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Dysregulation of *mprF* and *dltABCD* expression among daptomycin-non-susceptible MRSA clinical isolates

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Background: In small series or individual reports, SNPs within the *mprF* ORF and dysregulation of its expression in *Staphylococcus aureus* have been linked to daptomycin resistance (DAP-R) via a proposed gain-in-function mechanism. Similarly, dysregulation of *dltABCD* has also been associated with DAP-R.

Methods: Using 22 well-characterized, isogenic daptomycin-susceptible (DAP-S)/DAP-R clinical MRSA strain pairs, we assessed potential relationships of the DAP-R phenotype with: (i) regulation of *mprF* transcription; (ii) regulation of *dltABCD* transcription; (iii) expression of the two-component regulatory system, *graRS* (upstream regulator for both *mprF* and *dltABCD* transcription); (iv) SNPs within the *graRS* promoter or its ORF; and (v) altered *mprF* transcription and lysyl-phosphatidylglycerol (L-PG) synthesis.

Results: Enhanced expression of *mprF* occurred with SNPs in highly distinct and well-chronicled MprF domain 'hot spots' and rarely occurred without such mutations. Increased expression and/or dysregulation of *mprF* and *dltABCD* were not uncommon in DAP-R strains, occurring in 27% of strains for each gene. In these latter strains, neither *graRS* expression profiles nor polymorphic sequences within the *graRS* promoter or ORF could be significantly linked to altered transcription of *mprF* or *dlt*.

Conclusions: Although *graRS* can co-regulate *mprF* and *dltABCD* expression, loci outside of this regulon appear to be involved in dysregulation of these latter two genes and the DAP-R phenotype. Finally, DAP-R strains exhibiting significantly altered *mprF* transcription profiles produced significantly increased levels of L-PG.

Introduction

Prior investigations in our laboratory and others have linked SNPs within the *mprF* (multiple peptide resistance factor) locus of *S. aureus* with DAP-R [note—although the official term is 'daptomycin non-susceptibility', 'daptomycin resistance' (DAP-R) is used in this paper for ease of presentation].[1–3]

In a recent investigation,[1] using 22 well-characterized isogenic daptomycin-susceptible (DAP-S) and DAP-R MRSA clinical isolate pairs, we showed that all the *mprF* SNPs observed in the DAP-R strains were clustered within one of the two 'hot spot' MprF domains (central bifunctional domain or C-terminal synthase domain). Moreover, these *mprF* SNPs were correlated with excess cell membrane (CM) synthesis of lysyl-phosphatidylglycerol (L-PG) and enhanced surface positive charge.

In the current study, we utilized the same large well-characterized DAP-S and DAP-R isogenic clinical MRSA strain-pair collection ($n=22$ pairs) as that mentioned above to assess: (i) expression profiles of *mprF* and *dltABCD* transcription during both exponential-phase and stationary-phase growth; (ii) the relationships of such transcriptional patterns with expression and sequence profiling of the upstream two-component regulator of both *mprF* and *dltA*, i.e. *graRS*;[4–6] and (iii) correlation between dysregulation of *mprF* expression and L-PG production.

Materials and methods

Bacterial strains

We employed the previously described 22 DAP-S/DAP-R MRSA isogenic strain pairs of clinical bloodstream isolates randomly selected from the Cubist Pharmaceuticals Isolate Collection (Table 1).[1]

All strains were grown in either Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) or Mueller–Hinton broth (MH; Difco Laboratories) depending on the individual experiments. Liquid cultures were grown in Erlenmeyer flasks at 37°C with shaking (250 rpm) in a volume that was ≤10% of the flask volume. DAP-R was defined as an Etest MIC of ≥2 mg/L.[7]

DNA isolation and sequencing

Genomic DNA was isolated from the *S. aureus* study strains using the method of Dyer and Iandolo.[8] PCR amplification of the *graRS* ORFs was performed as previously described[4,6] and DNA sequencing of the *graRS* ORFs was kindly performed at City of Hope, Duarte, CA, USA. Sequencing of the *mprF* promoter (~500 bp upstream of the ATG start codon) was performed in the selected strain pairs using the primer pair mprF-pro-F (5′-CCCGAATTCTATGGTAATGATGTAGGTGAATATG-3′) and mprF-pro-R (5′-CCCTCTAGAGCTGTAGCAAACGTAATT-3′).

