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Failures in Clinical Treatment of *Staphylococcus aureus* Infection with Daptomycin Are Associated with Alterations in Surface Charge, Membrane Phospholipid Asymmetry, and Drug Binding▽

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Increasingly frequent reports have described the in vivo loss of daptomycin susceptibility in association with clinical treatment failures. The mechanism(s) of daptomycin resistance is not well understood. We studied an isogenic set of *Staphylococcus aureus* isolates from the bloodstream of a daptomycin-treated patient with recalcitrant endocarditis in which serial strains exhibited decreasing susceptibility to daptomycin. Since daptomycin is a membrane-targeting lipopeptide, we compared a number of membrane parameters in the initial blood isolate (parental) with those in subsequent daptomycin-resistant strains obtained during treatment. In comparison to the parental strain, resistant isolates demonstrated (i) enhanced membrane fluidity, (ii) increased translocation of the positively charged phospholipid lysyl-phosphotidylglycerol to the outer membrane leaflet, (iii) increased net positive surface charge ($P < 0.05$ versus the parental strain), (iv) reduced susceptibility to daptomycin-induced depolarization, permeabilization, and autolysis ($P < 0.05$ versus the parental strain), (v) significantly lower surface binding of daptomycin ($P < 0.05$ versus the parental strain), and (vi) increased cross-resistance to the cationic antimicrobial host defense peptides human neutrophil peptide 1 (hNP-1) and thrombin-induced platelet microbicidal protein 1 (tPMP-1). These data link distinct changes in membrane structure and function with in vivo development of daptomycin resistance in *S. aureus*. Moreover, the cross-resistance to hNP-1 and tPMP-1 may also impact the capacity of these daptomycin-resistant organisms to be cleared from sites of infection, particularly endovascular foci.

The rising incidence of invasive infections by resistant *Staphylococcus aureus* strains has created an urgent need for more-potent antistaphylococcal agents (5, 6, 45). Daptomycin is a cyclic lipopeptide approved by the FDA in 2003 for use in a wide variety of *S. aureus* infections. It has potent and uniform activity in vitro and in experimental infection models caused by methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) strains, including hetero-vancomycin-intermediate *S. aureus*, vancomycin-intermediate *S. aureus*, and vancomycin-resistant *S. aureus* (42, 45). A recent multinational randomized and controlled clinical trial of patients with *S. aureus* bacteremia and right-sided endocarditis confirmed the noninferiority of this compound compared to standard therapy (14). However, clinical *S. aureus* strains with a loss of daptomycin susceptibility in vitro (i.e., increased MICs) are now being reported in association with daptomycin treatment failures (15, 18, 32, 33, 44). Complicating such scenarios, recent in vitro studies have suggested that the initial treatment of MRSA infections with vancomycin may provide a foundation for the subsequent loss of daptomycin susceptibility (8, 41).

Upon complexing with calcium, daptomycin targets the *S. aureus* cell cytoplasmic membrane to initiate its antimicrobial activity (22, 43). Recent investigations have provided some insights into the potential mechanism(s) of daptomycin resistance, suggesting the involvement of staphylococcal genes regulating cell membrane (CM) surface charge (e.g., *mprF*) (39) and fatty acid biosynthesis (e.g., *yycFG*) (34). However, a detailed characterization of the phenotypic CM determinants of daptomycin resistance in *S. aureus* has not been reported to date.

We recently examined a well-defined series of *S. aureus* bloodstream isolates from a patient with endocarditis who failed both vancomycin and daptomycin therapies in association with increased daptomycin MICs in vitro. The current study was designed to identify possible changes in cell surface phenotypes relevant to daptomycin resistance, including fluidity, fatty acid content, phospholipid (PL) composition and asymmetry, surface charge, susceptibility to prototypic cationic antimicrobial peptides, membrane depolarization and permeabilization, binding of daptomycin and cationic peptides, and autolysis.

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MATERIALS AND METHODS

Bacterial strains. All strains in this investigation were MSSA bloodstream isolates from the above-described patient. We focused our studies on the following four strains: 616 (initial patient isolate), 629 (first isolate breaking through daptomycin therapy and demonstrating increased daptomycin MICs still within the susceptible range, termed the "transitional strain"), and 701 and 703 (subsequently isolated during daptomycin therapy and nonsusceptible to daptomycin). Pulsed-field gel electrophoresis using SmaI restriction enzymes confirmed that these isolates were clonal and did not represent new infecting strains acquired during daptomycin treatment (data not shown). Growth curves in Mueller-Hinton broth (Difco Laboratories, Detroit, MI) were virtually identical for the four study strains (data not shown). Their MICs to daptomycin, as determined by Etest, were 0.5, 0.75, 2, and 2 μg/ml for strains 616, 629, 701, and 703, respectively; the MICs of vancomycin were 2 μg/ml for all strains (21). All isolates were kept at −70°C, and there were no changes in their initial daptomycin susceptibility profiles upon storage. Since we identify the *mprF* gene as a putative target involved in daptomycin resistance (see below), the MICs to daptomycin were determined by Etest for a previously described isogenic strain pair: parental strain SA113 and its *mprF*-null deletion mutant (39).

Antimicrobial agents. Daptomycin was purchased from Cubist Pharmaceuticals (Lexington, MA) and reconstituted according to the manufacturer's recommendations. All daptomycin assays were done in the presence of 50 μg/ml calcium chloride as recommended by the manufacturer (20, 21).

Antimicrobial peptides (APs). Thrombin-induced platelet microbicidal protein 1 (tPMP-1) was purified by high-pressure liquid chromatography (27, 28, 47, 52, 53), and rational peptide 1 (RP-1, a tPMP-1 synthetic congener) was synthesized via multiplex synthesizer techniques (Rainin, Woburn, WA) as previously described (46, 50). Human neutrophil peptide 1 (hNP-1) (16, 30, 31) and gramicidin D (3, 4) were purchased from Peptide International (Louisville, KY) and Sigma Chemicals (St. Louis, MO), respectively.

