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Phenotypic and genotypic correlates of daptomycin-resistant methicillin-susceptible *Staphylococcus aureus* clinical isolates

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Abstract

Daptomycin (DAP) has potent activity *in vitro* and *in vivo* against both methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains. DAP-resistance (DAP-R) in *S. aureus* has been mainly observed in MRSA strains, and has been linked to single nucleotide polymorphisms (SNPs) within the *mprF* gene leading to altered cell membrane (CM) phospholipid (PL) profiles, enhanced positive surface charge, and changes in CM fluidity. The current study was designed to delineate whether these same genotypic and phenotypic perturbations are demonstrated in clinically-derived DAP-R MSSA strains. We used three isogenic DAP-susceptible (DAP-S)/DAP-R strain-pairs and compared: (i) presence of *mprF* SNPs, (ii) temporal expression profiles of the two key determinants (*mprF* and *dltABCD*) of net positive surface charge, (iii) increased production of *mprF*-dependent lysinylated-phosphatidylglycerol (L-PG), (iv) positive surface charge assays, and (v) susceptibility to cationic host defense peptides (HDPs) of neutrophil and platelet origins. Similar to prior data in MRSA, DAP-R (vs DAP-S) MSSA strains exhibited hallmark hot-spot SNPs in *mprF*, enhanced and dysregulated expression of both *mprF* and *dltA*, L-PG overproduction, HDP resistance and enhanced positive surface charge profiles. However, in contrast to most DAP-R MRSA strains, there were no changes in CM fluidity seen. Thus, charge repulsion via *mprF*- and *dlt*-mediated enhancement of positive surface charge may be the main mechanism to explain DAP-R in MSSA strains.

Keywords

Staphylococcus aureus; daptomycin resistance; *mprF*; single nucleotide polymorphism (SNP); host defense antimicrobial peptide

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Introduction

Staphylococcus aureus causes a wide array of local and systemic infections in humans and animals, and is among the most prevalent community-acquired and nosocomial pathogens. The ability of *S. aureus* to develop multi-antibiotic resistance, including to methicillin, vancomycin (VAN), and daptomycin (DAP) is raising significant public health concerns. Moreover, these latter two drugs are often antibiotics of last resort for many *S. aureus* infections.

DAP is a cyclic lipopeptide antibiotic with potent bactericidal activity against a wide range of Gram-positive pathogens, including MRSA and VAN-intermediate susceptibility (VISA) strains. However, there have been numerous recent reports of clinical *S. aureus* strains that have evolved DAP-resistance (DAP-R) *in vivo* during DAP treatment (Kaatz *et al.*, 2006; Jones *et al.*, 2008; Marco *et al.*, 2008; Murthy *et al.*, 2008; Bayer *et al.*, 2014, 2015). Previous studies have linked several characteristic phenotypes with DAP resistance in *S. aureus*: i) enhanced positive surface charge (Jones *et al.*, 2008; Yang *et al.*, 2010); ii) altered cell membrane (CM) phospholipid profiles, especially increased content of positively-charged phospholipids (lysyl-phosphatidylglycerol [L-PG]) (Mishra *et al.*, 2012b, 2014); iii) CM fluidity/rigidity. These phenotypes were often accompanied by a single or multiple genotypic changes such as upregulation of *mprF* and/or *dlt-ABCD* transcription (Yang *et al.*, 2009a, 2009b; Bayer *et al.*, 2016) and single nucleotide polymorphisms (SNPs) within the *mprF* open reading frame (ORF) (Murthy *et al.*, 2008; Bayer *et al.*, 2013, 2014, 2015, 2016; Yang *et al.*, 2013a). The *mprF* gene product, MprF, is a L-PG synthase which adds positively-charged lysine to the negatively-charged phosphatidylglyderol (PG) molecule within the staphylococcal CM (Peschel *et al.*, 2001; Staubitz *et al.*, 2004). It has been shown that the *mprF* SNPs were correlated with excess synthesis of L-PG in *S. aureus* CM and enhanced surface positive charge (Yang *et al.*, 2013a; Bayer *et al.*, 2014, 2015, 2016). More recently, it has been reported that all the *mprF* SNPs observed in the vast majority of DAP-R *S. aureus* strains are clustered within either one of the two 'hot spot' MprF domains: the central bifunctional domain or the C-terminal L-PG synthase domain (Ernst *et al.*, 2009; Bayer *et al.*, 2015). However, these latter data have mainly emerged from studies of DAP-R MRSA strains. Hence, to investigate phenotypic and genotypic correlates of DAP resistance in MSSA strains, we used three DAP-S/DAP-R isogenic clinical MSSA strain pairs to assess (i) presence of *mprF* SNPs in such DAP-R strains, (ii) the relationship of the SNPs to DAP-R phenotype, (iii) temporal transcriptional expression profiles of *mprF* and *dltABCD* genes, (iv) CM phospholipid profiles and fluidity, (v) cross-resistance to three cationic HDPs (LL-37, human cathelicidin; hNP-1, human neutrophil peptide-1; and tPMPs, thrombin-induced platelet microbicidal proteins), and (vi) positive surface charge metrics.