RNA isolation and quantitative real-time PCR

Fresh overnight cultures of *S. aureus* strains were used to inoculate TSB to an OD_{600} of 0.1. Total RNA was then obtained from either exponential (2.5 h) or late stationary (12 h) growth-phase cultures using previously described methods.[6]

For quantitative real-time PCR analyses, 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 an ABI PRISM 7000 Sequence Detection System in triplicate samples. The *mprF*, *dltA* and *gyrB* genes were detected using respective specific primers as described before.[6,9–11] Expression of the *graS* gene was detected using the primer pair qRT-F-graS (5′-GATGGGTTTTATTGGTGCAGA-3′) and qRT-R-graS (5′-CATTGGTATAGAACGG TTTTTGC-3′). Fold changes in expression levels of each target gene were quantified in relation to *gyrB*. A minimum of two independent runs were performed for each RNA sample.

CM phospholipid composition

The three major *S. aureus* CM phospholipids are phosphatidylglycerol, L-PG and cardiolipin.[12] To quantify the relative proportions of these three phospholipids in our strain sets, CM phospholipids were extracted from the selected *S. aureus* strain pairs as described previously.[13,14] The target phospholipids were separated and identified via two-dimensional thin-layer chromatography, then quantified by spectrophotometric assay, as described before.[13,14] At least three independent experiments were performed.

Statistical analysis

The Kruskal–Wallis ANOVA test with the Tukey *post hoc* correction for multiple comparisons was utilized where indicated. Significance was accepted at $P<0.05$.

Results and discussion

Expression of mprF and L-PG synthesis among DAP-R strains

As shown in Figure 1(a), quantitative real-time PCR analyses of *mprF* transcripts revealed that 10/22 DAP-R strains had enhanced *mprF* expression versus their respective isogenic DAP-S parental strains during either the exponential growth phase or the stationary growth phase. Among these 10 strains, this increase in *mprF* expression was statistically significant in 5 (50%). Of note, only the C37 strain exhibited significantly enhanced expression of *mprF* during both growth phases. These results suggest that, although altered expression of *mprF* among DAP-R MRSA isolates is relatively frequent, significantly altered *mprF* expression is less common. Although the C6, C8, C10, C25 and C37 strains had significantly increased *mprF* transcription, the *mprF* promoter sequences of these strains were identical to those of their respective