In vitro AP susceptibility testing. The MICs of gramicidin D in cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, MI) were determined by a microdilution technique according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (37). The MIC was defined as the lowest drug concentration preventing visible turbidity after 18 h of incubation at 37°C. The CLSI has established MIC guidelines only for daptomycin susceptibility (≤1 μg/ml) but not for daptomycin resistance. Thus, strains with MICs of ≥2 μg/ml have been termed "daptomycin-nonsusceptible" in this report.

Standard MICs for tPMP-1 and hNP-1 are not routinely determined since conventional nutrient media tend to mitigate the activity of these peptides. Thus, bactericidal assays were carried out with these APs (50–52). Briefly, *S. aureus* cells were diluted into the test AP solutions to achieve the desired final inocula (10^3 and 10^5 CFU/ml for tPMP-1 and hNP-1, respectively) (48, 49) and then incubated at 37°C. At 120 min of exposure, samples were removed and processed for quantitative culture to assess the extent of killing by each AP (48). Final data were expressed as mean surviving $\log_{10}$ CFU/ml (± standard deviations [SD]).

Population analyses. We performed population analyses of the strain set with both vancomycin and daptomycin by standard protocols (35). For vancomycin and daptomycin, the range of concentrations tested was 0.062 to 16 μg/ml to encompass sublethal-to-lethal drug levels, using an initial inoculum of ~10^9 CFU/ml.

Membrane fluidity. CM fluidity was determined by fluorescence polarization, using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene as described before (2). An inverse relationship exists between polarization index values and CM fluidity (i.e., lower polarization indices = greater CM fluidity) (2). These assays were performed a minimum of eight times for each strain.

CM fatty acid composition. Fatty acids of total lipids extracted from *S. aureus* were analyzed using gas-liquid chromatography after conversion to their methyl ester form (courtesy of Microbial ID Inc., Newark, DE) (2). All assays were performed a minimum of two times on separate days.

Membrane PL content and asymmetry: extraction, identification, and quantification of PLs. Phospholipids (PLs) were extracted from *S. aureus* cell pellets by standard methods (10, 13). The major PLs, phosphotidylglycerol (PG), cardiolipin (CL), and lysyl-PG (LPG), were separated by two-dimensional thin-layer chromatography (TLC) (11). LPG (positively charged) was identified by ninhydrin staining. PG, CL, and minor PLs were visualized by exposure of the TLC plate to iodine vapor (39). All standard PLs were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used to determine the positions of their spots on two-dimensional TLC plates.

For quantitative analysis, isolated PLs were digested and quantified spectrophotometrically at an optical density at 660 nm (OD_{660}) as previously described (11, 36). All assays were performed a minimum of three times on separate days.

LPG distribution. Fluorescamine, a fluorescent probe which specifically labels CM surface-exposed (outer leaflet), positively charged amino-PLs, was used to assay for CM LPG distribution (10, 36). Of note, once bound to LPG, fluorescamine alters its mobility characteristics and attenuates its ability to be detected by ninhydrin staining (19). After detection of the fluorescamine-labeled LPG spot on the TLC plate, its relative content (normalized with respect to total PL and total LPG) was quantified by the colorimetric assay described above.

Cell surface charge. Poly-L-lysine (PLL) is a polycationic molecule used to study the interactions of cationic peptides with charged bilayer membranes (17, 24). The fluorescein isothiocyanate-labeled PLL binding assay was performed using flow cytometric methods (FACSCalibur; Beckman Instruments, Alameda, CA) (36). Data were expressed as mean fluorescent units (± SD). The lower the residual cell-associated label, the greater the PLL repulsion and the more positively charged the *S. aureus* cell envelope (38).

Δψ. To monitor the membrane potential (Δψ), 1 μCi/ml of [^{3}H]tetraphenylphosphonium bromide (TPP^+; 26 Ci/mmol) was used as previously described (1). Counts were corrected for nonspecific binding of [^{3}H]TPP^+ by subtracting the radioactivity of 10% butanol-treated aliquots. For the calculation of Δψ, TPP^+ concentrations were applied to the Nernst equation $\Delta\psi = (2.3 \times R \times T/F)/\log([TPP^+_{in}]/[TPP^+_{out}])$, where R is the universal gas constant, T is the absolute temperature in Kelvin, F is the Faraday constant, $[TPP^+_{in}]$ is the molar concentration of TPP^+ inside the bacterial cells, and $[TPP^+_{out}]$ is the molar concentration of TPP^+ in the medium (1).

Membrane depolarization. The lipophilic dye 3,3-dipentyloxacarbocyanine ($DiOC_5$; Molecular Probes) incorporates into the cytoplasm of a cell and requires an intact Δψ for intracellular retention. *S. aureus* cells were grown to logarithmic phase in brain heart infusion broth, pelleted by centrifugation, washed in phosphate-buffered saline, and resuspended to a concentration of 5×10^7 CFU/ml in K^+ Eagle's minimal essential medium. Cells were incubated in darkness for 1 h with 0.05 μM $DiOC_5$. Organisms were pelleted by centrifugation, washed, resuspended in K^+ Eagle's minimal essential medium, and briefly sonicated. Cells were then incubated with Triton X-100 (10% [vol/vol]), RP-1 (40 μg/ml), or daptomycin (0.75 μg/ml; 6 μg/ml in the presence of 50 μg/ml $CaCl_2$). For flow cytometric analysis, the membrane potential-sensitive dye, $DiOC_5$, was excited at 488 nm and the emitted light was collected through a 525 ± 25 nm band-pass filter. Mean fluorescence was determined at 30-min intervals for up to 120 min. A reduction in mean $DiOC_5$ fluorescence represents depolarization of the staphylococcal membrane. A minimum of two experiments was performed for each agent to assess depolarization.

