Low-Level Resistance of *Staphylococcus aureus* to Thrombin-Induced Platelet Microbicidal Protein 1 In Vitro Associated with *qacA* Gene Carriage Is Independent of Multidrug Efflux Pump Activity


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Low-Level Resistance of \textit{Staphylococcus aureus} to Thrombin-Induced Platelet Microbicidal Protein 1 In Vitro Associated with \textit{qacA} Gene


\textit{Division of Infectious Diseases, Harbor-UCLA Medical Center, Torrance, California, and LA Biomedical Research Institute at Harbor-UCLA, Torrance, California; Geffen School of Medicine at UCLA, Los Angeles, California; and School of Biological Sciences, The University of Sydney, Sydney, New South Wales 2006, Australia}

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Thrombin-induced platelet microbial protein 1 (tPMP-1), a cationic antimicrobial polypeptide released from thrombin-stimulated rabbit platelets, targets the \textit{Staphylococcus aureus} cytoplasmic membrane to initiate its microbicidal effects. In vitro resistance to tPMP-1 correlates with survival advantages in vivo. In \textit{S. aureus}, the plasmid-carried \textit{qacA} gene encodes a multidrug transporter, conferring resistance to organic cations (e.g., ethidium [Et]) via proton motive force (PMF)-energized export. We previously showed that \textit{qacA} also confers a tPMP-1-resistant (tPMP-1\textsuperscript{r}) phenotype in vitro. The current study evaluated whether (i) transporters encoded by the \textit{qacB} and \textit{qacC} multidrug resistance genes also confer tPMP-1\textsuperscript{r} and (ii) tPMP-1\textsuperscript{r} mediated by \textit{qacA} is dependent on efflux pump activity. In contrast to tPMP-1\textsuperscript{r} \textit{qacA}-bearing strains, the parental strain and its isogenic \textit{qacB-} and \textit{qacC-containing} strains were tPMP-1 susceptible (tPMP-1\textsuperscript{i}). Efflux pump inhibition by cyanide \textit{m}-chlorophenylhydrazone abrogated Et\textsuperscript{r}, but not tPMP-1\textsuperscript{r}, in the \textit{qacA}-bearing strain. In synergy assays, exposure of the \textit{qacA-bearing} strain to tPMP-1 did not affect the susceptibility of Et (ruling out Et-tPMP-1 cotransport). The following cytoplasmic membrane parameters did not differ significantly between the \textit{qacA-bearing} and parental strains: contents of the major phospholipids; asymmetric distributions of the positively charged species, lysyl-phosphotidyl-glycerol; fatty acid composition; and relative surface charge. Of note, the \textit{qacA-bearing} strain exhibited greater membrane fluidity than that of the parental, \textit{qacB-}, or \textit{qacC-bearing} strain. In conclusion, among these families of efflux pumps, only the multidrug transporter encoded by \textit{qacA} conferred a tPMP-1\textsuperscript{r} phenotype. These data suggest that \textit{qacA-encoded} tPMP-1\textsuperscript{r} results from the impact of a specific transporter upon membrane structure or function unrelated to PMF-dependent peptide efflux.

Mammalian platelets appear to play a critical role in the innate host defense against the induction and progression of endovascular infections (6, 7, 22, 39, 43). This host defense property relates to the capacity of platelets to release a group of cationic antimicrobial peptides (CAMPs) collectively known as platelet microbicidal proteins (PMPs) at sites of endovascular damage or infection (39, 43). Both rabbit and human platelets release functionally and structurally similar PMPs (37, 45) with microbicidal activities in vitro against pathogens that commonly invade the bloodstream, including \textit{Staphylococcus aureus} (11, 12, 39). To date, the best-characterized PMPs are those released from platelets following stimulation by the procoagulant molecule thrombin (i.e., thrombin-induced PMPs \textit{tPMPs}) (11, 19, 20, 40, 45, 46). In humans and animals with cather-induced \textit{S. aureus} bacteremia, strains which are intrinsically susceptible to PMPs \textit{tPMP} in vitro are less likely to cause complicated endovascular infections, such as endocarditis (11, 12).