Materials and Methods

Bacterial strains

The three DAP-S/DAP-R MSSA study strain pairs used in the current study were clinical bloodstream isolates randomly selected and obtained courtesy of the Cubist Strain Collection: each strain pair included an initial pre-DAP treatment isolate and a post-DAP treatment isolate from the same patient that became DAP non-susceptible (Table 1). All the

three MSSA strain pairs were shown to be identical on pulsed-field gel electrophoresis (PFGE) analyses (data not shown). To further confirm the isogenicity of each strain pair, these isolates were genotyped by *spa*, inferred clonal complex and *agr* typing (Table 1).

All *S. aureus* strains were grown in either Tryptic Soy Broth (TSB; Difco Laboratories) or Meuller-Hinton broth (MH broth; Difco Laboratories) depending on the individual assays. Liquid cultures were grown in Erlenmeyer flasks at 37°C with shaking (250 rpm) in a volume that was no greater than 10% of the flask volume.

The MICs of the study strains to DAP, oxacillin (OX), and vancomycin (VAN) were determined by standard Etest (AB Biodisk) on MH agar plates, according to manufacturer's recommendation. DAP resistance was defined as an Etest MIC of ≥ 2 μg/ml (Boucher and Sakoulas, 2007). All MIC determinations were performed three times.

DNA isolation and *mprF* sequencing

Genomic DNA was isolated from the *S. aureus* strains using the method described previously (Dyer and Iandolo, 1983). PCR amplification of the *mprF* ORFs was performed as described previously, using the *mprF*-specific primer pair (Yang *et al.*, 2009a). DNA sequencing of the *mprF* ORFs was kindly performed at BIONEER, Daejeon, Korea. Multiple *mprF* sequence alignments were done using the BoxShade Server (http://www.ch.embnet.org/software/BOX_form.html).

Susceptibilities to host defense peptides

Purified LL-37 (found in human epithelial cells and neutrophils) and human neutrophil peptide-1 (hNP-1) were purchased from Peptide International. Thrombin-induced platelet microbicidal protein preparations (tPMPs) were obtained from fresh rabbit platelets as previously described (Yeaman *et al.*, 1992, 1994). This preparation is a mixture of the two predominant tPMPs seen in humans, tPMP-1 and tPMP-2, with a dominance of tPMP-1.

Since standard MIC testing in nutrient broth may underestimate cationic antimicrobial peptide activities (Yeaman *et al.*, 1992; Xiong *et al.*, 2005), *in vitro* susceptibility assays were performed with LL-37, hNP-1, and tPMPs as described previously using a two-hour microdilution method in Eagle's Minimal Essential Medium (Xiong *et al.*, 2005; Jones *et al.*, 2008). These assays were performed with LL-37 (1 μg/ml), hNP-1 (20 μg/ml), and tPMPs (2 μg/ml bioequivalents) using initial inocula of 5×10^3 CFU *S. aureus* cells (Xiong *et al.*, 2005; Jones *et al.*, 2008). These peptide concentrations were selected based on extensive pilot studies showing their inability to completely eradicate the starting inocula of the parental DAP-S strains over the two-hr time point. Data were calculated and expressed as the percent of survival (± SD) of peptide-exposed vs. peptide-unexposed *S. aureus* cells. A minimum of three independent studies in triplicate samples was performed for each peptide on distinct days.