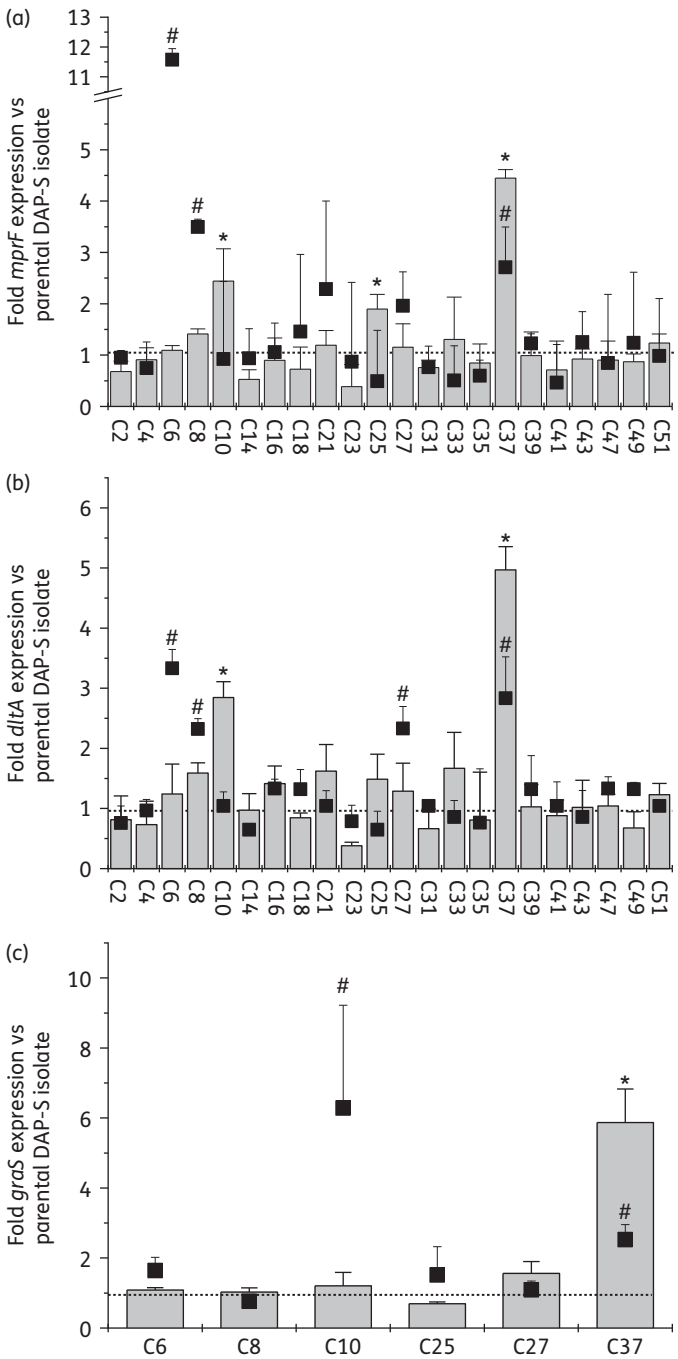

Figure 1. Transcription of *mprF* (a), *dltA* (b) and *graS* (c) genes among the study strains during exponential growth phase (bar graphs) and stationary growth phase (overlaid scatter plots with filled squares). Total cellular RNA samples from the strains grown in TSB medium were isolated at 2.5 h (exponential growth phase) or 12 h (stationary phase) post-inoculation and subjected to quantitative real-time PCR analyses. Each DAP-S parental *S. aureus* strain was normalized to 1. *$P<0.01$ versus respective DAP-S parental strains during exponential growth phase. #$P<0.01$ versus respective DAP-S parental strains during stationary phase.

DAP-S parental strains (data not shown). These data suggested that the increased *mprF* expression in these five DAP-R strains was not related to mutations within the *mprF* promoter.

Table 1. Daptomycin MICs, *mprF* SNPs and phospholipid (PL) profiles among the study strains[a]

			PL profiles (%, mean ± SD) among total PLs		
Strain	Daptomycin MIC (mg/L)[b]	*mprF* SNP[c]	L-PG	phosphatidylglycerol	cardiolipin
C1	0.19				
C2	2	L826F			
C3	0.5		23.5 ± 3.2	68.5 ± 4.2	8.0 ± 3.8
C4	4	P314L	25.6 ± 5.0	65.7 ± 10.5	8.7 ± 7.5
C5	0.25		14.2 ± 1.5	81.8 ± 1.5	4.0 ± 0.9
C6	3	T345A	19.2 ± 3.2*	77.7 ± 3.7	3.0 ± 1.1
C7	0.5				
C8	3	none			
C9	0.5		19.3 ± 7.1	76.0 ± 5.5	4.7 ± 1.7
C10	3	L826F	23.9 ± 5.6*	71.5 ± 6.5	4.7 ± 3.4
C13	0.75				
C14	4	T472K			
C15	0.75				
C16	4	M347R			
C17	0.5				
C18	4	L341S			
C19	0.38		14.9 ± 1.7	75.1 ± 3.2	10.0 ± 2.0
C21	4	L826F	30.9 ± 3.2*	50.5 ± 3.1*	18.6 ± 2.0*
C22	0.5				
C23	4	none			
C24	0.5				
C25	3	S295L			
C26	0.38		31.8 ± 13.9	62.9 ± 14.9	5.3 ± 2.5
C27	2	T345K	28.7 ± 0.8	68.9 ± 2.6	2.4 ± 1.8
C30	0.25				
C31	2	L826F			
C32	0.5		21.9 ± 3.6	71.2 ± 2.5	6.9 ± 3.8
C33	2	S337L	24.1 ± 3.1	73.1 ± 4.0	2.7 ± 2.0
C34	0.38				
C35	4	none			
C36	0.5		15.1 ± 4.7	81.5 ± 5.2	3.4 ± 2.6
C37	3	V351E	26.1 ± 4.6*	69.0 ± 4.3*	5.0 ± 2.3
C38	0.75				
C39	3	L826F			
C40	0.25		26.0 ± 5.2	68.4 ± 4.4	5.6 ± 1.9
C41	3	M347R	30.0 ± 3.1	59.7 ± 3.3*	10.3 ± 3.7*
C42	0.75				
C43	3	S337L			
C46	0.38				
C47	3	L826F			
C48	0.5				
C49	2	T345I			
C50	0.5				
C51	2	T345I			

[a]Pairs of isolates are represented by alternate no shading and shading, with the first strain in each pair being the DAP-S parental strain and the second being the DAP-R strain.
[b]These data have been published before.[1]
[c]Positions of nucleotide change within *mprF* ORFs.
*$P < 0.05$ versus respective DAP-S parental strain.