Previous studies indicated that tPMPs target and permeabilize the \textit{S. aureus} cytoplasmic membrane (CM) to initiate their microbicidal pathway (20, 44). Intracellular targets affecting macromolecular synthesis and autolytic enzyme pathways have also been implicated in the staphylocidal mechanisms of tPMPs (40, 42). However, the precise adaptations by which \textit{S. aureus} strains resist the microbicidal activities of tPMPs remain incompletely defined but appear to include enhanced membrane fluidity (3); reduced transmembrane potential (\(\Delta\psi\)) (17), and mutations of genes involved in generating the proton motive force (PMF) (2). In addition, we recently demonstrated that carriage of plasmids bearing genes encoding the \textit{QacA PMF-dependent transmembrane efflux pump specific for cationic compounds} was associated with low-level tPMP resistance \textit{tPMP} in vitro (21).

The current study was designed to further characterize the mechanism(s) of tPMP\textsuperscript{r} among strains bearing distinct multidrug efflux pumps, especially to delineate whether (i) the active export of the cationic tPMP was responsible for this phenotype and (ii) other transmembrane efflux pumps (i.e., \textit{qacB} and \textit{qacC}) also conferred tPMP\textsuperscript{r} in vitro.

(This study was presented in part at the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 17 to 20 September 2000 [L. I. Kupferwasser, R. A. Skurray, N. Firth, M. H. Brown, M. R. Yeaman, R. Prasad, M. Smriti, and A. S. Bayer, abstr. 2288].)
Materials and Methods

Bacterial strains. Table 1 lists the bacterial strains and plasmids used in this investigation. The parental staphylococcal strain SK982 is plasmid-free and tPMP-1 in vitro (21). Plasmids carrying either the qacA, qacB, or qacC multicid resistance determinants were transferred into SK982 by mixed-culture transfer as previously described (21). The multidrug transporter-bearing plasmids studied are members of different staphylococcal plasmid families, including pSK1 from S. aureus post buffered saline. Dilutions of tPMP-1 and night cultures of the tPMP-1 were determined by a time-kill assay as previously described (39). Over preparations was validated as previously published using a microbiologic assay tPMP-1 and from thrombin-stimulated rabbit platelets were prepared and tPMP-1 was expressed as a percentage of the number of CFU of the positive tPMP-1 phenotype was defined as and propagated human or the PMF. After these exposures, the MICs for Et under these various conditions combination with 20 mM glucose for 10 min to counteract the CCCP effects on (control) or to 50 /H9262 SK2727 SK982 pSK23 QacB 30 SK2714 SK982 pSK84 QacB 25 SK2719 SK982 pSK156 QacB 25, 30 SK5791 SK982 pSK5754 14 SK5871 SK982 pSK5640 This study SK5872 SK982 pSK5672 QacA This study SK5873 SK982 pSK5691 QacA This study

Table 1. Bacterial strains and plasmids

<table>
<thead>
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<th>Strain</th>
<th>Background</th>
<th>Plasmid</th>
<th>Transporter</th>
<th>Reference(s)</th>
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<td>QacC</td>
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<td>pSK23</td>
<td>QacB</td>
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<td>pSK84</td>
<td>QacB</td>
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<td>SK982</td>
<td>pSK156</td>
<td>QacB</td>
<td>25, 30</td>
</tr>
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</table>

Following a 24-h incubation period at 37°C. Data represent the means (±SD) of three independent experiments. In a parallel study, SK2355 cells (10^7 CFU final inoculum) were similarly prepared in the presence or absence of 50 µM CCCP with or without 20 mM glucose and then exposed to 2 µg/ml tPMP-1 for 2 h in MEM at 37°C. After tPMP-1 exposures, the percentages of survival were determined under the different exposure conditions. Since relative tPMP-1 can be induced by the PMF-lowering actions of CCCP against selected tPMP-1 strains (2), studies were conducted to distinguish this potential effect from that of CCCP-induced exfoliation in strain SK2355. Thus, we evaluated the tPMP-1 susceptibility profile of parental strain SK982 in the presence or absence of CCCP (12.5 to 100 mM) using the same assay as outlined above.