Cell membrane phospholipid (PL) composition

The three major staphylococcal CM phospholipids are phosphatidylglycerol (PG), cardiolipin (CL), and lysyl-phosphatidylglycerol (L-PG). To assess the relative proportions

of these three PLs in the study strains, CM PLs were extracted from the *S. aureus* strains as described previously (Mukhopadhyay *et al.*, 2007; Mishra *et al.*, 2009). These PLs were separated and identified via two-dimensional thin-layer chromatography (2D-TLC), then quantified by spectrophoto-metric analysis as described before (Mukhopadhyay *et al.*, 2007; Mishra *et al.*, 2009). A minimum of three independent experiments in triplicate samples were performed to analyze the proportion of PL content.

CM fluidity

The comparative CM fluidity of the three MSSA strain-pairs were determined by fluorescence polarization spectroscopy using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously (Bayer *et al.*, 2000; Mishra *et al.*, 2009). Data were expressed as the polarization index (PI), in which there is an inverse relationship between the PI and CM fluidity (i.e. higher PI equates less fluid cell membranes) (Xiong *et al.*, 2005; Jones *et al.*, 2008). These assays were repeated at least five times independently.

Positive surface charge

The cytochrome *c* binding assay was performed to measure the relative net positive surface charge of the strain-pairs as described previously (Yang *et al.*, 2009a, 2010, 2012). Briefly, *S. aureus* cells were grown overnight in TSB, washed with 20 mM MOPS buffer (pH 7.0) four times, and resuspended in the MOPS buffer at OD_{600} of 1.0. Next, cells were incubated with 0.5 mg/ml cytochrome *c* for 15 min and the amount of cytochrome *c* remaining in the supernatant was measured by determining absorbance at 530 nm (the absorption maximum if the prosthetic group) (Peschel *et al.*, 1999). The more unbound cytochrome *c* that was detected in the super-natant, the more net positively charged the bacterial surface. At least three independent runs in triplicate samples were performed on separate days.

RNA isolation and qRT-PCR analysis

For RNA isolation, fresh overnight cultures of *S. aureus* strains were used to inoculate TSB to OD_{600} of 0.1. Cells were harvested during both exponential growth (2.5 h) and stationary phase (12 h). Total RNA was isolated from the cell pellets by using the RNeasy kit (Qiagen) and the FASTPREP FP120 instrument (BIO 101), according to the manufacturer's recommended protocols.

Quantitative real time PCR assay was carried out as detailed previously (Bertsche *et al.*, 2011; Yang *et al.*, 2013b). Briefly, 2 μg of DNase-treated RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's protocols. Quantification of cDNA levels was performed following the instructions of the Power SYBR green master mix kit (Applied Biosystems) on a LineGene 9620 fluorescent quantitative detection system (BIOER technology) in triplicate samples. The *mprF*, *dltA*, and *gyrB* genes were detected using respective specific primers as described before (Yang *et al.*, 2009b, 2012). Fold changes in expression levels of *mprF* and *dltA* genes were determined in relation to the housekeeping gene, *gyrB*. The expression profiling was performed at both exponential and stationary growth phases. At least two independent runs were performed for each RNA samples.

Statistical analysis

The Kruskal-Wallis ANOVA test with the Tukey *post hoc* correction for multiple comparisons was utilized. Significance was determined at P value of < 0.05.