Membrane permeabilization. Membrane permeabilization of the strain set was detected via release of the preloaded fluorescent probe, calcein (12, 26, 49). Calcein-loaded *S. aureus* cells (final inoculum, 10^6 CFU/ml) were diluted into the sublethal concentrations of test AP or daptomycin solutions (i.e., 0.5 μg/ml tPMP-1, 10 μg/ml hNP-1, 32 μg/ml gramicidin D, and 1.5 μg/ml daptomycin) based on our pilot studies and incubated at 37°C for 120 min. At selected time points, calcein retention was quantified using a Turner digital fluorometer (model 450; Barnstead/Thermolyne Corp., Dubuque, IA) (49). Cobalt (Co^{2+}; 2 μM chloride salt; Sigma) was used to quench the fluorescence of calcein released into the extracellular supernatant (49). Each experiment included a concomitant quantitative culture to ensure that calcein loading did not alter *S. aureus* susceptibility to the APs. Experiments were repeated independently at least three times on separate days.

Autolysis. Logarithmic-phase *S. aureus* cells at a final OD_{630} of 0.7 were exposed to 0.05% (vol/vol) Triton X-100, daptomycin (6 μg/ml), or vancomycin (50 μg/ml) and incubated at 35°C with gentle agitation. Serial samples were obtained during a 24-h exposure, and the OD_{600} was measured. Autolysis was quantified as a percent of the initial OD_{600} remaining at each sampling time point. Data in the figures represent the means of two separate assays.

AP and daptomycin binding. To determine peptide binding to *S. aureus* cells, AP or daptomycin (40 μg/ml RP-1, 20 μg/ml hNP-1, 6 μg/ml daptomycin, or 32 μg/ml gramicidin D [in dimethyl sulfoxide]) was added to 10^8 CFU of each *S. aureus* strain, incubated for 10 min, and centrifuged (10,000 rpm). Supernatants were then removed, lyophilized, and stored at 4°C until assayed by radial diffusion (51). RP-1 was used instead of tPMP-1 in this assay because of its better nutrient agar diffusibility. An agar diffusion plate was prepared by seeding 10^6 CFU/ml of *Bacillus subtilis* ATCC 6633 for RP-1, hNP-1, and daptomycin or *Staphylococcus aureus* strain COL for gramicidin D into 20 ml of 10 mM 2-(*N*-morpholino)ethane sulfonic acid (pH 5.5) supplemented with 1% molecular

TABLE 1. In vitro susceptibilities of the study strains to APs

Strain	% Survival (mean ± SD) after 2-h exposure to:	
	1 μg/ml tPMP-1	20 μg/ml hNP-1
616 (parental)	7 ± 5	2 ± 2
629 (transitional)	29 ± 14	8 ± 8
701 (daptomycin nonsusceptible)	79 ± 13	29 ± 13
703 (daptomycin nonsusceptible)	81 ± 7	22 ± 8

grade agar and 0.03% glucose (Sigma, St. Louis, MO). To derive a standard curve, AP or daptomycin was added to the wells at a range of concentrations, incubated for 3 h at 37°C, and then removed by aspiration. Then, 10 ml of brain heart infusion agar was overlain, and plates were incubated overnight at 37°C. Zone sizes (mm) were measured using SpotDense software within the FluorChem 8900 imaging system (Alpha Innotech, San Leandro, CA). The amounts of supernatant AP or daptomycin were determined from the standard curves described above. The concentrations of bound AP or daptomycin were then calculated based on the initial AP or daptomycin concentrations in each reaction mixture and the residual supernatant drug and expressed as mean μg/ml (± SD) bound. At least three independent runs were performed on separate days.

Statistics. Data were analyzed by the Kruskal-Wallis analysis of variance, with corrections made for multiple comparisons where appropriate. A P value of ≤0.05 was considered significant.

RESULTS

Antimicrobial susceptibilities to APs. As shown in Table 1, parental strain 616 was highly susceptible to both tPMP-1 and hNP-1, while both daptomycin-nonsusceptible strains were ~10-fold more resistant to both APs. Strain 629 (the transitional isolate) exhibited intermediate susceptibility to tPMP-1 while remaining fully susceptible to hNP-1. For gramicidin D, all four isolates had MICs of 32 μg/ml, likely reflecting the unique mechanism of action of this peptide that differs from that of tPMP-1 and daptomycin (49).

The daptomycin MICs for the laboratory-derived parental strain SA113 and its *mprF* null mutant were 0.38 and 0.094 μg/ml, respectively, as determined by Etest.

Population analyses. Vancomycin population analyses of strains 616 and 629 were virtually identical, revealing heterogeneous subpopulations surviving exposures of between 0.25 and 1 μg/ml vancomycin (Fig. 1A). In strain 701 (and 703 [data not shown]), a similar heterogeneous subpopulation was detected over the same vancomycin range, although the extent of these subpopulations was greater than in strains 616 and 629. In strains 616 and 629, daptomycin population analyses were also virtually identical, with heterogeneous subpopulations surviving exposures of between 0.5 and 1 μg/ml daptomycin (Fig. 1B). For daptomycin-nonsusceptible strains, 701 and 703 (data not shown), the population curve was shifted to the right, with heterogeneous subpopulations surviving exposures of 0.5 to 4 μg/ml daptomycin.

Membrane fluidity. Both daptomycin-nonsusceptible strains (701 and 703) exhibited substantially greater CM fluidity (0.33 ± 0.001 postinfection) than the parental strain (0.36 ± 0.001 postinfection; $P = 0.03$ and $P = 0.02$, respectively, versus the parental strain). The transitional strain (629) also had more fluid membranes than the parental strain, although this trend did not reach statistical significance (0.34 ± 0.001 postinfection; $P = 0.14$).

Membrane fatty acid composition. Fatty acid analyses revealed no substantive differences among the strain set in terms of unsaturation indices, acyl chain length profiles, the proportion of branched-chain fatty acids, or iso- versus anteiso-branched-chain fatty acid content (data not shown).

Membrane PL content and asymmetry. Table 2 shows the relative proportions of the three major membrane PLs of the four study strains. The proportions of the negatively charged PL, CL, were very similar among the strains. In contrast, the proportion of the negatively charged PG was ~10% to 14% lower in the two daptomycin-nonsusceptible strains, although this difference was not statistically significant. The total amounts of the positively charged PL, LPG, were similar in the parental strain and the two daptomycin-nonsusceptible strains (23 to 29%). However, the total amount of LPG that was translocated to the outer membrane leaflet was ~2-fold higher in both nonsusceptible isolates than in the parental strain. Further, the outer-to-inner membrane leaflet LPG ratio was ~2:1 to 4:1 for the two nonsusceptible strains, compared with ~1:1 for the parental strain. For the transitional strain, 629, although the total amount of membrane LPG observed was less than in the other isolates, ~3- to 4-fold more was distributed to its outer membrane leaflet than to its inner membrane leaflet.