Impact of QacA substrate–tPMP-1 coincubations on antistaphylococcal activity. Fractional inhibitory concentration (FIC) analysis was carried out in microtiter plates using 10^7 CFU of SK2355 cells with combinations of two QacA substrates, Et and chlorhexidine (Chlx), or tPMP-1. Increasing increments of each compound (3.125 µg/ml to 250 µg/ml for Et or Chlx and 1 µg/ml to 12 µg/ml for tPMP-1) were combined using the checkerboard procedure (30). Microtiter plates were incubated for 48 h at 37°C. The Et, Chlx, and tPMP-1 MICs were determined as described above. The FIC index was calculated as x/MIC + y/MIC, where x and y represent the lowest concentration of each compound (in combination) at which there is no growth and MIC, and MIC, represent the individual MIC of each compound. FIC indices have been defined as being indicative of the following: ≤0.50, synergy; 0.51 to 2.00, additivity; 2.01 to 4.00, indifference; and >4, antagonism (10, 27, 32). Data represent means (±SD) from three independent experiments.

FA analyses. Fatty acids (FAs) from the total amount of lipids extracted from the S. aureus constructs were analyzed using liquid gas chromatography after mild acetylation starring capped 5% Microbial ID Inc. Newark, DE). In brief, 10ng of bacterial cells was collected and saponified in boiling water with 1 ml of 50% alkaline methanol (15% NaOH) for 30 min. The tubes were cooled, and 2 ml of 45% methanol containing 3 N HCl was added for methylation. The methyl esters were extracted using a 1:1 mixture of hexane and methyl tert-buty1 ether. The aqueous (lower) phase was discarded, and the organic phase (containing FAs) was washed with 0.3 N NaOH. The purified ester mixture was separated by isolation analyses (Sherlock microbial identification system; Microbial ID, Newark, DE). A 25-mm by 0.2-mm phenyl methyl silicone–fused-silica capillary column with a flame ionization detector and autosampler was used for detection. Each FA-methyl ester composition was compared to database standards (straight-chained saturated fatty acids from 9 to 20 carbons in length and five hydroxy acids) using Sherlock pattern recognition software (Microbial ID, Newark, DE). The unsaturation profiles as well as the relative compositions of iso- versus anteiso-branched- chain fatty acids were calculated. All assays were performed a minimum of two times on separate days.

Pl analyses. (i) Extraction, identification, and quantification of PLs. Phospholipids (PLs) were extracted from S. aureus by standard methods (8). Briefly, cells were grown for 18 h in a brain heart infusion broth, harvested by centrifugation at 2000 × g, 4°C, and washed in buffer A (100 mM potassium phosphate, 5 mM EDTA, pH 7.2) and then in buffer B (100 mM potassium phosphate, 600 mM potassium chloride, pH 8.2). Cells (0.6 g [wet weight], ∼1 × 10^8 cells/ml) were suspended in 3 ml of buffer B and transferred to a 25-mg glass conical flask and cooled to 4°C with gentle swirling. The suspension was centrifuged and the pellet washed with buffer C (200 mM potassium acetate, 600 mM potassium chloride, pH 4.5) at 4°C.