Results

MIC testing

Previous studies have suggested that VAN treatment in humans might be an important conditioning for subsequent increase of DAP MICs during DAP treatment (Cui *et al.*, 2006; Pillai *et al.*, 2007). Of note, two (C12 and C45) of the three DAP-R strains exhibited moderate increase in VAN MICs (Table 1). Susceptibilities to OX has also been moderately increased in all the three DAP-R strains.

mprF SNPs among DAP-R MSSA strains

As shown in Table 1, *mprF* sequencing analyses revealed that all three DAP-R MSSA strains had a nonsynonymous mutation in the *mprF* ORF compared with their respective DAP-S parental strains, causing distinct amino acid substitutions. The C12 and C29 DAP-R strains had a P314L substitution, while the C45 DAP-R strain had a S337L substitution. These two *mprF* SNPs were previously shown to be associated with DAP-R among MRSA isolates (Bayer *et al.*, 2015, 2016). Based on previous topographical studies (Ernst *et al.*, 2009; Bayer *et al.*, 2015), these amino acid substitutions were located in the central bifunctional domain of the MprF protein.

HDP susceptibility profiles

As shown in Table 2, all three DAP-R MSSA strains exhibited an overall higher survival profile vs. their respective DAP-S parental strains against all HDPs tested. The C12 strain had most substantial differences in HDP killing profiles vs the DAP-S parental C11 strain for all three HDPs.

Positive surface charge and CM phospholipid (PL) content

Cytochrome *c* binding assays revealed that all three DAP-R strains had significantly increased positive surface charge versus their respective DAP-S parental strains (Fig. 1).

Since increases in L-PG synthesis and/or translocation usually result in enhanced positive surface charge, we compared the proportion of total L-PG within the overall PL content of the three DAP-S/DAP-R MSSA strain pairs. As noted in Table 3, all three DAP-R strains contained significantly more L-PG than their respective DAP-S parental strains. This outcome was associated with the significant reduction in PG content in the three DAP-R strains, although this only reached statistical significance in the C11-C12 strain-pair.

CM fluidity

For all three pairs, CM fluidity analyses revealed no significant differences in the polarization indices (PI values), indicating that the CMs of the three DAP-R strains had no differences in their CM order profiles (Table 3).

Expression of *mprF* and *dltABCD*

Quantitative real-time PCR analysis of *mprF* transcripts during exponential and stationary growth phases revealed that C12 and C29 DAP-R strains demonstrated enhanced *mprF* expression vs their respective isogenic DAP-S strains during the both growth phases (Fig. 2A and B). Although not statistically significant, the C45 DAP-R strain had a moderate increase in *mprF* expression vs. its DAP-S parental strain only during exponential growth phase.

As shown in Fig. 3A, all the three DAP-R strains showed enhanced expression of *dltA* versus their respective DAP-S strains during exponential growth phase. In contrast, at stationary growth, when *dltABCD* espression is generally minimal (Yang *et al.*, 2009a, 2010), only the C29 DAP-R strain exhibited ~2-fold increase in *dltA* expression as compared to its C28 DAP-S parental strain (Fig. 3B). Of note, the two DAP-R strains, C12 and C45, showed significant decrease in *dltA* expression vs. their respective DAP-S parental strains during stationary growth phase.

Discussion

It has been previously shown that the *mprF* SNPs correlated with perturbed growth phase-dependent *mprF* expression in a number of DAP-R MRSA isolates (Yang *et al.*, 2009a, 2009b; Bayer *et al.*, 2014, 2015). The*mprF* dysregulations have been correlated with phenotypic 'gains-in-function' readouts of MprF, such as increased L-PG synthesis and elevated L-PG translocation to the outer CM leaflet, resulting in increased surface positive charge (Jones *et al.*, 2008; Ernst *et al.*, 2009, 2015; Yang *et al.*, 2009a, 2009b). In addition, we previously found that upregulation in the expression of the gene responsible for d-alanylation of wall teichoic acid (*dlt-ABCD*) also accounted for enhancement in positive surface charge (Yang *et al.*, 2009a).

In the current investigation, we used three isogenic DAP-S/DAP-R MSSA clinical strain-pairs to evaluate and extend DAP-R mechanisms in MSSA isolates in terms of both phenotypic and genotypic correlates.