Next, to assess the correlation between altered expression of *mprF* and L-PG synthesis, eight DAP-S and DAP-R pairs were selected for phospholipid compositional analyses depending on their *mprF* expression profiles (Figure 1a): (i) DAP-R strains expressing significantly increased *mprF* transcripts in the exponential and/or stationary phase; (ii) DAP-R strains displaying no changes

in *mprF* expression; (iii) DAP-R strains expressing marginally increased *mprF* transcripts during both growth phases; and (iv) DAP-R strains expressing marginally increased *mprF* transcript only during the exponential growth phase.

The proportion of total L-PG within the overall phospholipid content was significantly increased in the DAP-R strains (C6, C10 and C37) exhibiting significantly increased *mprF* transcription in exponential, stationary or both growth phases versus the DAP-S parental strains (Figure 1a and Table 1). In contrast, no significant changes in L-PG synthesis were observed in the two DAP-R strains (C4 and C41) that did not show any changes in *mprF* transcription in either growth phase versus the respective parental DAP-S strains. Of the two DAP-R strains (C21 and C27) that exhibited a marginal increase in *mprF* transcription during both growth phases, only C21 displayed significantly increased L-PG synthesis. The C33 strain, which displayed a marginal increase in *mprF* transcription only during the exponential growth phase, showed no increase in L-PG production versus the DAP-S parental strain (C32) (Table 1).

Our current data were generally in line with prior observations,[2,11,15] showing that only when *mprF* expression is significantly enhanced in either growth phase is L-PG production significantly increased. Interestingly, when *mprF* transcription was increased by an insignificant amount, increased L-PG production was occasionally observed among DAP-R isolates.

Expression of dltABCD among DAP-R strains

The *dltABCD* operon also contributes to the staphylococcal net positive surface charge by D-alanylating cell-wall teichoic acids through distinct effector mechanisms.[16] As shown in Figure 1(b), 14 of the 22 DAP-R strains exhibited enhanced *dltA* expression profiles during exponential or stationary growth phases as compared with their respective DAP-S parental strains. This increased *dltA* expression in DAP-R versus DAP-S strains was significant in five isolates (23%) (C6, C8, C10, C27 and C37). Importantly, in four of these five isolates (C6, C8, C10 and C37) enhanced *mprF* expression was also seen, suggesting a co-regulation scenario by the upstream transcriptional regulator(s), *graRS*.[4–6]

Overall analyses of *mprF* and *dltA* expression in the 22 clinical DAP-R MRSA strains revealed that, among the 22 strain pairs, six DAP-R isolates (~27%) exhibited significantly altered expression of *mprF* and/or *dltABCD* during exponential- and/or stationary-phase growth. Of interest, five of these six strains contained *mprF* SNPs within the central bifunctional domains ($n=4$) or within the synthase domain ($n=1$). One isolate had no *mprF* SNP (Table 1).

The six DAP-R strains with significantly enhanced *mprF* and/or *dltA* expression were grouped and compared for surface positive charge profiles using the previously published cytochrome *c* binding data[1] versus the remaining 16 DAP-R strains; there was no significant difference in surface charge profiles between the two groups ($P=0.6$; data not shown). These data suggest that dysregulation of *mprF* and/or *dltABCD* expression alone is not enough to explain the enhanced surface positive charge observed in some clinical DAP-R strains.

Expression of graRS among DAP-R strains

Since 6/22 DAP-R strains exhibited enhanced expression of *mprF* and/or *dltA* genes, we assessed the potential role of *graRS* induction in the increased *mprF* and *dltABCD* transcription in these 6 DAP-R isolates. As shown in Figure 1(c), only strains C10 and C37 showed enhanced expression of *graS* during either exponential- or stationary-phase growth. To assess whether the enhanced expression of *graRS* in these two DAP-R strains was linked to altered promoter structure, *graRS* promoter regions were sequenced; this revealed that the *graRS* promoter sequences of these two DAP-R strains were identical to those of their respective DAP-S parental strains (data not shown).

We next sequenced the *graRS* ORFs of these six DAP-R strains to assess the potential correlation of DAP-R, enhanced *mprF*/*dltABCD* expression and the presence of SNPs within the *graRS* ORF. ORF sequencing analyses revealed that these six DAP-R strains displaying increased *mprF*/*dltABCD* expression had *graRS* ORF sequences identical to those of their respective DAP-S parental strains (data not shown). Thus, dysregulation and enhanced expression of *mprF* and *dlt* could not be linked to mutations within either the promoter or the ORF regions of *graRS*. Therefore, future studies should endeavour to extend knowledge of regulatory mechanisms/factors affecting *mprF* and *dltABCD* expression beyond the *graRS* system.

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Transparency declarations

None to declare.

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