The cell pellet was then extracted using 2.1 (vol/vol) chloroform-methanol, followed by washing with 0.9% NaCl to remove nonlipid contaminants (21). The extracted organic layer was evaporated to dryness under nitrogen and stored at −20°C until analysis. The major PL species, phosphatidylglycerol (PG), cardiolipin (CL), and blysophosphatidylglycerol (LPG), were separated by two-dimen sional thin-layer chromatography (2D-TLC) using Silica Gel 60 F254 high-performing TLC plates (Merck, Darmstadt, Germany) and subsequently developed with chloroform-methanol-25% ammonium hydroxide (65:25:6, by volume) in the vertical orientation and chloroform-acetone-acetic acid-methanol-water (45:16:9:8:4, by volume) in the horizontal orientation, as detailed previously (9, 15). LPG (positively charged) was identified by ninhydrin staining (33). PG, CL, and other minor PLs were visualized by exposure of the TLC plate to iodine vapor. All PLs were purchased from Avanti Polar Lipids, Inc. (Ala baster, AL) and used as internal standards to determine the positions of their spots on 2D-TLC plates.

For quantitative analysis, isolated PLs were individually recovered from TLC plates and digested at 180°C for 3 h with 0.3 ml of 70% perchloric acid. The digested samples were incubated with a colorimetric reagent (10% ascorbic acid, 2.5% ammonium molybdate, 5% perchloric acid [1:1:8, vol/vol/vol]) for

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Table 2: tPMP-1 susceptibility phenotypes of the study strains

<table>
<thead>
<tr>
<th>Concentration of tPMP-1 (µg/ml)</th>
<th>SK2355 (pSK2355, qacA)</th>
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<th>SK2727 (pSK108, qacA)</th>
<th>SK2371 (pSK2371, qacA)</th>
<th>SK2416 (pSK2416, qacA)</th>
<th>SK2718 (pSK2718, qacA)</th>
<th>SK2719 (pSK2719, qacA)</th>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>4</td>
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ND: not done.

RESULTS

tPMP-1 susceptibility phenotypes. Table 2 depicts the susceptibility profiles of the study strains to tPMP-1 across a concentration range of 2 to 12 µg/ml. Based on the arbitrary breakpoint of 50% survival at 2-h exposures, the qacA-bearing strain SK2355 exhibited low-level resistance over the 2- to 4-µg/ml range of tPMP-1. In contrast, the qacB- and qacC-bearing strains were tPMP-1-resistant across the entire concentration range, paralleling data for the parental strain, SK982 (Table 2). These results, irrespective of the plasmid type carrying the multidrug efflux genes, implied that the differences observed in tPMP resistance were due solely to the efflux protein. Thus, one representative strain for each transporter type was used in subsequent detailed studies (SK2355 qacA, SK2725 qacB, and SK2416 qacC).

Previous studies using pSK1 and deletion derivatives established that the presence of qacA was sufficient to confer a tPMP-1 phenotype (3, 21). However, qacR, which encodes the multidrug-binding transcriptional regulator of qacA (5, 14), was also present on these plasmids. Therefore, to determine if
qacR directly contributes to tPMP-1", we constructed pSK5754, a pSK1 derivative, in which qacA was replaced with a chloramphenicol resistance gene. This plasmid lacked qacA but still possessed a fully intact qacR determinant (and all other determinants of pSK1) and did not confer tPMP-1 (tested only at 2 μg/ml) (Table 2). Additionally, the qacA-negative plasmid vector-bearing strain SK5871 was tPMP-1", whereas the qacA-qacR plasmid-bearing construct (SK5872) and the qacA-alone plasmid-bearing construct (SK5873) each had approximately twofold-increased tPMP-1 resistance. These data support the notion that this is a qacA-specific effect and that the presence or absence of qacR has no bearing on the observed tPMP-1 resistance phenotype.