First, as has been shown previously in MRSA strains (Yang *et al.*, 2010; Bayer *et al.*, 2014, 2015), *mprF* ORF sequencing revealed that all the three DAP-R MSSA strains contained SNPs within the bifunctional domain of MprF (Ernst *et al.*, 2009; Bayer *et al.*, 2015). The two amino acid sequence substitutions within MprF (P314L and S337L) have been shown to be associated with enhanced L-PG synthesis in the context of DAP-R phenotype in MRSA strains (Bayer *et al.*, 2015). As shown in Fig. 2A and B, C12 and C29 DAP-R strains had enhanced *mprF* expression versus their respective isogenic DAP-S parental strains during both growth phases. The C45 strain also had moderate, albeit non-significant, increases in *mprF* expression during exponential growth phase. The *mprF* promoter sequences of these three DAP-R strains were identical to those of their respective DAP-S parental stranis (data not shown), indicating the increase in *mprF* expression in the three DAP-R strains was not related to point mutations within the *mprF* promoter

Next, to assess the correlation among *mprF* SNPs, altered expression of *mprF* transcription, and L-PG synthesis, phospholipid (PL) compositional analyses were performed. As expected, the proportion of total L-PG among the overall PL content was significantly increased in the all three DAP-R MSSA strains (Table 3). The increased synthesis of cell membrane L-PG in DAP-R MSSA strains was well correlated with enhanced net positive surface charge in these strains (Fig. 1). These findings are generally in line with prior observations in MRSA strains (Bayer *et al.*, 2014; Mishra *et al.*, 2014; Bayer *et al.*, 2015, 2016), suggesting that hot spot SNPs within the *mprF* ORF of DAP-R MSSA are gain-in-function mutations which translate into increased L-PG production and coincident enhancement of positive surface charge.

Second, altered *dltA* expression profiles were similar to those of *mprF* in all three DAP-R MSSA strains during exponential growth phase (Fig. 3A). Moreover, the DAP-R C29 strain displayed increased expression of *dltA* during stationary growth phase (Fig. 3B). It should be noted that although the DAP-R C29 strain exhibited significantly enhanced expression of both *mprF* and *dltABCD* during both growth phases, the extent of its increased surface positive charge was similar to the other two DAP-R MSSA strains. These data indicate that *mprF* and *dltABCD* expression profiles alone are not enough to explain the surface positive charge regulation in *S. aureus*. Present investigations are in progress to further elucidate genetic factors linked to the DAP-R phenotype, including *graRS*, *yycFG*, and *rpoB* (Friedman *et al.*, 2006; Bertsche *et al.*, 2011; Cheung *et al.*, 2014).

Third, recent publications have suggested that DAP-R MRSA strains frequently display cross-resistance to HDPs and other cationic molecules (Peschel *et al.*, 2001; Yang *et al.*, 2010, 2013a; Mishra *et al.*, 2011b, 2012a; Bayer *et al.*, 2015). In agreement with these observations in MRSA strains, all three DAP-R MSSA strains in our study exhibited significantly enhanced resistance to killing by one or more of the HDPs tested vs their respective DAP-S parental strains (Table 2). It has been proposed that there are two major HDP resistance mechanisms in *S. aureus*: (i) enhanced positive surface charge leading to a charge-repulsive milieu (Jones *et al.*, 2008; Yang *et al.*, 2009a, 2009b) and (ii) alteration of CM biophysical order (fluidity/rigidity) to perturb the optimal interactions of the peptide-of-interest with the target CM (Mukhopadhyay *et al.*, 2007; Mishra *et al.*, 2009, 2011a). Data from the current study would suggest that mainly charge-mediated mechanisms are in-play among MSSA DAP-R strains.

