v) DMT (drug/metabolite transporter) superfamily, and (vi) PACE (proteobacterial antimicrobial compound efflux) (Poole, 2007; Hassan et al., 2015). Of these, MFS pumps constitute the majority of efflux transporters encoded in *B. cereus* group strains, typically more than 50 per strain. The MFS comprises secondary transporters that use the electrochemical gradient of protons or sodium ions across the cell membrane to energize substrate transport, including drug efflux (Pao et al., 1998; Saier et al., 1999; Reddy et al., 2012). The ‘transporter classification system’ (see <http://www.tcdb.org/>) classifies the MFS into 82 families. With respect to drug efflux pumps, the drug:H⁺ antiporter families (DHA)1 to 3 are the largest and best investigated drug exporter families in the MFS (Saier et al., 2014).

In this study, we characterize the phylogenetic and some functional properties of the putative multidrug transporter BC3310 from *B. cereus* ATCC 14579. BC3310 was classified by *in silico* analysis as a member of the major facilitator superfamily and the phylogenetic relationship within this group was determined. A deletion mutant of *bc3310* was constructed and overexpression of BC3310 allowed for functional characterization in a heterogenous host as well as purification and partial biochemical characterization *in vitro*.

Materials and Methods

Bioinformatics Analyses

Bacterial sequence information was collected using the IMG homepage from the Joint Genome Institute (Markowitz et al., 2012). Sequence alignments were performed using MEGA MUSCLE alignment with default settings (Tamura et al., 2013) and the phylogenetic tree was constructed using MrBayes (Ronquist et al., 2012). Prediction of the transmembrane helices was done by submitting the primary protein sequence of BC3310 (UniProt Q81B77) to HMMTOP (Tusnady and Simon, 2001).

Construction of *B. cereus* bc3310 Deletion

Mutant

A markerless mutant of *bc3310* was constructed as described (Simm et al., 2012) in the *B. cereus* ATCC 14579 wild type according to the method of Janes and Stibitz (2006) and using the primers listed in Table 1. The *B. cereus* plasmid pBClin15 was lost during the process of making the markerless mutant and therefore a plasmid cured strain was used for phenotypic comparison as in previous investigations (Voros et al., 2013). The presence of the deletion was confirmed by sequencing. *B. cereus* was grown in LB medium at 30°C, unless otherwise stated.

Escherichia coli BC3310 Expression Constructs

The expression levels of genes cloned into pTTQ18-based plasmids are inducible by isopropyl β-D-thiogalactopyranoside (IPTG). Furthermore, the genes are fused with a sequence coding for a C-terminal (His)₆ tag for identification and

TABLE 1 | Primers used in this study.

Primer for	Sequence (5' → 3')
Overexpression in pTTQ18	
pTTQ18-bc3310F	CATGGATCCATCGCTTTACTTTGGATTATGG
pTTQ18-bc3310R	CCGCCTGCAGCGTTGTTGTATGCC
D105 mutants	
bc3310_D105N_f	GATTCTAGTGGAGTTGGAAATCATATGCTTCATGTC GGAAC
bc3310_D105N_r	GTTCCGACATGAAGCATAATGATTCCAATCTCAACTAG AAATC
bc3310_D105A_f	GATTCTAGTGGAGTTGGAGCTATATGCTTCATGTC GGAAC
bc3310_D105A_r	GTTCCGACATGAAGCATAATGAGCTCCAATCTCAACTAG AAATC
bc3310_D105E_f	GATTCTAGTGGAGTTGGAGAACATATGCTTCATGTC GGAAC
bc3310_D105E_r	GTTCCGACATGAAGCATAATGTTCTCCAATCTCAACTAG AAATC
Deletion mutant	
dbc3310_5'_f	CGCGGATCCATGAACAAAATATATTAC
dbc3310_5'_r	CAATTCCTCCAAAAAGTAAACGCAT
dbc3310_3'_f	GTTTACTTTGGAGGGAAATTGAAGTAA
dbc3310_3'_r	ACGCGTCGACTAGTTGATACCTGTTC

purification of the expressed protein. The plasmid construct pTTQ18-bc3310 (pbc3310) was made by general molecular biology techniques according to Sambrook and Russell (2001) by amplifying the gene *bc3310* from genomic DNA of *B. cereus* ATCC 14579 using the primers listed in Table 1. The plasmids for expressing BC3310 D105 mutants pbc3310D105A, pbc3310D105N, and pbc3310D105E were made using sequence and ligation-independent cloning (Li and Elledge, 2007). The presence of each mutation was confirmed by sequencing. The *E. coli* strain DH5 α $\Delta acrAB$ (Simm et al., 2012) carrying pTTQ18 empty vector or the overexpression plasmids was made for minimal inhibition concentration (MIC) testing. For protein purification the *E. coli* strain BL21 was transformed with pbc3310.

Escherichia coli strains harboring plasmids were grown in 50 or 250 ml LB medium with ampicillin ($100 \mu\text{g ml}^{-1}$) at 37°C and 180 rpm in 250 ml or 1 l baffled flasks or on LB agar plates at 37°C , unless otherwise stated.

MIC Tests

Overnight cultures of *B. cereus* ATCC 14579 (without pBClin) and *B. cereus* $\Delta 3310$ or *E. coli* DH5 α $\Delta acrAB$ (Simm et al., 2012) with relevant plasmid were inoculated 1:100 and grown to an OD₆₀₀ between 0.8 and 1.0 at 37°C and 180 rpm. These pre-cultures were diluted to a final OD₆₀₀ of 0.02. The test was performed at least three times in duplicate in microtiter plates and antibiotics were added in a 2-fold serial dilution. For susceptibility assay using *E. coli* strains $100 \mu\text{g ml}^{-1}$ ampicillin and 0.01 mM IPTG were added to all cultures. The cultures were incubated at 37°C for 20–24 h and visually inspected for growth. The lowest concentration, at which no growth was observed, was determined as the MIC.