Effect of QacA efflux pump inhibition on *S. aureus* Et and tPMP-1 susceptibility phenotypes. The QacA membrane protein uses the PMF to drive substrate export and is inhibitable by the membrane energy uncoupler CCCP (28). Figure 1 depicts the impact of CCCP (in the presence or absence of glucose) on the Et susceptibility profiles of the qacA-bearing strain SK5875. As noted, preexposure to CCCP reduced the Et MIC by approximately fourfold ($P < 0.05$ versus the MIC for non-CCCP-treated cells), an effect that was reversed by coexposures to both CCCP and glucose (Fig. 1). In contrast, CCCP preexposures had only modest impacts on the tPMP-1 phenotype of the strain SK2355 (Fig. 2). In addition, exposing the plasmid-free parental control strain SK982 to CCCP (12.5 to 100 mM) caused no changes in its tPMP-1 profile (data not shown).

Impact of QacA substrate–tPMP-1 coincubations on net antimicrobial activity. Simultaneous exposure of qacA-bearing strains to two or more substrates microbicidal for the efflux system should saturate this pump and lead to a reduced efflux of the individual substrate molecules. This, in turn, should lead to enhanced inhibition of growth of the organism. This effect has been confirmed for a number of QacA-substrate combinations (27). To test whether Et and tPMP-1 are cotransported by QacA, we performed an FIC analysis using combinations of the two QacA substrates, Et and Chlx, or tPMP-1. The coincubation of Et and Chlx reduced the MICs for each compound by approximately fourfold compared to those for each compound alone (Fig. 3a) (FIC index, 1; $P < 0.05$). This interaction was interpreted as additive. In contrast, the coincubation of Et with tPMP-1 had little effect on the antistaphylococcal activity of either agent alone (Fig. 3b) (FIC index, 2 [indifference]).

Fatty acid analyses. There were no substantive differences observed in total fatty acid contents, unsaturation distributions, iso- or anteiso-branched-chain fatty acid contents, or ratios between the study strains (data not shown).

Phospholipid analyses. Fluorescamine, a fluorescent probe which specifically labels CM surface-exposed (outer-leaflet) positively charged amino PLs was used to assay the CM LPG distribution (2, 15). For both the parental SK982 and qacA-bearing SK2355 strains, PG was the predominant CM phospholipid.
pholipid (~67% for each strain). This mirrors data generated from our laboratory using other genetic background strains of *S. aureus* (41). There were no substantial differences in the relative proportions among the three major membrane phospholipids, PG, CL (~7% for each strain), and LPG (~23% for each strain), in a comparison of the parental strain, SK982, with *qacA*-bearing SK2355. Moreover, the distributions of LPG within the outer versus the inner CM leaflets did not differ between strains (~5 to 6% of the total LPG was distributed to the outer CM leaflet).

**CM fluidity measurements.** DPH is a probe that localizes in the hydrophobic core of the lipid bilayer and is highly fluorescent when bound therein. Fluorescence polarization studies using DPH revealed that the CMs of the *qacA*-bearing strain SK2355 (p.i., 0.293 ± 0.01) were more fluid than those of the parental strain (p.i., 0.31 ± 0.13) and those of the strains carrying *qacB* and *qacC*-containing plasmids (p.i., 0.315 ± 0.02 and 0.308 ± 0.01, respectively).

**Cytochrome c binding.** Cytochrome c is a cationic protein derived from equine heart. It has been used previously to estimate the relative surface charge of the cell envelope of isogenic *S. aureus* strain pairs (34). Cytochrome c binding assays showed that there were no differences in levels of binding to the cell surface of the parental strain SK982, SK2725, or the tPMP-1* strain SK2355 (28% ± 3%, 31% ± 14%, and 30% ± 4%, respectively). Although the binding of cytochrome c to the *qacC*-bearing strain SK2416 was somewhat higher than that of the other three constructs (46% ± 14%), this difference did not reach statistical significance.