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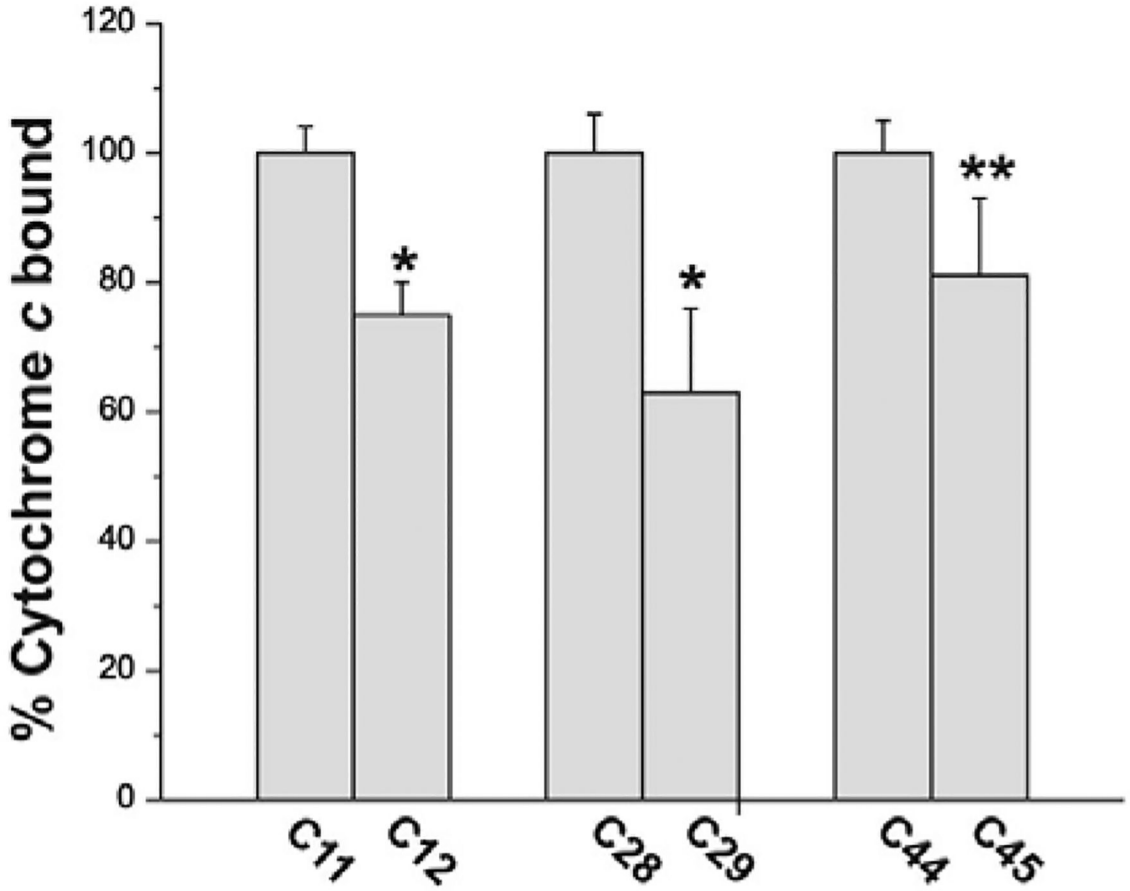

Fig. 1. Relative positive surface charge by cytochrome *c* binding.

The graph shows percent of cytochrome *c* bound after 15 min of incubation with *S. aureus* cells at room temperature. Each DAP-S parental *S. aureus* strain was normalized to 100%. Data represent the means and standard deviations from three independent experiments. $*P < 0.01$; $**P < 0.05$ vs DAP-S parental strains.

