Enhanced Expression of $dltABCD$ Is Associated with the Development of Daptomycin Nonsusceptibility in a Clinical Endocarditis Isolate of \textit{Staphylococcus aureus}.

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Using isogenic clinical bloodstream \textit{Staphylococcus aureus} strains from a patient relapsing endocarditis, we investigated the transcriptional profiles of the \textit{mprF} and \textit{dlt} genes in the context of cell-surface charge and daptomycin nonsusceptibility. As in prior studies, a point mutation within \textit{mprF} was observed in the daptomycin-nonsusceptible strain. However, neither the transcriptional profile of \textit{mprF} nor the membrane phospholipid analyses were compatible with the anticipated \textit{mprF} gain-in-function phenotype. In contrast, we demonstrated enhanced \textit{dlt} expression coincident with increased positive surface charge and reduced daptomycin binding.

With the increasing use of daptomycin in clinical practice, there have been a number of recent reports of strains of \textit{Staphylococcus aureus} (especially methicillin-resistant \textit{S. aureus}) developing daptomycin nonsusceptibility during therapy with this agent [1, 2]. Some, but not all, of these daptomycin-nonsusceptible clinical strains exhibit distinct single-nucleotide polymorphisms (SNPs) within the \textit{mprF} gene, in association with 

a “gain-in-function” phenotype [1, 3]. There are 2 major functions of the \textit{mprF} gene product in modifying organism net surface charge: (1) lysinylation of membrane phosphotidylglycerol (PG) to generate lysyl-PG (LPG) and (2) translocation of this positively charged phospholipid to the outer membrane leaflet. In addition to \textit{mprF}, the \textit{dltABCD} operon also contributes to the net positive surface charge by \textit{d}-alanylation of teichoic acids through distinct effector mechanisms [4]. Thus, greater net positive surface charge as mediated by \textit{mprF} and/or \textit{dltABCD} mechanisms would theoretically reduce the overall access of calcium-decorated daptomycin to its putative membrane target [4, 5].

In the present study, we examined an isogenic pair of clinical bloodstream \textit{S. aureus} strains from a patient experiencing daptomycin therapy failure in which the relapse strain exhibited daptomycin nonsusceptibility in vitro. We focused our investigations on the transcriptional profiles of the \textit{mprF} and \textit{dltABCD} genes in the context of cell membrane surface charge and phospholipid profiles. These factors were hypothesized to affect interaction with and susceptibility to the calcium-decorated functional form of daptomycin, as well as susceptibility to innate cationic antimicrobial peptides (CAPs) involved in innate host defense [4, 6].

\textbf{Methods.} The \textit{S. aureus} strains (BOY755 and BOY300) used in this study were methicillin-susceptible bloodstream isolates from a patient with prosthetic mitral valve endocarditis. BOY755 was the initial patient isolate, and BOY300 was subsequently isolated during daptomycin therapy and found to be nonsusceptible to daptomycin. Pulsed-field gel electrophoresis (PFGE) (with \textit{Smal}, \textit{agr} sequencing (type 2), and \textit{spa} typing (type 2) confirmed that these 2 isolates were clonal (data not shown). The minimum inhibitory concentrations (MICs) to daptomycin, as determined by standard micro-E-test, were 0.5 and 2 \mu g/mL for strains BOY755 and BOY300, respectively, using stationary-phase cells. The MICs for vancomycin were 1 \mu g/mL for both strains (the patient was not treated with vancomycin because of allergies to vancomycin and penicillin, although nafcillin therapy was eventually used after desensitization). Population analyses were performed with both daptomycin and vancomycin by standard protocols. Both strains exhibited equivalent growth curve kinetics and ultimate colony-forming unit (CFU) yields over a 24-h period (data not shown).