**DISCUSSION**

We have previously shown that the capacity of *S. aureus* strains to acquire low-level resistance to tPMP-1 enables the organism to persist in both experimental infections and human endovascular infections (1, 6, 7, 11). Five mechanisms have been described which are associated with reduced in vitro susceptibility to tPMPs in *S. aureus*. (i) Small-colony variants, either gentamicin-induced or created by the mutagenesis of the *menD* locus (19), maintain a substantially lowered Δψ than parental strains due to such electron transport chain defects (19, 20). (ii) Variants selected following serial passages of tPMP-1* *S. aureus* strains in this peptide (43), like small-colony variants, exhibit a diminished Δψ (A. S. Bayer, M. R. Yeaman, H.-G. Sahli, D. Bra, and R. A. Proctor, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. A-106, p. 19, 1997). (iii) Strains with transposon disruptions in the complex I enzyme function result in the defective generation of the Δψ. We have recently shown that mutations within the snoA-snoG operon (which encodes an NADH oxidoreductase) render such strains relatively tPMP-1* (2). Importantly for CAMPs, a threshold Δψ appears to be critical for their interactions with target microbial membranes. Thus, the diminished Δψ observed in these three strain types likely contributes to their tPMP-1* phenotype. (iv) Strains showing relative increases in cell surface positive charge, as demonstrated by Peschel et al. (33, 34), have cell membranes and cell walls that contain high proportions of positively charged phospholipids and teichoic acids species, respectively. This renders such strains relatively resistant to killing by a number of CAMPs, including tPMP-1. Lysinylation of phosphatidylglycerol and d-alanylation of teichoic acid appear to be responsible for this phenotype. These data suggest that the charge-based surface repulsion of CAMPs by a less negatively charged surface envelope could contribute to CAMP resistance (33, 34). (v) We also recently showed that *S. aureus* strains carrying plasmid determinants (e.g., pSK1) encoding the PMF-dependent, multidrug export pump QacA of the major facilitator superfamily of transport proteins also exhibit reduced susceptibility to tPMP-1 in vitro, compared to that of the isogenic parental or a *qacA* mutant construct (21). The current study was designed to further investigate the mechanisms by which *qacA* carriage confers relative tPMP-1 resistance and whether this phenotype is specific for *qacA* or is a general mechanism conferred by the presence of other multidrug exporter proteins.