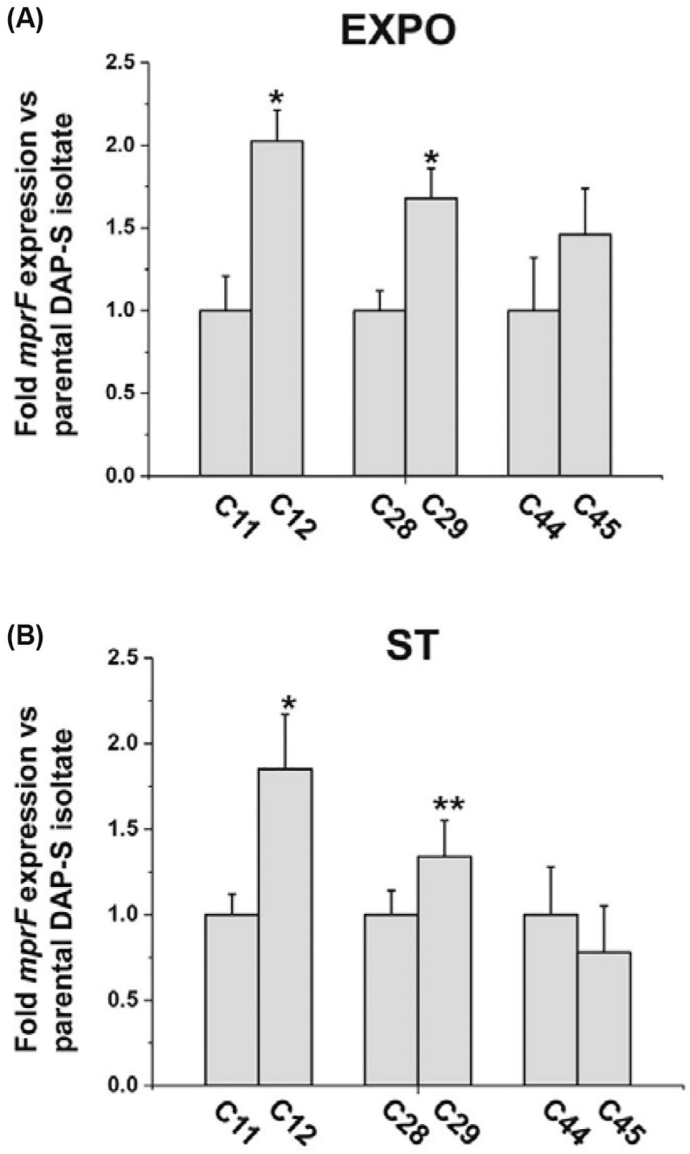

Fig. 2. Relative transcription level of *mprF* during exponential (A) and stationary (B) growth phase.

RNA samples were isolated from exponential-and stationary-phase cultures of the strains and were subjected to qRTPCR to detect transcription of *mprF* and *gyrA*. EXPO = exponential growth phase; ST = stationary growth phase; Fold expression = compared to *gyrB* gene, with parental strain fold-expression set at "1". **P* < 0.01 vs DAP-S parental strains.