\textit{S. aureus} strains were grown in either tryptic soy broth (Difco Laboratories) or Mueller-Hinton broth (Difco Laboratories). To determine the impact of growth phase–dependent \textit{dlt} expression on daptomycin MICs, the above-described micro-E-test was used. For stationary-phase cells, overnight cultures...
were used. For exponential-phase cells, overnight cultures of *S. aureus* were diluted to an optical density of 0.1 (read at 600 nm) in fresh medium and incubated for 3 h at 37°C.

All daptomycin (Cubist Pharmaceuticals) assays were done in the presence of 50 μg/mL calcium, as recommended by the manufacturer. Human neutrophil peptide 1 (hNP-1) from human polymorphonuclear leukocytes was purchased from Peptide International. The predominant CAP in mammalian platelets, thrombin-induced platelet microbicidal protein 1 (tPMP-1), was prepared from rabbit blood as described elsewhere [7]. RP-1 (a synthetic congener modeled after the microbicidal domains of platelet microbicidal proteins) was prepared and bioassayed as detailed elsewhere [8, 9]. Synthetic RP-1 has a mechanism of action that recapitulates that of tPMP-1 and was substituted for tPMP-1 in assays requiring relatively large amounts of peptide (eg, surface-binding assays).

For transcription analyses, total RNA was isolated from cell pellets by means of an RNeasy kit (Qiagen) and a FastPrep FP120 instrument (BIO 101), in accordance with the manufacturer’s recommended protocols. Reverse-transcription polymerase chain reaction (RT-PCR) was performed as described elsewhere [10]. The *dlt* cDNA products were detected using the primer pair dlt-F (5′-ATATGATTGTTGGATGATTGGTGCCA-3′) and dlt-R (5′-ACATATGGTCACAACCTGAAGCTACG-3′). The *mprF* cDNA products were detected using the primer pair mprF-F (5′-GTAGTAATCAGTATGTGACG-3′) and mprF-R (5′-GATGCATCGAAACATGGGAATAC-3′).

With respect to CAP susceptibility testing, previous studies

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**Figure 1.** Population analyses of study strains with respect to vancomycin (A) or daptomycin (B) and expression of *mprF* (C) and the *dlt* operon (D). Population analyses data are means ± standard deviations for at least 2 separate assays. Total cellular RNA samples were isolated from exponential-phase and stationary-phase cultures of BOY755 (lane 1) and BOY300 (lane 2) and were subjected to reverse-transcription polymerase chain reaction to detect expression of *mprF*, *dltA*, and *gyrA*.
have shown cross-resistance among daptomycin-nonsusceptible strains with both hNP-1 and tPMP-1 [1]. In vitro bacterial assays were conducted with tPMP-1 and hNP-1 as described elsewhere, using a 2-h microdilution method [1, 6].

To determine peptide binding to S. aureus cells, RP-1 or daptomycin (40 and 6 μg/mL, respectively) was added to 1×10^8 CFUs of each strain. Supernatants were then analyzed for residual unbound peptide by a radial diffusion assay and standard curve techniques, as described elsewhere [1, 7], and the amount of bound peptide was calculated. For the daptomycin assay, the within-day and between-day variations were 25% and 10%, respectively. For the RP-1 binding assay, both the within-day and between-day variations were <5%.

The binding of fluorescein isothiocyanate (FITC)–labeled cationic poly-L-lysine (PLL) to the staphylococcal surface was used to quantify the relative net cell-surface charge, using a flow cytometry assay that has been detailed elsewhere [1, 11]. A total of 10,000 events were counted, and 4 separate experiments were analyzed using the FACSCalibur system (Becton-Dickinson Labware).

To determine membrane phospholipid content and asymmetry, membrane phospholipids were extracted from S. aureus cell pellets by standard methods [11]. The 3 major membrane phospholipids—PG, LPG, and cardiolipin (CL)—were separated by 2-dimensional thin-layer chromatography, removed from the plates, and then quantified spectrophotometrically by a chemical assay that has been described elsewhere [1, 11].