Several interesting observations emanated from this study. First, among the three multidrug export proteins QacA, QacB, and QacC, only QacA was associated with low-level tPMP-1 resistance at multiple peptide concentrations. These three multidrug export proteins all confer resistance to monovalent cationic antimicrobials, for example Et. In addition, QacA confers resistance to bivalent cationic compounds, such as Chl (24, 27). In fact, studies have shown that QacA mediates resistance to more than 30 cationic, lipophilic antimicrobials belonging to 12 distinct chemical families, although no resistance to trivalent cations or to anionic compounds has been observed (5, 27, 28). QacC is a small-membrane protein that belongs to a separate family of transport proteins, the small, multidrug resistance family (31), and shares no structural homology to QacA or QacB. In contrast, the QacA and QacB export proteins are highly related; there are only seven amino acid differences between them, and one of these differences, at position 323, is responsible for the resistance to bivalent cations demonstrated by QacA but not QacB (29). Second, resistance to CAMPs via active efflux has been described to exist in a number of bacteria. For example, in *Yersinia enterocolitica*, the Ros system, composed of RosA and RosB, a major facilitator superfamily multidrug export protein and a potassium efflux protein, respectively, mediates resistance to CAMPs using an energy-dependent process (4). Similarly, in *Neisseria* spp., Shafer et al. and Tzeng et al. have demonstrated the presence of the PMF-driven Mtr multidrug efflux system which modulates susceptibility to a range of CAMPs (36, 38). However, despite the fact that tPMP-1 is a lysine-rich cationic peptide (net charge, +5 [46]), making it a potential candidate for export by QacA, two lines of evidence reported here are against this hypothesis. (i) Interference with QacA pump function via the PMF inhibitor CCCP did not significantly impact the tPMP-1 susceptibility phenotype and (ii) coinfection of Et and tPMP-1 in the *qacA*-bearing strain did not substantially affect the in vitro activity of either agent, suggesting that QacA-mediated cotransport of these two antimicrobials did not occur. Furthermore, CCCP might induce a relative tPMP-1* phenotype by its nonspecific reduction in the PMF, which might be compensated for by a putative QacA-mediated tPMP-1 efflux in strain SK2355. Therefore, we evaluated the impacts of CCCP on the parental strain’s (SK982) tPMP-1 susceptibility profile. The finding that CCCP did not affect the tPMP-1 susceptibility of strain SK982 strongly suggests that the QacA-associated...
tPMP-1' phenotype observed in SK2355 is not attributable to a combined specific efflux and a nonspecific PMF reduction. As the cytoplasmic membrane is a principal target for initiating the staphylococcal pathway of tPMP-1, we carried out several analyses of the membrane structure and function in a comparison of the parental strain with strains expressing the three different multidrug export pumps. Only the qacA-bearing strain exhibited an increase in membrane fluidity compared to that of the other members of this isogenic strain set. Of note, enhanced membrane fluidity has been a consistent feature of several other tPMP-1' S. aureus strains, including the above-mentioned transposon and serial-passage variants. We recently studied the potential biophysical mechanisms of increased membrane fluidity in the transposon-derived sno mutant of S. aureus (K. Mukhopadhyay, W. Whitmire, J. Molden, Y. Q. Xiong, A. Peschel, P. McNamara, R. A. Procotor, J. Adler-Moore, M. R. Yeaman, and A. S. Bayer, Abstr. 105th Gen. Meet. Am. Soc. Microbiol., abstr. A-002, p. 1, 2005). In this construct, the basis for enhanced fluidity was related to an increased proportion of shorter acyl chain length fatty acids and a shift in branched-chain fatty acids from iso-branched to anteiso-branched species (16). Of particular importance, more-fluid membranes tend to leak protons, with a resulting decrease in Δψ (17). Since a threshold Δψ appears to be required for the optimal microbialicidal efficacy of tPMP-1 (20, 44), this is one potential explanation for the linkage between enhanced membrane fluidity and relative tPMP-1 resistance. In addition, extremes of CM fluidity or rigidity are likely to impact the interactions of CAMPs with the target membrane (26, 41). It should be emphasized that relatively small changes in CM characteristics (such as fluidity) have a major impact upon interactions with CAMPs (35). Of note, we could not detect any substantial differences in membrane fatty acid composition in the current stain set. An additional mechanism by which CM structure may influence tPMP-1 susceptibility profiles relates to its PL content and distribution (17). Several laboratories have shown that the overall content of the uniquely positively charged membrane phospholipid species, LPG, may substantially affect the overall surface charge of the organism. Peschel et al. (33, 34) have recently shown that knockouts of the genes responsible for the lysoylation of PG (mprF) or the α-0-lylyation of cell wall teichoic acid (dltA) render the surface envelope of such constructs more negatively charged. This enables a number of CAMPs to bind to the surfaces of these strains more avidly, making them more peptide susceptible in vitro. In recent studies, we confirmed that the transposon-derived tPMP-1' snoD mutant displayed a significantly increased proportion of outer-leaflet-localized LPG compared to that of its tPMP-1' parental strain. These characteristics provided the mutant with an enhanced repulsive cell surface toward the cationic molecule, cytochrome c, and perhaps tPMP-1. Similar analyses in the current study could not confirm either the increased overall LPG content or the enhanced outer-leaflet LPG asymmetry for the qacA-bearing construct. In summary, our investigations delineate a strong correlation between the presence of the QacA transmembrane efflux pump and a tPMP-1' phenotype that is not explicable by the transport of this cationic peptide. Ongoing studies in our laboratories are focusing upon (i) analyses of tPMP-1 binding among these distinct constructs and (ii) investigations to define the mechanism(s) of enhanced membrane fluidity in tPMP-1' strains in the context of qacA carriage.

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