Cell surface charge. The binding of positively charged PLL to the parental strain was ~2-fold greater than that to either of the two daptomycin-nonsusceptible strains ($P < 0.05$; ~650 versus 300 to 340 fluorescence units, respectively). In addition, PLL binding to the transitional strain (629) was substantially less (~450 fluorescence units) than to the parental strain, although this difference did not reach statistical significance.

$\Delta\psi$. The time profiles of the decline and reestablishment of the $\Delta\psi$ during exponential- and postexponential-growth phases were virtually identical for the strain set. The $\Delta\psi$ started at ~−100 mV at time zero, dropping to ~−70 mV during logarithmic growth and then reestablishing at ~−120 mV in the postexponential period (data not shown).

Membrane depolarization. Triton X-100 caused depolarization of all four strains (curve shift to the left; Fig. 2). The parental strain was depolarized by 60 min of exposure to daptomycin, while neither the transitional strain nor either daptomycin-nonsusceptible strain was depolarized by daptomycin. RP-1 yielded no depolarization, consistent with our prior observations with tPMPs (51).

Membrane permeabilization. By 120 min of exposure, sublethal concentrations of tPMP-1 (0.5 μg/ml) yielded a trend toward reduced membrane permeabilization in the daptomycin-nonsusceptible strains compared to that in the parental strain (Fig. 3A). However, this trend did not reach statistical significance. Sublethal hNP-1 concentrations (10 μg/ml) caused significantly reduced membrane permeabilization in the two daptomycin-nonsusceptible strains as well as in the transitional strain compared to that in the parental isolate at this same time point ($P < 0.05$) (Fig. 3B). Similarly, sublethal daptomycin exposure (1.5 μg/ml) for 120 min was associated with reduced membrane permeabilization in the nonsusceptible and transitional strains compared to that in the parental strain ($P = 0.07$ for strain 703) (Fig. 3C).

Autolysis. The rate of Triton X-100-induced autolysis appeared to be lower in both daptomycin-nonsusceptible strains

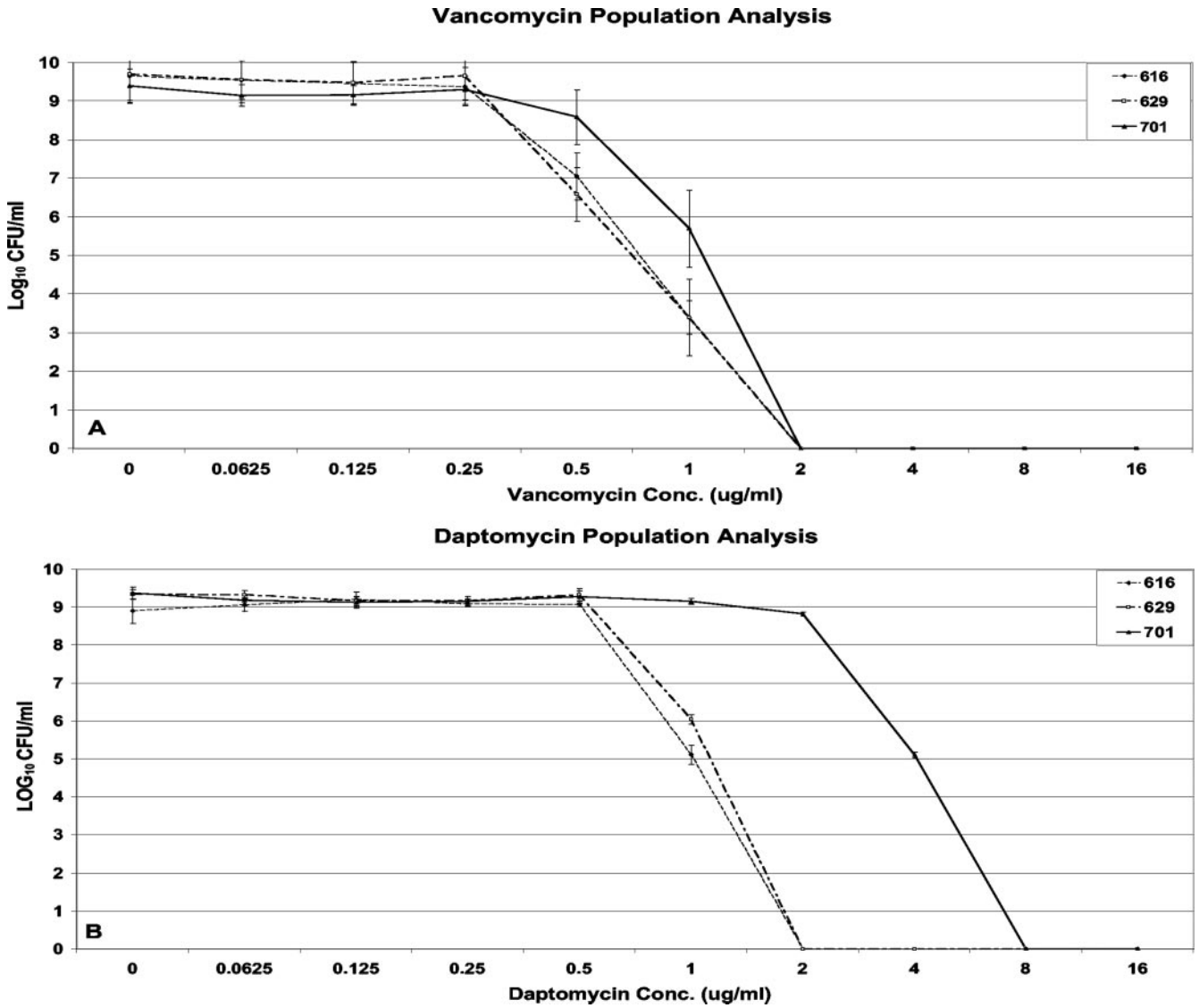

FIG. 1. Population analyses of study strains upon exposure to a range of vancomycin (A) or daptomycin (B) concentrations (conc.). These data represent the means (± SD) for two separate assays. See the text for details.