Ethidium Bromide Accumulation Assay

Escherichia coli strains DH5 α $\Delta acrAB$ with the plasmids pTTQ18 and pbc3310 were grown on LB agar plates supplemented with $100 \mu\text{g ml}^{-1}$ ampicillin and 0.01 mM IPTG at 37°C overnight. Cells were collected with a loop and resuspended in PBS supplemented with 0.4% glucose (pH 7.7.4) to an OD₆₀₀ of 1.000 (± 0.005). These cells were applied on a microtiter plate and, where appropriate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to achieve an end concentration of 200 μM . Thereafter, ethidium bromide was added to an end concentration of 25 μM and the fluorescence change was measured over 60 min in a Safire spectrophotometer (Tecan, Crailsheim, Germany) with excitation and emission wavelength of 518 and 605 nm, respectively. Duplicate measurements were recorded on at least two cultures.

Heterologous Expression of BC3310 and Its Mutants with (His)₆-tag and Western Blot

Overnight cultures of *E. coli* DH5 α $\Delta acrAB$ carrying pbc3310, the empty vector (pTTQ18) or plasmids encoding the *bc3310* mutants (pbc3310D105A, pbc3310D105N, or pbc3310D105E) were transferred to fresh LB (amp) medium and grown to

an OD₆₈₀ between 0.4 and 0.6. Expression was induced with 0.75 mM IPTG and the cultures were grown for another 3 h. For quantification of expression, Western blot assays were performed. One milliliter of the overexpression cultures was harvested by centrifugation at 15000 g, 4°C for 5 min. The pellet was washed (20 mM Tris-HCl pH 7.6, 100 mM NaCl, 5% glycerol, 1 mM phenylmethanesulfonylfluoride (PMSF)) and resuspended depending on cell mass in ice-cold lysis buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5% glycerol, 5 mM β -mercaptoethanol, 1 mM PMSF, 1 $\mu\text{g ml}^{-1}$ DNase). Cells were lysed by continuous sonication for 25 min in a cold water bath. SDS-PAGE and Western blots were performed as described in Sambrook and Russell (2001). (His)₆-tag detection was done using a mouse anti-(His)₆ antibody (Qiagen, Hilden, Germany) and a horse anti-mouse horseradish peroxidase-labeled secondary antibody (New England Biolabs. ECL advanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Pittsburgh, PA, USA) was used and chemiluminescence was measured by using the Analyzer Universal hood (Bio Rad, München) and the Quantity one 4.6.6 Software. Quantification was performed by pixel counting of five biological replicates on five different Western blots.

Purification of the BC3310 Protein by Affinity Chromatography

For protein expression and purification, the method described by Ward et al. (2000) was used. In short, *E. coli* strain BL21 pbc3310 was grown in 2TY medium (1.6% tryptone, 1% yeast extract, 0.5% sodium chloride, pH 7) and expression was induced at an OD₆₈₀ between 0.4 and 0.6 with 0.75 mM IPTG. The culture was grown for another 3 h and cells were harvested. For inner membrane preparation, *E. coli* cells were resuspended in 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA and kept frozen at -80°C . After thawing, cells were disrupted with a Continuous Flow Disruptor (Constant Systems, UK) and inner membranes isolated by sucrose gradient centrifugation. Samples were kept at -80°C in Tris-HCl (pH 7.5) and EDTA.

Inner membranes were solubilized in 20 mM CAPSO (pH 10.0), 300 mM sodium chloride, 20% glycerol, 1% *n*-dodecyl β -D-maltoside (DDM), 20 mM imidazole (pH 10.0). Immobilized metal affinity chromatography (IMAC) was performed using 20 mM CAPSO (pH 10.0), 10% glycerol, 0.05% DDM, 20 mM imidazole (pH 10.0) as wash buffer and 20 mM CAPSO (pH 10.0), 200 mM imidazole, 5% glycerol, and 0.05% DDM as elution buffer.

Circular Dichroism Measurement

Purified protein was washed using a spin concentrator with 20 mM CAPSO (pH 10.0), 5% glycerol and 0.05% DDM until imidazole-free. CD spectral analysis was performed from 270 to 195 nm in a 1 nm step resolution using a spectropolarimeter (Jasco J-715) with constant nitrogen flushing and a scan rate of 10 nm min^{-1} . Response time was set at 1 s with a sensitivity of 100 mdeg and 10 nm bandwidth. The data comprised an accumulation of 20 scans, from which the buffer contribution was subtracted.