Data were analyzed by the Kruskal-Wallis analysis of variance. Differences for which were considered statistically significant. For daptomycin, the BOY300 population curve was shifted substantially to the right, with heterogenous subpopulations surviving exposures of between 0.5 and 16 μg/mL (Figure 1A). Area under the curve (AUC) values for daptomycin population analyses were ∼4-fold greater in the nonsusceptible strain than in the susceptible strain (mean ± standard deviation [SD], 89.45 ± 1.04 for BOY755 and 20.67 ± 1.52 for BOY300). Vancomycin population analyses of strains BOY755 and BOY300 were virtually identical (mean ± SD AUC values, 6.53 ± 0.12 vs 6.75 ± 0.34), revealing heterogenous subpopulations surviving exposures of between 0.25 and 1 μg/mL (Figure 1B).

In agreement with our previous findings [1], the parental strain (BOY755) was highly susceptible to both tPMP-1 and hNP-1, whereas the BOY300 strain was ∼2-fold and >40-fold more resistant to tPMP-1 and hNP-1, respectively (Table 1).

The binding of positively charged PLL to the parental strain (BOY755) was significantly greater than that of the daptomycin-nonsusceptible strain (BOY300) (mean ± SD, 668 ± 79 vs 506 ± 29 fluorescence units; P<.05). This finding suggests increased repulsion of daptomycin, putatively due to increased net positive cell-surface charge in BOY300. Paralleling the PLL binding data, the binding of both RP-1 (the synthetic surrogate for tPMP-1) and daptomycin was reduced in the daptomycin-nonsusceptible strain (BOY300) (P<.05 (Table 1).

The proportions of the negatively charged phospholipids, PG and CL, were very similar between the 2 strains (Table 1). There was a moderate increase in the total amounts of the positively charged membrane phospholipid, LPG, in the nonsusceptible strain (∼26% vs 32%), although this difference was not statistically significant. Of note, the inner to outer membrane leaflet LPG ratio (as a measure of mprF translocase activity) was 1:1 for both strains.

A single-point mutation (C to A) was observed at position 884 in the mprF gene in the daptomycin-nonsusceptible strain (BOY300). This mutation yields a serine-to-leucine amino acid substitution at position 295 (S295L) (data not shown). In contrast, sequencing analyses of mprF and dlt promoter regions (537-bp and 721-bp regions upstream of the ATG start codon, respectively) revealed no differences between the 2 strains (data not shown). Sequencing studies were performed at the City of Hope Medical Center (Duarte, CA).

As shown in Figure 1C, RT-PCR analysis revealed that mprF is transcribed maximally and to an equivalent extent during early exponential growth in both strains. In contrast to mprF expression, transcription of the dltABCD operon was notably enhanced in the daptomycin-nonsusceptible strain compared with the parental strain during exponential growth (Figure 1D).

Table 1. Phospholipid Profiles, Peptide Binding, and In Vitro Susceptibilities of the Strain Set

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percent survival after 2-h exposure to a</th>
<th>Amount of drug bound, μg</th>
<th>Total phospholipid content, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 μg/mL tPMP-1</td>
<td>20 μg/mL tPMP-1</td>
<td>RP-1 (40 μg)</td>
</tr>
<tr>
<td>BOY755 (parental)</td>
<td>28.5 ± 12</td>
<td>1 ± 1</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>BOY300 (DAP nonsusceptible)</td>
<td>47.5 ± 14.8b</td>
<td>46 ± 3.3b</td>
<td>0.64 ± 0.32b</td>
</tr>
</tbody>
</table>

NOTE. Data are means ± standard deviations. CL, cardiolipin; DAP, daptomycin; hNP-1, human neutrophil peptide 1; LPG, lysyl-PG; PG, phosphotidylglycerol; RP-1, synthetic congener modeled after the microbicidal domains of platelet microbicidal proteins; tPMP-1, thrombin-induced platelet microbicidal protein 1.

a Experiments were repeated a minimum of 4 times.  
b P<.05 vs the parental strain.
For the daptomycin-nonsusceptible strain (BOY300), the MICs in both the exponential-growth and stationary-growth phases were 2 μg/mL, compared with 0.25 and 0.5 μg/mL, respectively, for the daptomycin-susceptible parental strain (BOY755). This increased MIC profile in strain BOY300 paralleled its enhanced expression profile of dlt but not mprF.