than in the parental strain over the first 3 hours of the assay. However, this difference was significant only at the 2-h time point for both nonsusceptible strains ($P < 0.05$ versus the parental strain). Between 4 and 24 h, the rates of autolysis seemed to be equivalent among the strain set (Fig. 4A). In addition, daptomycin-induced lysis rates were lower in both daptomycin-nonsusceptible strains than in the parental strain between 4 and 24 h of exposure, although this reached statistical significance only at the 6- and 7-h time points ($P < 0.05$) (Fig. 4B). Further, vancomycin-induced lysis appeared slower in the daptomycin-nonsusceptible and transitional strains than in the parental strain over the 24-h exposure (Fig. 4C). However, these differences were significant only for the 18-h time point and only for strain 703 ($P < 0.05$).

TABLE 2. Phospholipid composition and asymmetry of LPG for the study strains

Strain	% of total phospholipid content (mean ± SD)			
	Inner leaflet LPG	Outer leaflet LPG	PG	CL
616 (parental)	12 ± 4	11 ± 4	66 ± 5	13 ± 5
629 (transitional)[a]	3 ± 2	10 ± 3	63 ± 4	14 ± 11
701 (daptomycin nonsusceptible)[a]	5 ± 1	20 ± 9	55 ± 6	12 ± 7
703 (daptomycin nonsusceptible)	9 ± 6	20 ± 8	59 ± 10	12 ± 6

[a] TLC identified a small proportion of unknown phospholipids for this strain.

AP and daptomycin binding. As noted in Table 3, binding of RP-1 (the surrogate for tPMP-1) or gramicidin D was reduced in the daptomycin-nonsusceptible strains, although this reached statistical significance only in strain 703, compared to that in the parental strain ($P < 0.05$). Likewise, hNP-1 binding was reduced in both daptomycin-nonsusceptible strains and the transitional strain, 629 ($P < 0.05$ versus the parental strain).

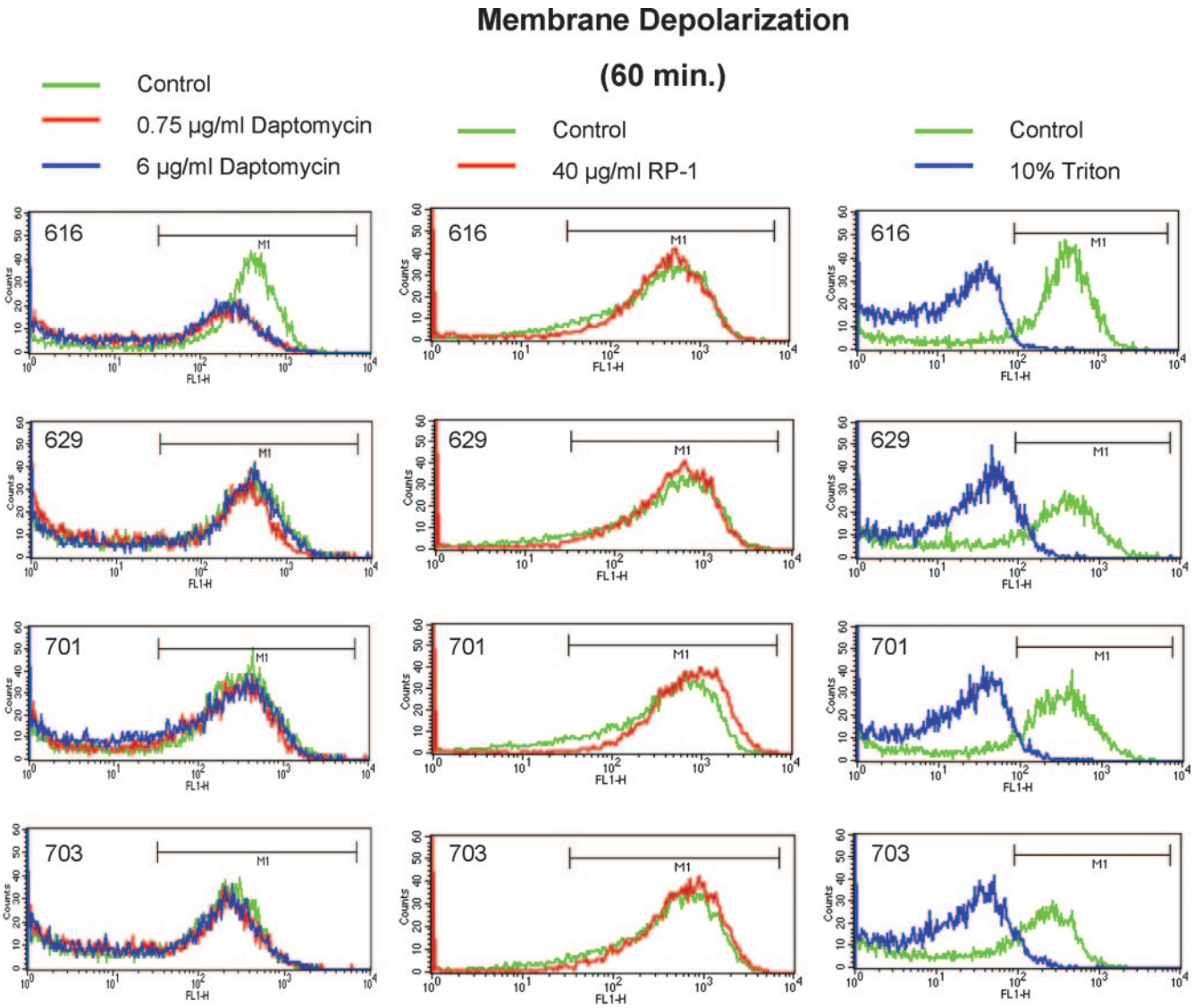

FIG. 2. Membrane depolarization by daptomycin, RP-1, and Triton X-100 by flow cytometry at 60-min exposures. These data represent the means for three separate assays. See the text for details.