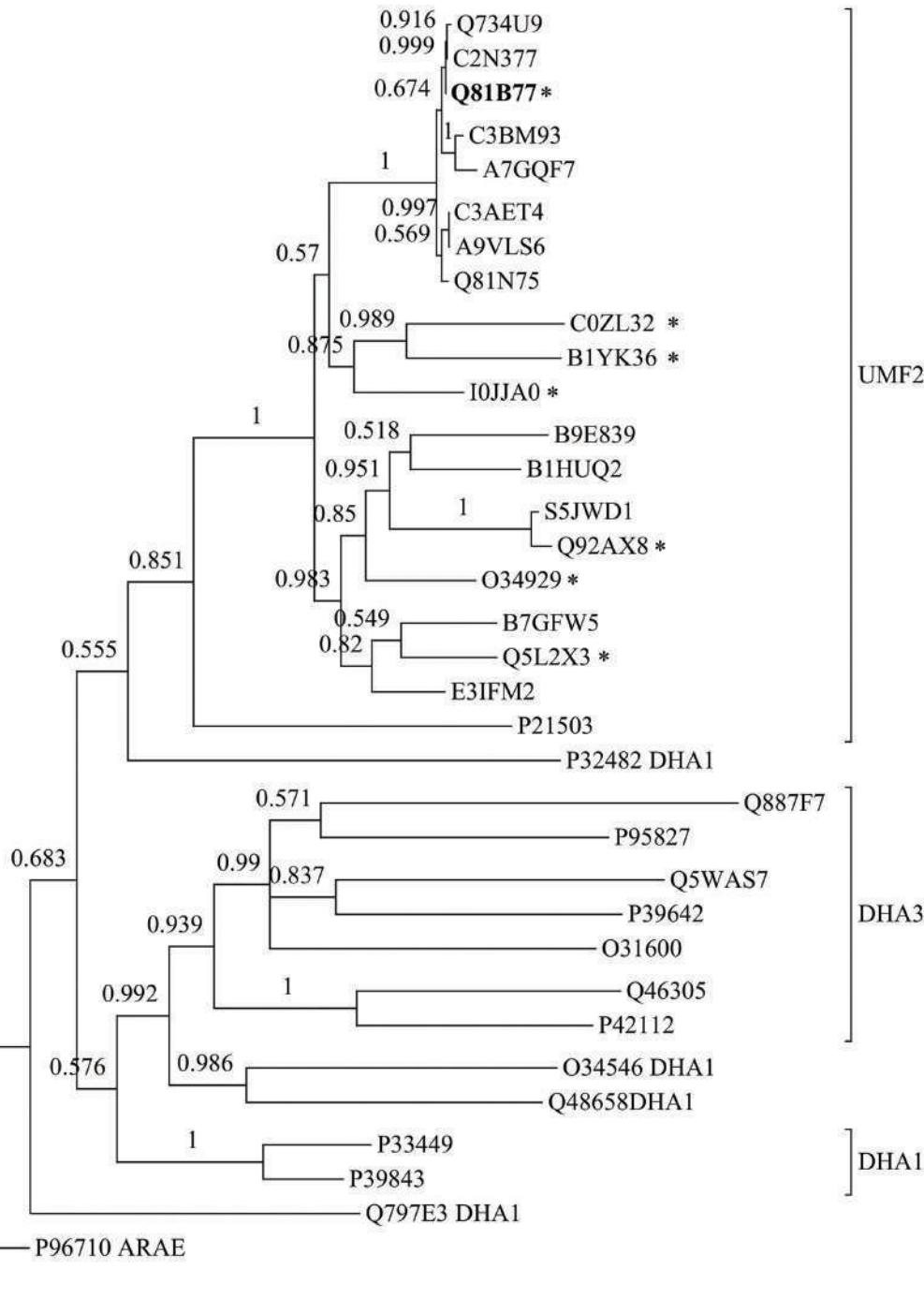


FIGURE 1 | Dendrogram comparing BC3310 from *Bacillus cereus* ATCC 14579 with orthologous proteins and other multidrug transporters from the DHA1 and DHA3 families. BC3310 from *B. cereus* ATCC 14579 (UniProt accession number: Q81B77; bold font) and orthologous proteins from *B. cereus* ATCC 10987 (Q734U9), *B. cereus* ATCC 10876 (C2N377), *B. anthracis* str. Ames (Q81N75), *B. cereus* ssp. cytotoxis (A7GQF7), *B. weihenstphanensis* (A9VLS6), *B. mycoides* (C3AET4), *B. pseudomycoides* (C3BM93), *Geobacillus* sp. Y4.1MC1 (E3IFM2), *Halobacillus halophilus* (I0JJA0), *B. subtilis* (O34929), *Listeria innocua* (Q92AX8), *Listeria monocytogenes* (S5JWD1), *Geobacillus kaustophilus* (Q5L2X3), *Lysinibacillus sphaericus* (B1HUQ2), *Exiguobacterium sibiricum* (B1YK36), *Anoxybacillus flavithermus* (B7GFW5), *M. caseolyticus* (B9E839), *Brevibacillus brevis* (C0ZL32), *Escherichia coli* (P21503), and DHA1 proteins from *Lactococcus lactis* (Q48658), *B. subtilis* (Q797E3, O34546, P39843, P33449), *Pseudomonas aeruginosa* (P32482) and DHA3 proteins from *Streptococcus pyogenes* (P95827), *B. subtilis* (P39642, O31600, P42112), *B. clausii* (Q5WAS7), *Pseudomonas syringae* (Q887F7), *Clostridium perfringens* (Q46305) and the sugar transporter AraE from *B. subtilis* (P96710) as an outgroup were used to build the tree. Posterior probability values are shown at each node and the bar represents the expected number of amino acid substitutions per site. The seven protein sequences marked with * were aligned in Figure 5.

Results

BC3310 is Conserved in the *B. cereus* Group

To date, 228 strains of the *B. cereus* group of bacteria have been sequenced (Markowitz et al., 2012). A BLASTP search showed that the protein BC3310 is highly conserved within this group. In 225 strains BC3310 orthologs with >91% amino acid identity were identified. The predicted ortholog from the reduced genome sized *B. cereus* cytotoxin NVH 391-98 displayed 88% identity. The two strains (*B. anthracis* 3154 and *B. anthracis* A2012) in which no BC3310 ortholog was found are draft genomes which display a gap at the relevant genomic position (data not shown). Orthologs of the BC3310 protein are also found in other bacteria of the order Bacillales including *B. subtilis* (51% amino acid identity), *Listeria innocua* (47% amino acid identity), *Geobacillus kaustophilus* (47% amino acid identity), *Lysinibacillus sphaericus* (50% amino acid identity), *Exiguobacterium sibiricum* (39% amino acid identity), *Anoxybacillus flavithermus* (49% amino acid identity), *Macroccoccus caseolyticus* (42% amino acid identity), *Brevibacillus brevis* (41% amino acid identity). The phylogenetic relationship of BC3310 to a selection of orthologs is depicted in a dendrogram (Figure 1). BC3310 clusters very closely with orthologous proteins from other *B. cereus* group members, thus forming a distinct cluster separate from the orthologs of other Bacillales species.

B. cereus Δbc3310 is More Susceptible to Ethidium Bromide Compared to the Wild Type

To examine the role of BC3310 in conferring drug tolerance in *B. cereus* ATCC 14579 a microbroth dilution test was conducted comparing the *B. cereus* wild type to its isogenic markerless knock-out mutant. Growth of the strains in twofold serial dilutions of ten compounds, including antibiotics from different

classes, was tested. The susceptibility of the $\Delta bc3310$ mutant only differed from the susceptibility of the wild type strain for one of the 10 tested compounds. *B. cereus* $\Delta bc3310$ was two times more susceptible to ethidium bromide compared to the wild type (Table 2). It is possible that redundancy among efflux transporters masks the substrate range of the BC3310 transporter or that the transporter is not expressed under the conditions studied. Hence, a heterologous *E. coli* expression system with a hypersensitive *E. coli* strain and IPTG-inducible BC3310 expression was used to further investigate possible substrates.