Discussion. The current study focused on phenotypic and genotypic analyses of a daptomycin-susceptible and daptomycin-nonsusceptible strain pair from the bloodstream of a patient with endocarditis who experienced daptomycin treatment failure. Of note, the current isogenic strain pair was isolated from a different hospital in a distinct geographic area than a similar previously reported strain pair [1, 3]. As opposed to our prior published study, the current patient did not receive vancomycin therapy before daptomycin was used. Moreover, the genotypic profiles of the current strain pair were also distinct from the previously reported strain pair in terms of PFGE pattern and spa typing. Despite these distinct genotypic profiles, the daptomycin nonsusceptible strain (BOY300) demonstrated increased positive surface charge, reduced daptomycin and CAP binding, and cross-resistance to both platelet (tPMP-1) and neutrophil (hNP-1) CAPs, paralleling the results of our prior report [1]. This finding led us to postulate that, as before [3], the current daptomycin-nonsusceptible strain would demonstrate point mutations in mprF, enhanced expression of this gene, and evidence of an mprF gain-in-function phenotype, using membrane phospholipid profiling as the readout.

Interestingly, in the current study a specific SNP within the mprF open reading frame was identified in the daptomycin-nonsusceptible strain that resulted in an identical amino acid substitution (S295L) in the putative mprF translocase domain, as was described in our previously reported daptomycin-nonsusceptible S. aureus strain series [1, 3]. However, despite this similarity the current daptomycin-nonsusceptible strain (BOY300) did not exhibit an mprF gain-in-function phenotype. Thus, this latter strain exhibited only a modest increase in total LPG production (Table 1) and similar inner versus outer membrane LPG distribution ratios, relative to its daptomycin-susceptible parental strain. Furthermore, unlike the previously characterized daptomycin-nonsusceptible S. aureus strains [3, 12], BOY300 failed to show increased mprF transcription relative to its susceptible counterpart strain (BOY755). In light of these data, it is suggested that the mere presence of a point mutation within the putative translocase domain of mprF is not sufficient to yield a gain-in-function phenotype in LPG translocation. This concept would suggest the requirement for the coincident enhanced expression of the mutated form of mprF to provide a substantive contribution to the daptomycin-nonsusceptible phenotype.

Because increased net positive surface charge and, thus, reduced CAP and daptomycin binding in the daptomycin-nonsusceptible strain (BOY300) could not be explained by total LPG production or translocation differences, expression of the dltABCD operon was also determined. Interestingly, RT-PCR analysis revealed increased dltABCD expression in the daptomycin-nonsusceptible BOY300 relative to BOY755 during exponential growth. This correlated with increases in daptomycin MICs in strain BOY300 during both the exponential and stationary phases of growth compared with the parental strain. These findings strongly support the concept that the enhanced surface positive charge in BOY300 likely results from dltABCD-mediated increases in alanylation of wall teichoic acids, with resultant charge-dependent repulsion of daptomycin. Thus, the current data are the first, to our knowledge, to document an mprF-independent and dltABCD-dependent pathway for daptomycin nonsusceptibility in clinical isolates. Furthermore, analyses of the dlt promoter region showed no difference in sequence between the BOY755 and BOY300 strains (data not shown), indicating that increased expression of the dlt operon in BOY300 likely results from the influence of regulatory factor(s) other than a cis-acting element.

Studies are in progress to further examine the detailed genetic regulatory pathway(s) that control mprF and dltABCD expression in the context of daptomycin nonsusceptibility. Of interest to the current findings, it has been recently shown that, in certain strains of S. aureus, the aps (graRS) genes appear to positively regulate mprF and dltABCD expression in response to specific CAPs [13].

References


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