For daptomycin, there was a significant reduction in binding to both daptomycin-nonsusceptible strains as well as to the transitional strain compared to that in the parental strain ($P < 0.05$).

DISCUSSION

The mechanism(s) of daptomycin resistance in *S. aureus* remains incompletely understood. Friedman et al. (15) have recently demonstrated a predictable series of genetic perturbations induced by in vitro passages, including mutations in (i) *mprF* (a gene which contributes to membrane charge through lysinylation of PG) (39), (ii) *yycG* (a histidine kinase gene of multiple functions, including impacts on membrane fatty acid biosynthesis) (34), and (iii) *rpoB* and *rpoC* (subunits of RNA polymerase). Importantly, similar genetic defects were found in three sets of clinical *S. aureus* isolates from patients failing daptomycin therapy, although no phenotypic membrane function data were provided. Kaatz et al. (23) recently reported on the emergence of daptomycin resistance in a patient with MRSA tricuspid valve endocarditis who failed daptomycin treatment. Interestingly, the daptomycin-resistant strain exhibited (i) reduced daptomycin binding to both the whole cells and the cytoplasmic membranes and (ii) reduced membrane depolarization. In addition, the resistant isolates demonstrated the loss of an 81-kDa membrane protein postulated to represent a daptomycin membrane "chaperone." Finally, Silverman et al. (43) have confirmed that some daptomycin-resistant bacterial strains selected in vitro have altered membrane potential, a feature shared by selected AP-resistant *S. aureus* strains (1, 27).

The present study was designed to provide a detailed analysis of cytoplasmic membrane correlates of in vitro daptomycin resistance in a well-characterized strain set from an endocar-

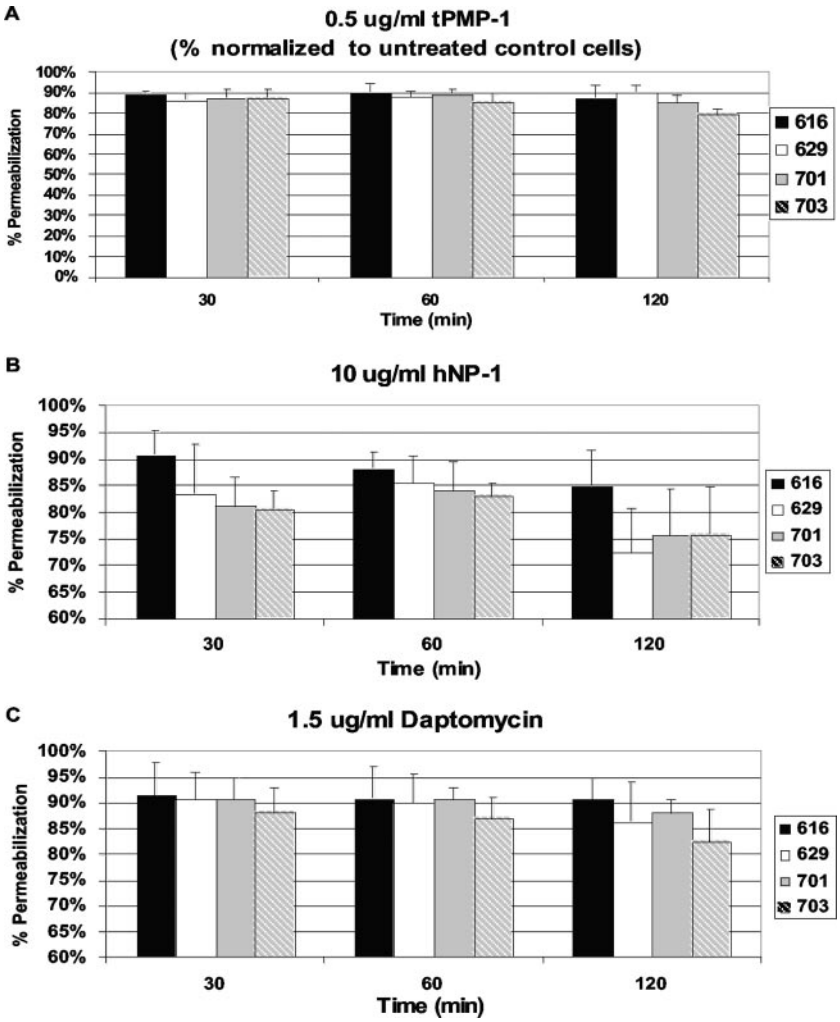

FIG. 3. Membrane permeabilization of the study strains by sublethal concentrations of two study antimicrobial peptides (tPMP-1 [A] and hNP-1 [B]) and daptomycin (C) by using the calcein release assay. These data represent the means (± SD) for three separate assays. See the text for details.

ditis patient failing daptomycin therapy. A number of interesting observations emanated from this study. Although most of the daptomycin-resistant strains reported in the recent literature are MRSA strains, the development of daptomycin resistance in this community-acquired MSSA strain demonstrates that daptomycin resistance may occur rapidly in these isolates as well. Of interest, population analyses of the initial bloodstream isolate prior to antimicrobial therapy did reveal the presence of heterogeneously distributed subpopulations to both vancomycin and daptomycin. However, all heterogeneous subpopulations fell within the range of susceptibility for each agent ($\leq$2 μg/ml).

Detailed analysis of membrane structure and function identified a cadre of phenotypes that readily distinguished the daptomycin-susceptible strains from the daptomycin-nonsusceptible strains. For example, the CMs of the daptomycin-nonsusceptible isolates were significantly more fluid than those of the daptomycin-susceptible parental strain. Importantly, such enhanced membrane fluidity has been correlated with in vitro resistance to killing by cationic APs (e.g., PMPs) (1, 2). The mechanism(s) underlying this enhanced membrane fluidity in daptomycin-nonsusceptible isolates remains to be determined. Fatty acid analyses did not identify prototypic changes in the daptomycin-nonsusceptible strains which are usually associated with enhanced fluidity profiles, such as longer acyl chain lengths, increased unsaturation indices, and shifts in iso- to anteiso-branched-chain fatty acid content (25). It is notable that the daptomycin-resistant isolates described by Friedman et al. exhibited point mutations within the *yycFG* operon, a global regulator involved in fatty acid biosynthetic pathway coordination (15).