Expression of BC3310 Protein in *E. coli*

The ability of *E. coli* to heterologously express intact BC3310 protein was investigated. The *bc3310* gene was cloned into the expression vector pTTQ18 as described (Saidijam et al., 2006, 2011; Szakonyi et al., 2007). BC3310 was expressed with a C-terminal RGSHis₆ tag and detected by Western blotting using an antibody against the RGSHis₆ tag (Figure 2). The protein was solubilized from the inner membrane fraction with DDM and purified by affinity chromatography (Figure 2). The major band on the Coomassie stained gel was subjected to Edman degradation and confirmed to contain the first eight predicted amino acids of BC3310. Topology analysis with HMMTOP predicted 12 transmembrane helices in the BC3310 transport protein. Circular dichroism measurements of the purified protein resulted in a spectrum with nodes at 210 and 222 nm (Figure 3), indicating a prevailing α -helical structure (Wallace et al., 2003) and thus confirming the integrity of the heterologously produced protein.

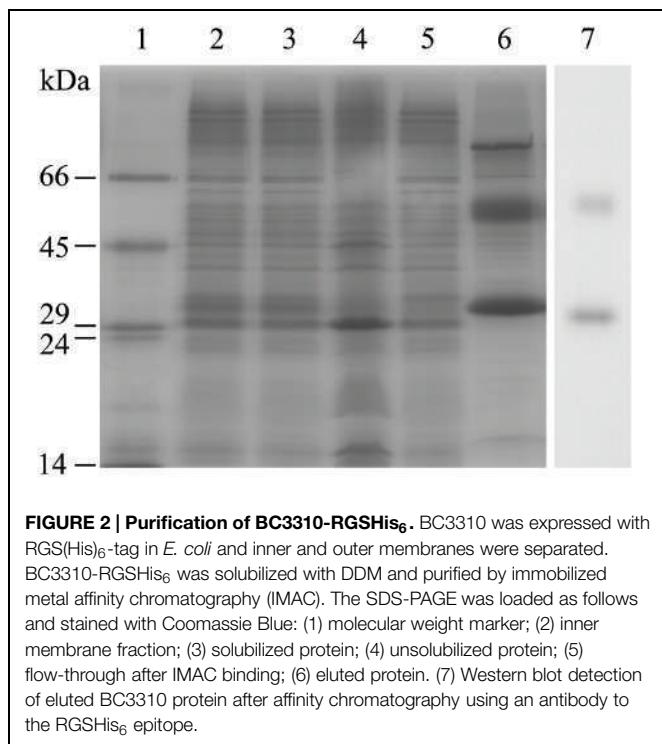
Thereafter the substrate range of heterologously expressed BC3310 was determined. A susceptibility assay was performed using *E. coli* DH5 α $\Delta acrAB$ in which the major multidrug efflux

TABLE 2 | Minimal inhibition concentration (MIC) of *E. coli* DH5 α $\Delta acrAB$ expressing BC3310 (pbc3310) compared to empty vector control (pTTQ18) and *Bacillus cereus* ATCC 14579 $\Delta bc3310$ ($\Delta bc3310$) compared to *B. cereus* ATCC 14579 (wild type).

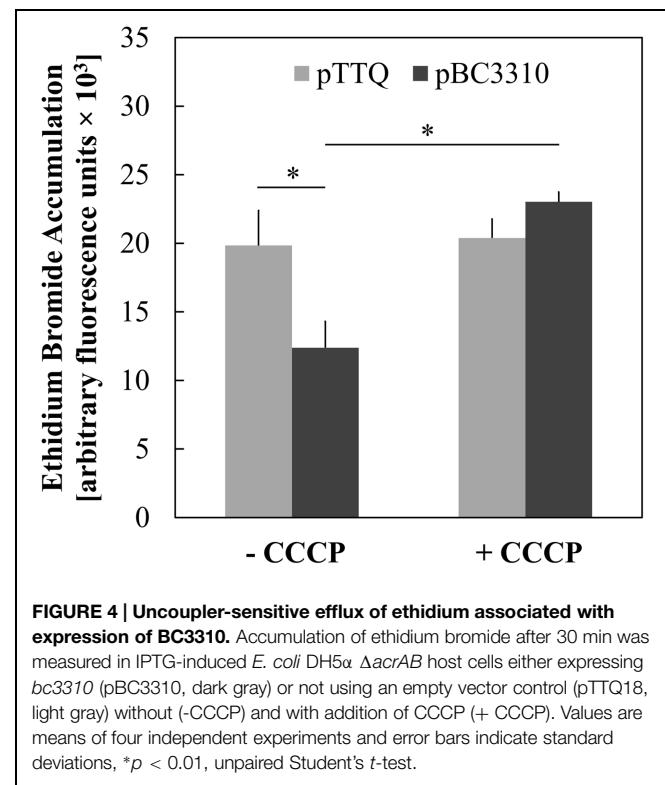
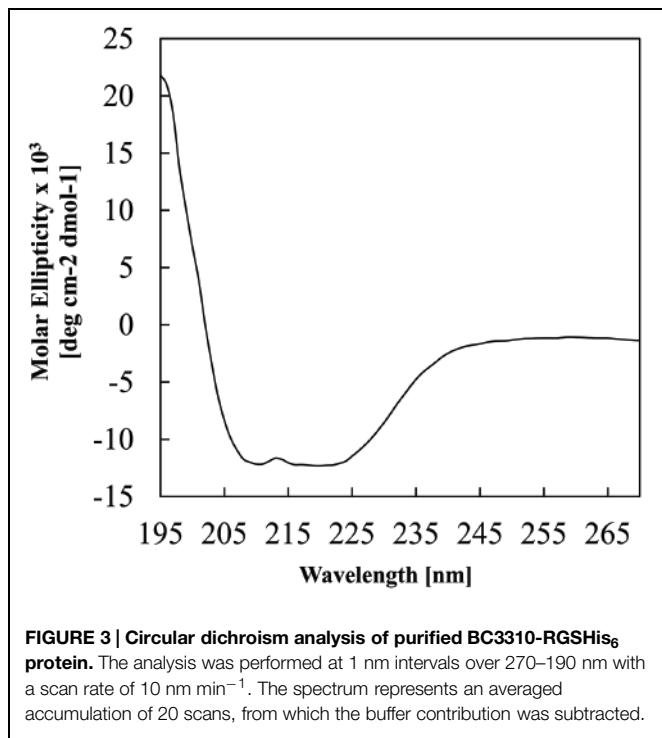
Compound	MIC [$\mu\text{g ml}^{-1}$]					
	<i>E. coli</i> DH5 α $\Delta acrAB$			<i>B. cereus</i> ATCC 14579		
	Empty vector	pbc3310	§	Wild type	$\Delta bc3310$	§
Apramycin	n.d.*	n.d.		12.5	12.5	1
Chloramphenicol	1.25	1.25	1	3.13	3.13	1
Erythromycin	12.5	12.5	1	0.25	0.25	1
Kanamycin	2.5	2.5	1	12.5	12.5	1
Lincomycin	400	400	1	n.d.	n.d.	
Nalidixic acid	n.d.	n.d.		5	5	1
Novobiocin	1.25	1.25	1	n.d.	n.d.	
Phleomycin	n.d.	n.d.		50	50	1
Tetracycline	1.25	1.25	1	1.25	1.25	1
Ethidium bromide	3.13	12.5	4	50	25	0.5
SDS	100	400	4	100	100	1
Silver nitrate	1.3	2.7	2	0.43	0.43	1