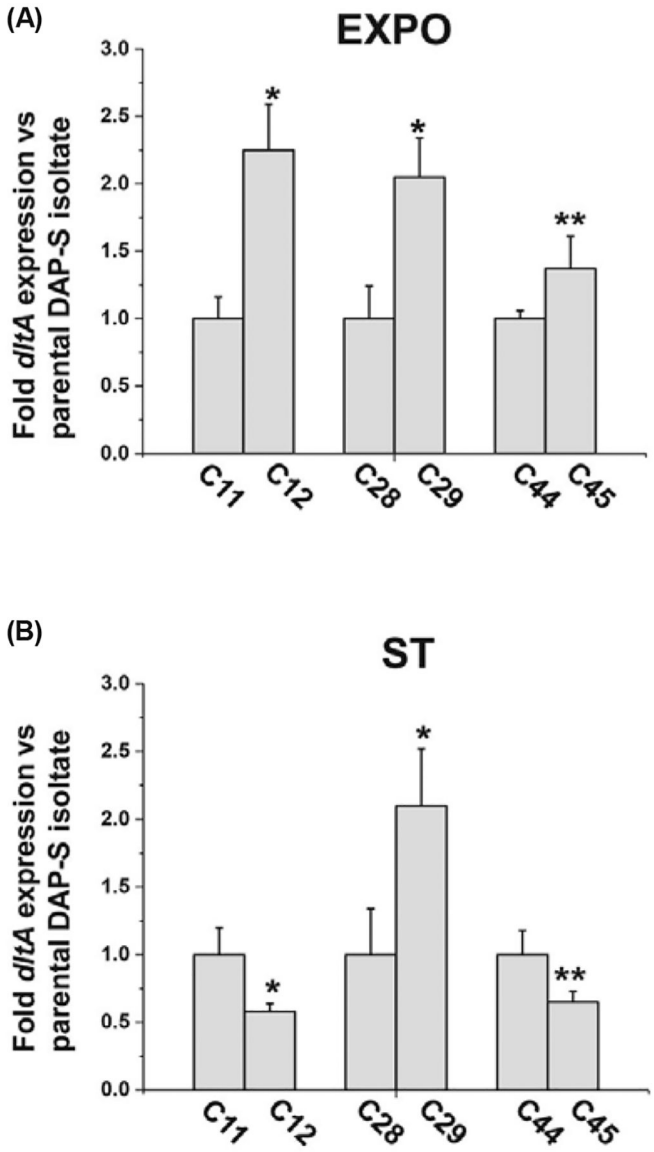

Fig. 3. Relative transcription level of *dltA* during exponential (A) and stationary (B) growth phase.

RNA samples were isolated from exponential-and stationary-phase cultures of the strains and were subjected to RT-PCR to detect transcription of *dltA* and *gyrA*. EXPO, exponential growth phase; ST, stationary growth phase; Fold expression, compared to *gyrB* gene, with parental strain fold-expression set at "1". $*P < 0.01$; $**P < 0.05$ vs DAP-S parental strains.

Table 1.

Bacterial strains examined in the current studies

Strain	*spa* type	CC type[a]	*agr* type	DAP MICs (μg/ml)	OX MICs (μg/ml)	VAN MICs (μg/ml)	*mprF* SNPs	
							Nucleotide position	Amino acid change
C11	454	121	IV	0.38	0.75	1	CCT → CTT (941)	P314L
C12	454	121	IV	3	0.5	2		
C28	2	5	II	0.12	1.0	1	CCT → CTT (941)	P314L
C29	2	5	II	2	0.38	1		
C44	410	5	II	0.38	1.0	1	TCA → TTA (1010)	S337L
C45	410	5	II	4	0.75	2		

OX, oxacillin; DAP, daptomycin; VAN, vancomycin; SNPs, single nucleotide polymorphisms.

[a]Clonal complex (CC) types were inferred by comparing MLST data base.

Table 2.

In vitro susceptibility to killing by cationic host defense peptides

Strain	% Survival (mean ± SD) after 2-h exposure to:		
	10 μg/ml LL-37	20 μg/ml hNP-1	2 μg/ml tPMPs
C11	21.8 ± 11.2	34.2 ± 8.6	4.9 ± 3.2
C12	56.2 ± 22.6**	42.7 ± 6.4*	34.7 ± 15.4*
C28	5.8 ± 3.7	33.4 ± 9.8	43.5 ± 17.1
C29	40.2 ± 23.2*	37.6 ± 7.4	46.6 ± 21.7
C44	42.5 ± 19.7	49.8 ± 22.2	13.6 ± 6.7
C45	60.3 ± 25.4	59.3 ± 16.7	37.3 ± 14.1*

* $P < 0.01$;

** $P < 0.05$ vs the DAP-S parental strains

Table 3.

Comparative cell membrane phospholipid profiles and cell membrane fluidity of study strains

Strains	% of total phospholipids ± SD			CM fluidity
	Total LPG	PG	CL	
C11	18 ± 2.3	78 ± 3.6	5 ± 2.0	0.208 ± 0.05
C12	25 ± 4.4*	68 ± 6.3*	7 ± 4.4	0.193 ± 0.02
C28	15 ± 3.1	79 ± 7.1	6 ± 5.7	0.233 ± 0.03
C29	23 ± 5.8**	70 ± 10.5	7 ± 5.8	0.234 ± 0.04
C44	20 ± 4.1	74 ± 3.2	5 ± 2.4	0.223 ± 0.02
C45	26 ± 3.8**	68 ± 7.5	6 ± 5.0	0.202 ± 0.02

Abbreviations: LPG, lysyl-phosphatydylglycerol; PG, phosphatidylglycerol; CL, cardiolipin.

*$P < 0.01$;

**$P < 0.05$ vs respective DAP-S parental strains.