Definable differences were observed between membrane PL profiles of daptomycin-susceptible strains and those of daptomycin-nonsusceptible strains. Despite overall similar contents of the three major membrane PLs (PG, CL, and LPG), the daptomycin-nonsusceptible isolates exhibited increased outer leaflet LPG translocation compared to the daptomycin-susceptible isolates. LPG is a positively charged PL which is unique to *S. aureus*, accounting for ~10 to 30% of the total PL content of its cytoplasmic membrane (36, 39, 49). The LPG content con-

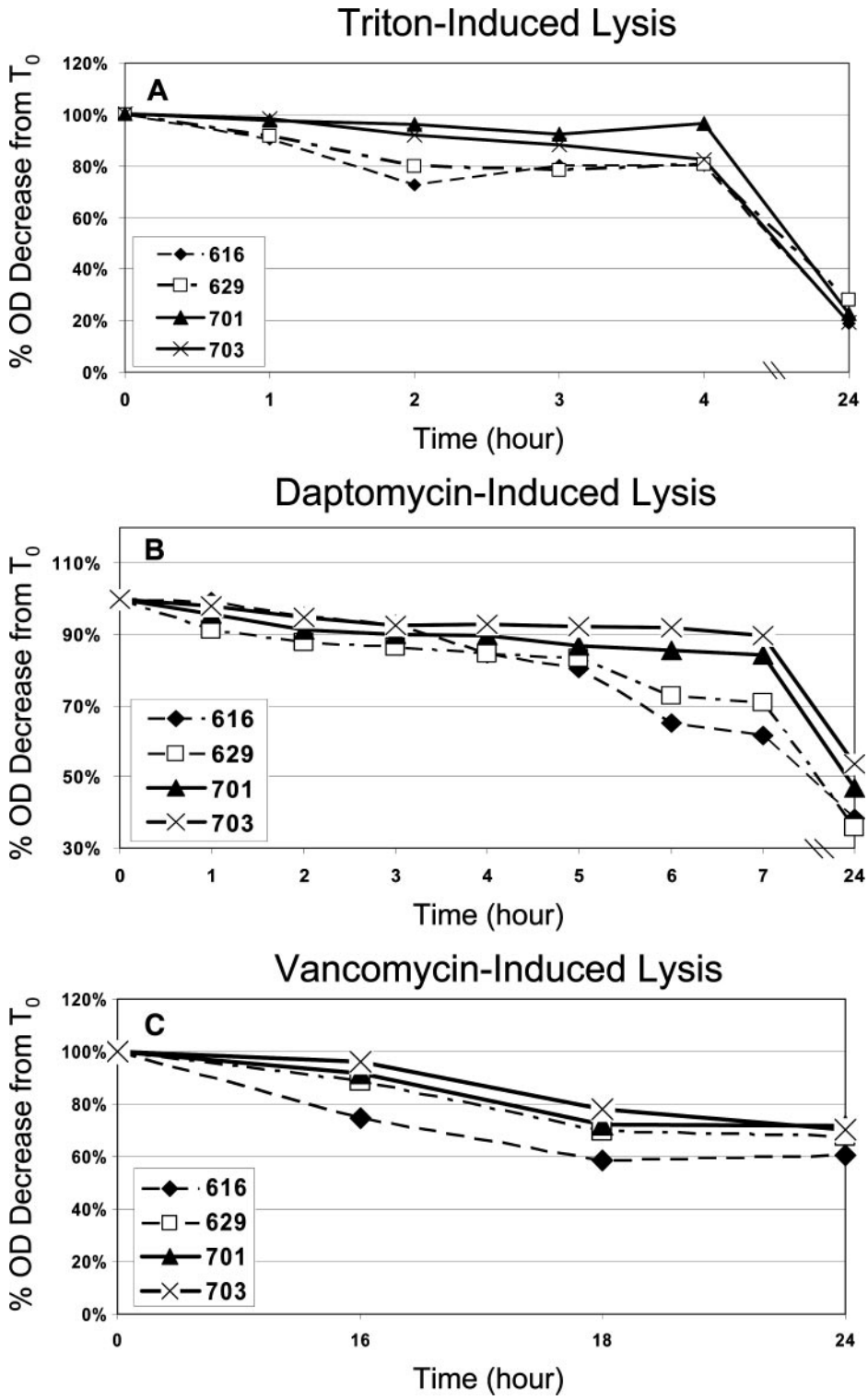

FIG. 4. Autolysis of the study strains, as determined by the change in OD_{600} over time for Triton X-100 (A), daptomycin (B), and vancomycin (C). Data represent the means for at least two separate runs. Data are depicted as the percent change from the time zero (T_0 [preexposure]) value, set at 100%. See the text for details.

tributes to the relative positive charge characteristics of the staphylococcal cell surface (39). Thus, it was not unexpected that this enhanced translocation of LPG to the outer membrane leaflet was associated with increased surface repulsion of the cationic marker, PLL, in these strains. The synthesis and outer leaflet translocation of LPG are regulated by *mprF* (39). The deletion of *mprF* yields staphylococcal cells with reduced outer leaflet LPG translocation, reduced cell surface positive

TABLE 3. Peptide binding to strain set

Strain	Concn of drug bound (μg/ml [mean ± SD])[a]			
	RP-1 (40 μg)	Gramicidin D (32 μg)	hNP-1 (20 μg)	Daptomycin (6 μg)
616 (parental)	12.4 ± 0.48	12.0 ± 3.0	13 ± 1.2	5.4 ± 0.83
629 (transitional)	11.7 ± 0.10	10.0 ± 1.8	4.0 ± 0.5*	1.61 ± 1.3*
701 (daptomycin nonsusceptible)	11.7 ± 0.15	11.0 ± 3.8	8.0 ± 1.1	2.32 ± 1.23*
703 (daptomycin nonsusceptible)	8.0 ± 0.34*	9.0 ± 2.7*	8.0 ± 1.6	2.24 ± 1.07*