§represents fold difference between *E. coli* or *B. cereus* strains, experiments were conducted at least three times in duplicate.

* denotes not determined.



complex was disrupted. The MICs of different compounds for the strain expressing BC3310 from pTTQ18 were compared to the MICs for the empty vector control. The *E. coli* strain expressing BC3310 showed a fourfold higher MIC for ethidium bromide and SDS and a twofold higher MIC for silver nitrate (Table 2).



Ethidium Bromide Efflux of BC3310 is Disrupted by CCCP

Major facilitator superfamily efflux proteins are secondary active transporters that utilize the electrochemical gradient across the cell membrane to extrude compounds. The BC3310 protein sequence displays motifs characteristic of an MFS transporter (see below) and so the ability of BC3310 to confer resistance to ethidium bromide by means of drug efflux was investigated further. A whole cell ethidium bromide accumulation assay with the *E. coli* DH5α ΔacrAB strain expressing BC3310 was performed. Ethidium bromide fluoresces upon binding to double-stranded DNA, and the fluorescence intensity correlates with the accumulation of ethidium bromide. The *E. coli* strain expressing *bc3310* (pBC3310) showed less fluorescence compared to the empty vector control (pTTQ18), thereby implying that BC3310 exports ethidium bromide (Figure 4). Addition of the protonophore CCCP led to an increase in fluorescence intensity in the strain expressing *bc3310* to approximately the control level (pTTQ18) (Figure 4, dark gray bars). This increase indicates the inability of BC3310 to export ethidium bromide due to the disruption of the electrochemical gradient.

Mutation of the Conserved Aspartic Acid Residue (D105) Abolishes Ethidium Bromide Efflux

Proton or substrate translocations by transport proteins often require acidic residues within transmembrane helices (Paulsen et al., 1996a; Edgar and Bibi, 1997; Sanderson et al., 1998;

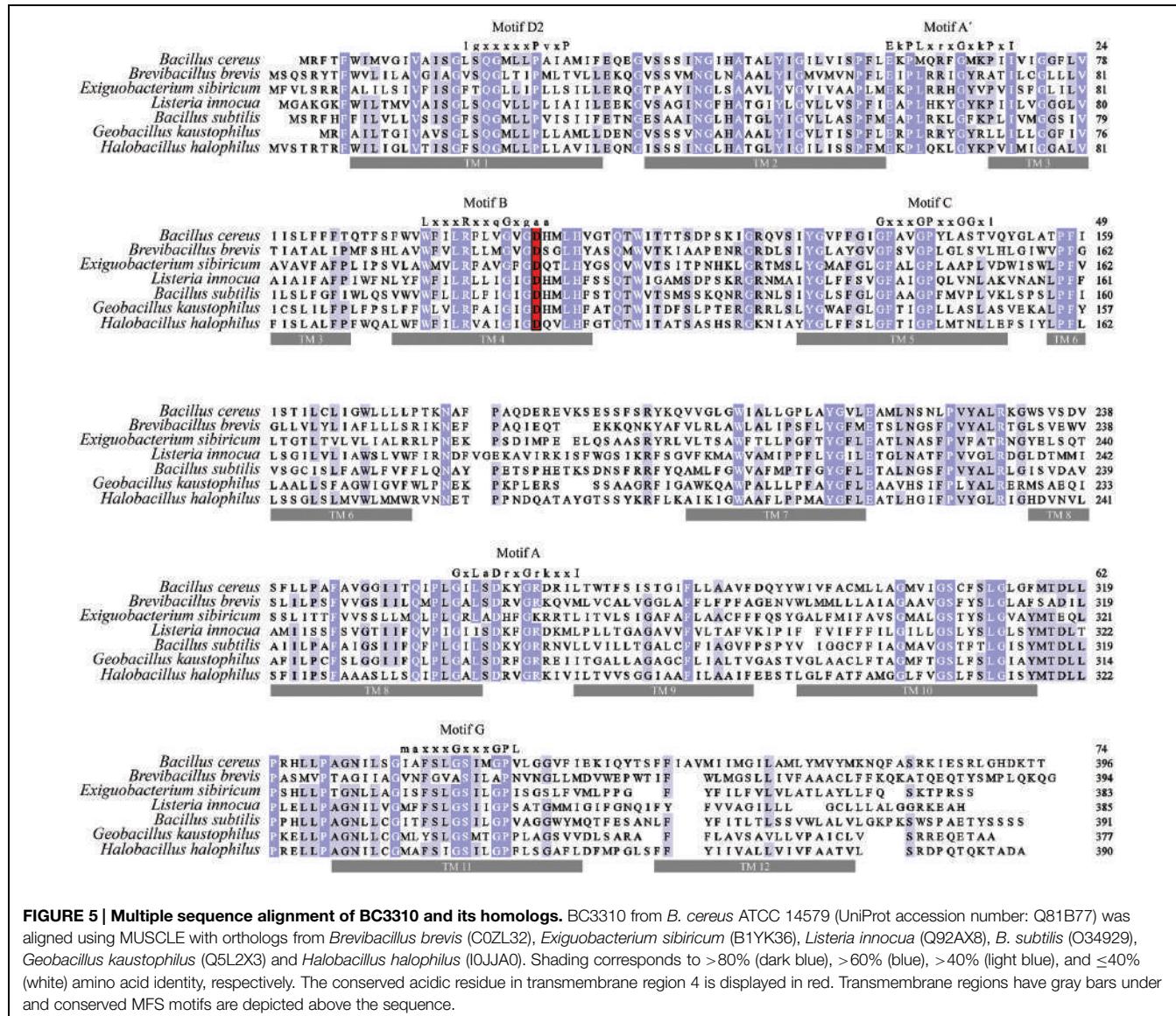


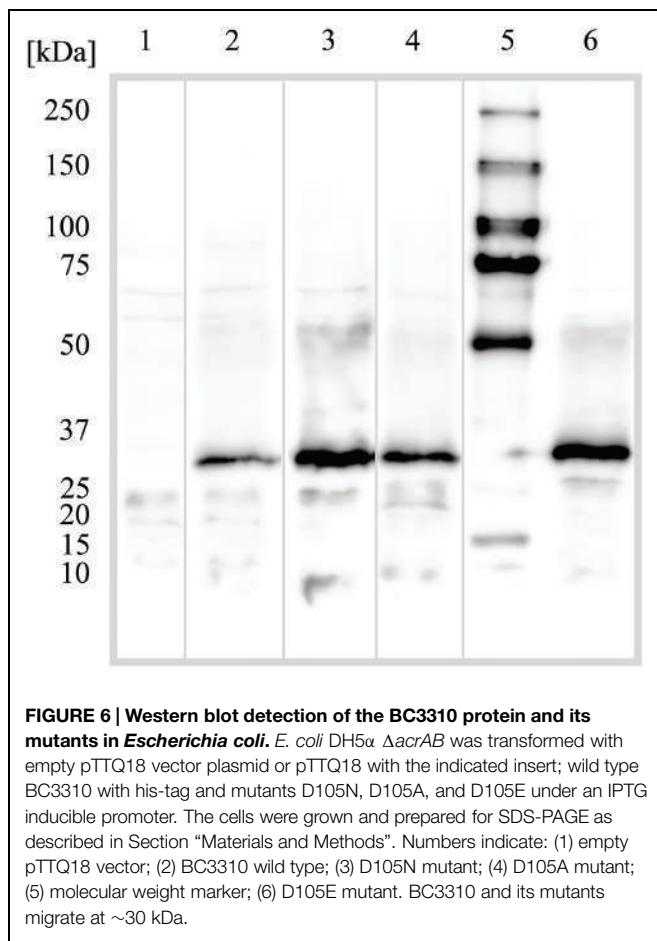
FIGURE 5 | Multiple sequence alignment of BC3310 and its homologs. BC3310 from *B. cereus* ATCC 14579 (UniProt accession number: Q81B77) was aligned using MUSCLE with orthologs from *Brevibacillus brevis* (COZL32), *Exiguobacterium sibiricum* (B1YK36), *Listeria innocua* (Q92AX8), *B. subtilis* (O34929), *Geobacillus kaustophilus* (Q5L2X3) and *Halobacillus halophilus* (I0JJAO). Shading corresponds to >80% (dark blue), >60% (blue), >40% (light blue), and ≤40% (white) amino acid identity, respectively. The conserved acidic residue in transmembrane region 4 is displayed in red. Transmembrane regions have gray bars under and conserved MFS motifs are depicted above the sequence.

Dang et al., 2010). Sequence alignment of BC3310 with orthologous proteins revealed a conserved acidic residue in putative TMS 4 (Figure 5). In order to investigate the importance of this conserved aspartate residue (D105) for efflux activity, mutational analyses were conducted. Three constructs were made in which the aspartate residue was mutated to glutamate (D105E), asparagine (D105N), or alanine (D105A). The expression of the mutant proteins was detected and quantified by Western blot (Figure 6). This showed that the expression of all mutant proteins was three to four times higher compared to the expression of wild type protein. MIC determination of ethidium bromide and silver nitrate was performed to investigate the functionality of the mutant BC3310 proteins (Table 3). Even though more mutant protein was expressed, the susceptibility of strains expressing mutant BC3310 was reduced to levels approximating those of the empty vector control-strain. Thus, mutational change of the

aspartate residue to another acidic or a structurally similar residue abolished the efflux ability of BC3310 for ethidium bromide and silver nitrate, indicating that both the size and charge of the side chain at position 105 are important for protein function.

BC3310 Belongs to the UMF-2 Family of the MFS

BC3310 showed prevailing α-helical structure in our CD analysis and is predicted to be a 12-TMS multidrug transporter belonging to the MFS. Most of the 12 TMS-containing MFS proteins that efflux several drugs are members of the drug:H⁺ antiporter families DHA1 and DHA3. To determine if BC3310 belongs to one of these families within the MFS, a multiple alignment of sequences orthologous to BC3310 and sequences from the well described DHA1 and DHA3 families was performed. From this alignment a dendrogram was built



which showed clustering of BC3310 and orthologs in a distinct clade separate from the DHA1 and DHA3 family proteins included in the analysis (**Figure 1**). This analysis supported the transporter classification database (TCDB) division of YfkF, the BC3310 ortholog in *B. subtilis*, into a separate family,

the unknown major facilitator family-2 (UMF-2) (Saier et al., 2014).