[a] *, $P < 0.05$ versus the parental strain.

charge, and increased susceptibility in vitro to a number of cationic APs, including PMPs, hNP-1, and gramicidins (36, 39, 49). Paradoxically, the phenotype of the current daptomycin-nonsusceptible strains (increased outer leaflet LPG translocation, increased relative surface positive charge, and increased resistance to cationic APs) suggests a "gain in *mprF* function." Of note, the deletion of *mprF* resulted in a fourfold decrease in daptomycin MICs in the strain set studied in the present investigation, supporting the linkage of this operon with daptomycin susceptibility profiles. Genotypic studies in our laboratories are currently examining the status of the *mprF* gene in parental clones versus that in daptomycin-nonsusceptible clones. It is certainly possible that mutations within the *mprF* operon or in other key regulatory elements may underlie such gains in function, similar to the point mutations within *mprF* for daptomycin-resistant MRSA strains, as documented by Friedman et al. (15). Of interest, Pillai et al. (40) have recently documented the development of daptomycin nonsusceptibility in three clinical hetero-vancomycin-intermediate *S. aureus* isolates with neither daptomycin exposures nor *mprF* point mutations.

The current mode-of-action model for daptomycin assumes that it complexes with calcium and then functions like a typical cationic AP. Therefore, it was logical to delineate the comparative abilities of the strain set to generate a transmembrane potential ($\Delta\Psi$) of sufficient magnitude to drive the activity of daptomycin. Typically, when *S. aureus* grows in fresh, glucose-containing media, the $\Delta\Psi$ initially drops due to rapid glucose consumption and substrate phosphorylation. It rises again when lactate and acetate are subsequently consumed. In the current study, all four study strains showed such typical behavior, excluding the notion that the loss of daptomycin susceptibility was based on reduced basal membrane energetics.

Of note, daptomycin caused only partial depolarization of the cytoplasmic membrane of the parent strain, while neither the transitional nor the nonsusceptible variants were depolarized at all. This raises questions about membrane poration and subsequent depolarization being the primary lethal mechanisms for daptomycin (22). Indeed, the capacity of daptomycin to cause rapid leakage of potassium ions from the cytoplasm is very low compared to that of the lantibiotic nisin (H.-G. Sahl, unpublished data); this indicates that membrane depolarization may be a relevant yet not the major component of daptomycin killing. Nevertheless, perturbations of such a "minor component" of daptomycin activity (i.e., depolarization) may be sufficient to explain, at least in part, the loss of daptomycin susceptibility in the current study.

Other membrane events may also be contributing to the daptomycin-nonsusceptible phenotype in our strain set. For example, the ability of calcium-complexed daptomycin (and other cationic peptides, such as PMPs and hNP-1) to associate with the surface of daptomycin-nonsusceptible strains was significantly reduced compared to its ability to associate with the surface of the parental strain. This correlated with a reduced capacity of daptomycin to permeabilize the daptomycin-nonsusceptible isolates compared to its capacity to permeabilize the daptomycin-susceptible isolates.

The documented cross-resistance to the cationic, membrane-active, host defense cationic peptides hNP-1 and tPMP-1 among the daptomycin-nonsusceptible isolates in our study should be emphasized. Experimental and human data confirm that in vitro resistance to tPMP-1 correlates with an increased prevalence of human endocarditis and a reduced capacity to clear *S. aureus* strains from valvular vegetations in experimental endocarditis, respectively (29). Thus, cross-resistance to such host defense peptides may well contribute to the failure of daptomycin therapy in patients with "high-inoculum" infections, such as endocarditis (9).

We did not analyze the daptomycin-nonsusceptible study strains for possible cell wall perturbations, although these have previously been described by other groups (7). It is conceivable that such changes could contribute to the net surface charge differences we observed. Of interest, Kosowska-Shick et al. have recently shown substantial reductions in O acetylation of muramic acid within the peptidoglycan of daptomycin-nonsusceptible clinical isolates compared to that of their respective daptomycin-susceptible parental strains (28a). This was postulated to impact surface hydrophobicity and/or charge (28a).

Finally, daptomycin-nonsusceptible strains in this study demonstrated lower rates of autolysis than the daptomycin-susceptible parental strain. Since the autolytic pathway in *S. aureus* appears to contribute to full execution of the lethal pathway of cationic APs such as PMPs (48), it is not surprising that daptomycin resistance is associated with defective autolysis. The mechanism(s) of defective autolysis in daptomycin-resistant strains remains to be defined.

In comparing the membrane phenotypes of daptomycin-susceptible and daptomycin-nonsusceptible isolates, it was interesting to note that the "transitional" strain exhibited most (although not all) of the perturbations observed in the daptomycin-nonsusceptible strains. Yet, the transitional strain did not achieve the higher-level resistance to daptomycin observed in subsequent strains. These findings suggest that although membrane alterations appear to antedate daptomycin resistance, additional phenotypic or genotypic events are required. Current studies in our laboratories are designed to test this hypothesis, focusing on genetic characterization of daptomycin resistance, including (i) comparative sequencing of *mprF* and

other putative target genes involved in such resistance, (ii) comparative genome hybridizations, and (iii) differential gene expression profiles by microarray analyses. Of interest, preliminary data (unpublished) have shown no sequence differences in the *mprF* promoter regions of the current strain set; in contrast, both daptomycin-nonsusceptible strains exhibit a single nucleotide polymorphism in the *mprF* open reading frames compared to the parental strain.

Thus, in summary, at least four phenotypic membrane alterations correlate with daptomycin resistance in *S. aureus*: increased fluidity, increased positive surface charge resulting in reduced drug binding, reduced permeabilization capacities, and reduced depolarization. It is likely that one or more of these phenotypes are interrelated. Further, it should be emphasized that although differences in selected parameters between the parental and mutant strains did not reach statistical significance (e.g., LPG content, LPG asymmetry, and membrane depolarization), it is highly likely that they contributed to the composite mechanism of daptomycin nonsusceptiblity in this strain set.

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