Transport Proteins within the UMF-2 Family Contain a Variant of the MFS Signature Motif A

Sequence alignment revealed that amino acid sequence motifs characteristic for MFS transporters, namely motif A, B, C, and G were conserved in BC3310 and orthologous proteins (**Figure 5**) (Henderson and Maiden, 1987; Griffith et al., 1992; Paulsen et al., 1996b). Motif A is conserved in the loop region between transmembrane segments (TMS) 2 and 3, and has been called the MFS signature motif due to its conservation across the superfamily. In the majority of MFS transporters, including the DHA1 family proteins, the motif A consensus sequence is G-x-L-a-D-r/k-x-G-r/k-r/k-x-x-I (x indicating any amino acid; capital and lower case letters representing amino acid frequency of >70% and 40–70%, respectively; Henderson and Maiden, 1987; Griffith et al., 1992; Paulsen et al., 1996b). However, a functional variant of this motif has been described in the *Clostridium perfringens* DHA3 family tetracycline efflux protein TetA(P): E-x-P-x-x-x-x-x-D-x-x-R-K (bold letters overlap with D, r/k,r/k of the canonical motif A) (Bannam et al., 2004). In BC3310 and its orthologs a modified motif A (motif A') was identified, which represents a hybrid of the canonical motif A and the TetA(P) motif A (**Table 4**) (Paulsen et al., 1996b; Bannam et al., 2004). The N-terminal sequence of motif A' in BC3310 orthologs resembles the TetA(P) (DHA3) motif A, with E and P conserved in both motifs, whereas the C-terminal sequence corresponds to the DHA1 motif A. This results in the BC3310 modified motif A' sequence E-r/k-P-L-x-r/k-x-G-x-r/k-P-x-I (bold letters correspond to sequences of the previously described motif A sequences).

As in other MFS transporters, a second motif A-like sequence is present between TMS 8 and TMS 9 in BC3310 (consensus sequence: G-x-L-S-D-r/k-x-G-R-r/k-x-x-i/l). This sequence coincides more with the signature motif A compared to the motif A' sequence between TMS 2 and TMS 3 (Henderson and Maiden, 1987; Griffith et al., 1992).

Discussion

Heterologous expression of BC3310 in a drug hypersusceptible *E. coli* strain increased the tolerance of the bacteria to AgNO₃, SDS, and ethidium bromide, indicating that it has a role in resistance to multiple drugs. Whole cell accumulation assays of ethidium bromide in *E. coli* expressing bc3310 demonstrated CCCP-sensitive efflux of ethidium in the drug hypersusceptible *E. coli* strain confirming a function as a drug efflux protein. Hence, BC3310 is an energy-dependent multidrug efflux pump. Inactivation of bc3310 in *B. cereus* ATCC 14579, also resulted in increased susceptibility to ethidium bromide, but not to SDS or AgNO₃, suggesting, low basal expression of bc3310 under the conditions used in our experiments. It has, however, previously been reported that addition of 1 mM AgNO₃ to exponentially growing cultures of *B. cereus* ATCC 14579

TABLE 3 | Relative expression rate and relative MIC of *E. coli* strains producing no BC3310, BC3310 wild type, D105N, D105A, or D105E mutant protein.

<i>E. coli</i> DH ₅ α ΔacrAB producing	Relative expression ^b [%]	Relative resistance to [%] ^a	
		Ethidium bromide	Silver nitrate
BC3310 wild-type	100	100	100
No BC3310	NA ^c	20	60
D105N	440	20	50
D105A	330	25	50
D105E	380	30	60

^aMICs were determined in *E. coli* DH₅α ΔacrAB pTTQ18 expressing bc3310 in LB media or LB media without NaCl (for silver nitrate) supplemented with 0,04 mM IPTG and 75 µg ml⁻¹ carbenicillin.

^baverage of five different Western blots of five different cultures.

^cNA, not applicable.

TABLE 4 | Consensus sequences of motif A variants found in MFS drug export families.

MFS family	Consensus sequence of motif A variants															
DHA1				G	x	L	a	D	r/k	x	G	r/k	r/k	x	x	I
TetA(P) (DHA3)	E	x	P	x	x	x	x	D	x	x	x	R	K			
BC3310 (UMF-2)	E	r/k	P	-	-	-	L	x	r/k	x	G	x	r/k	P	x	I

x indicates any amino acid; capital, and lower case letters represent an amino acid frequency occurrence of >70 and 40–70%, respectively; bold letters indicate an overlap with conserved amino acids of the DHA1 or DHA3 family.

induced expression of *bc3310* (Babu et al., 2011) and we detected AgNO₃-induced temporal expression of *bc3310* by qRT-PCR under our experimental settings (data not shown). Therefore, although BC3310 seems to have a role in transport of Ag⁺ and/or NO₃⁻ it is not essential in conferring AgNO₃ resistance under the conditions tested, but may be important under specific circumstances. *B. cereus* ATCC 14579 contains 93 genes annotated as drug transporter which corresponds to 1.7% of the protein coding genes in the genome (Saidijam et al., 2006, 2011; Ren et al., 2007). In comparison, *B. subtilis* and *E. coli* display 32 and 37 genes encoding drug transport proteins, respectively, which correspond to 0.8 and 0.9% of the protein coding genes (Nishino and Yamaguchi, 2001; Ren et al., 2004). Considering the high number of annotated drug transporter genes in the genome of *B. cereus*, it is possible that one or more transporters compensate for the loss of BC3310, thereby concealing a potential effect of a gene disruption.

The efflux of ethidium bromide by BC3310 is dependent on a conserved aspartate residue, which could not be replaced by another acidic or hydrophobic amino acid. This indicates an important role of the aspartate residue at position 105 (D105) in the putative TMS 4. This residue is also conserved in BC3310 orthologs. Even though this aspartate residue is not reported to be one of the conserved residues, it falls into the boundaries of motif B. The motif B sequence of BC3310 and orthologs is W-x-x-L-R-x-x-G-x-G-D-x which overlaps to a large degree with canonical motif B L-x-x-x-R-x-x-q-G-x-g-a-a (bold letters indicate matching amino acids, underlined letter is D105 in BC3310). Motif B contains an absolutely conserved basic amino acid residue which is proposed to play a role in proton transfer (Paulsen and Skurray, 1993). This residue is also conserved in BC3310 (R98).

Sequence analyses classified BC3310 into the UMF-2 family of the MFS which is distinct from the well characterized drug efflux families DHA1 and DHA3 and consists of previously uncharacterized proteins. We have thus described the first functional data for a member of the UMF-2 family and showed that it includes multidrug efflux proteins. Previously transporters belonging to (at least) five of the 82 different families have been implicated in multidrug efflux. Besides the mentioned DHA1 and DHA3 families with 12 TMS-containing transporters, multidrug efflux proteins have been described for the Organic Cation Transporter family (2.A.1.19) (Koepsell, 2013). In addition, the DHA2 family is known to contain multidrug efflux proteins with 14 TMS (Paulsen et al., 1996b)

and the gene encoding MdrA in *Streptomyces coelicolor*, classified into the Acriflavin Sensitivity family (2.A.1.36), is regulated by a TetR repressor that recognizes multiple drugs (Hayashi et al., 2013).

Interestingly, BC3310 and its orthologs contain an alternative motif A' consensus sequence E-r/k-P-L-x-r/k-x-G-x-r/k-P-x-I between putative TMS 2 and 3. We propose that this consensus sequence can be used as a marker to distinguish the UMF-2 family from other MFS families. The presence of a second motif A in BC3310 is likely due to the duplication of 6 TMS during the evolution of the 12-TMS MFS transporters (Paulsen and Skurray, 1993). Similarly, motif G relates to a duplication of motif C (antiporter motif) (Paulsen et al., 1996b). Motif C is only conserved in exporters and not in importers (Paulsen and Skurray, 1993). This motif is also found with a high similarity (including the functionally important GP dipeptide; De Jesus et al., 2005) in BC3310 and orthologs which is in line with the efflux function of BC3310. Little similarity to MFS motif D2 is observed in the sequence alignment of BC3310 orthologs. As reported previously, motif D2 does not appear to be highly conserved in recently investigated 12-TMS MFS transporters and a function has not yet been assigned (Paulsen et al., 1996b; Kapoor et al., 2009).

The gene encoding the BC3310 transporter is highly conserved in the genomes of the *B. cereus* group members indicating that *bc3310* belongs to the core genome of the *B. cereus* group. Comparison of the *bc3310* genomic region of *B. cereus* ATCC 14579 with the equivalent regions of selected *B. cereus* group members, *B. cereus* ATCC 10987, *B. cereus* ATCC 10876, *B. anthracis* Ames Ancestor A2084, *B. thuringiensis* sv. kurstaki YBT-1520, and *B. mycoides* ATCC 6462 showed the same gene organization. The different species of the *B. cereus* group inhabit many different niches and display a high number of efflux transporter genes in the genome compared to other bacteria which could account for the different lifestyles (Saidijam et al., 2006, 2011). Thus, genes conserved in the genomes of the *B. cereus* group might play a role in the fundamental maintenance of physiological functions. Preliminary phenotypic microarray data using BIOLOG, however, did not reveal significant differences between *B. cereus* ATCC 14579 wild type and $\Delta bc3310$ mutant. Condition-dependent transcriptome analyses of the *bc3310* ortholog, *yfkF*, in *B. subtilis* revealed relatively constant transcriptional activity across the conditions investigated (Nicolas et al., 2012). The highest level of gene expression was observed in cells within stationary (OD₆₀₀ ~2)

or transition ($OD_{600} \sim 1.4$) growth phases in LB medium or LB medium supplemented with glucose as well as on LB agar. Ethanol stress conditions revealed the lowest expression of this gene. Furthermore *yfkF* is predicted to be under the control of the housekeeping sigma factor *SigA* (Nicolas et al., 2012). Transcription of genes encoding multidrug transporters with a major role in protecting the cell against toxic compounds is generally activated by transcription factors that recognize toxic compounds or stress signals, such as *AcrR*, *SoxS*, *MarR*, and *Rob* in the case of *AcrAB* of *E. coli* (Ma et al., 1996; Sulavik et al., 2001; Randall and Woodward, 2002; Rosenberg et al., 2003). This fact and the minor intrinsic susceptibility against toxic compounds in the *B. cereus* $\Delta bc3310$ deletion mutant indicate that BC3310 is not a potent multidrug transporter with a main role in protecting the cell against toxic xenobiotics. It rather hints to an ancient and maybe general function in the normal physiology of the *B. cereus* group of bacteria. To further elucidate the role of this transporter the inactivation of other efflux proteins might be required.

Taken together, we have performed the first phylogenetic and functional characterization of a member of the UMF-2. The amino acid sequence of BC3310 comprises known motifs of the 12-TMS MFS transporters with a modified motif A' between TMS 2 and TMS 3. BC3310 is a multidrug transporter with confirmed predominant α -helical structure. It confers resistance to ethidium bromide, SDS, and silver nitrate when expressed in *E. coli*. The export of ethidium bromide is energy dependent and requires a conserved aspartate residue in TMS 4. The

deletion of *bc3310* in *B. cereus* resulted in increased susceptibility to ethidium bromide under the conditions tested. The high conservation of *bc3310* within the *B. cereus* group genomes indicates that it is part of the core genome. We hypothesize that the intrinsic role of BC3310 is not as a typical multidrug transporter, but rather as an important component in the normal physiology of the bacteria, under conditions that still remain to be identified.

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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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