

## In Vitro Resistance of *Staphylococcus aureus* to Thrombin-Induced Platelet Microbicidal Protein Is Associated with Alterations in Cytoplasmic Membrane Fluidity

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Received 15 December 1999/Returned for modification 18 February 2000/Accepted 17 March 2000

Platelet microbicidal proteins (PMPs) are small, cationic peptides which possess potent microbicidal activities against common bloodstream pathogens, such as *Staphylococcus aureus*. We previously showed that *S. aureus* strains exhibiting resistance to thrombin-induced PMP (tPMP-1) in vitro have an enhanced capacity to cause human and experimental endocarditis (T. Wu, M. R. Yeaman, and A. S. Bayer, *Antimicrob. Agents Chemother.* 38:729–732, 1994; A. S. Bayer et al., *Antimicrob. Agents Chemother.* 42:3169–3172, 1998; V. K. Dhawan et al., *Infect. Immun.* 65:3293–3299, 1997). However, the mechanisms mediating tPMP-1 resistance in *S. aureus* are not fully delineated. The *S. aureus* cell membrane appears to be a principal target for the action of tPMP-1. To gain insight into the basis of tPMP-1 resistance, we compared several parameters of membrane structure and function in three tPMP-1-resistant (tPMP-1<sup>r</sup>) strains and their genetically related, tPMP-1-susceptible (tPMP-1<sup>s</sup>) counterpart strains. The tPMP-1<sup>r</sup> strains were derived by three distinct methods: transposon mutagenesis, serial passage in the presence of tPMP-1 in vitro, or carriage of a naturally occurring multiresistance plasmid (pSKI). All tPMP-1<sup>r</sup> strains were found to possess elevated levels of longer-chain, unsaturated membrane lipids, in comparison to their tPMP-1<sup>s</sup> counterparts. This was reflected in corresponding differences in cell membrane fluidity in the strain pairs, with tPMP-1<sup>r</sup> strains exhibiting significantly higher degrees of fluidity as assessed by fluorescence polarization. These data provide further support for the concept that specific alterations in the cytoplasmic membrane of *S. aureus* strains are associated with tPMP-1 resistance in vitro.

*Staphylococcus aureus* is a major human pathogen, both in community- and nosocomially acquired infections (3, 9, 10, 27). Our laboratories and others have characterized an array of cationic, antimicrobial peptides from mammalian platelets, termed platelet microbicidal proteins (PMPs) (4, 45, 46). Among these is thrombin-induced PMP-1 (tPMP-1) which is released from platelets stimulated with thrombin (46, 47). This peptide exerts potent microbicidal effects in vitro against pathogens that commonly gain access to the bloodstream, including *S. aureus* (43). The antimicrobial host defense functions of platelets against endovascular infections like endocarditis have been proposed to result from, in part, their capacity to release tPMP-1 in response to physiological stimuli generated at damaged endovascular surfaces. Since the isolation of organisms such as *S. aureus* from the bloodstream of patients is relatively common (10, 27) yet *S. aureus* endocarditis is comparatively uncommon (10), it is likely that tPMP-1 plays a key role in preventing such endovascular infections. Implicit in this notion is an intrinsic susceptibility of the pathogen to the antimicrobial effects of tPMP-1. In contrast, tPMP-1-resistant (tPMP-1<sup>r</sup>) organisms may have a distinct survival advantage at

sites of endovascular damage. Our laboratories have recently confirmed that tPMP-1<sup>r</sup> strains of *S. aureus* exhibit an enhanced propensity to induce both human and experimental endocarditis (1, 5, 6) and are associated with a more severe form of this infection compared to tPMP-1-susceptible (tPMP-1<sup>s</sup>) counterpart strains (5, 6).

Our previous studies have identified the *S. aureus* cytoplasmic membrane as a principal target for the microbicidal actions of PMPs, leading to rapid depolarization, permeabilization, and eventual cell death (14). The cytoplasmic membranes of tPMP-1<sup>r</sup> strains of *S. aureus* appeared to be more resistant to these perturbations than genetically related tPMP-1<sup>s</sup> counterpart strains (14, 45; T. M. Wu, M. R. Yeaman, C. C. Nast, C. Itatani, and A. S. Bayer, *Abstr. 96th Gen. Meet. Am. Soc. Microbiol.* 1996, abstr. A72, 1996). These data suggest that one mechanism of tPMP-1 resistance in *S. aureus* relates to alterations in cytoplasmic membrane structure and/or function.

As noted above, thrombin, a key platelet agonist generated at sites of endothelial cell damage or microbial colonization (7, 44) prompts the release of antimicrobial peptides (tPMPs) from rabbit and human platelets (40, 47). As shown by acid-urea gel electrophoresis and reverse-phase high-performance liquid chromatography, the predominant tPMP released by thrombin from rabbit platelets is tPMP-1 (46, 47). The present study was designed to compare the membrane characteristics of genetically related strains of *S. aureus* exhibiting tPMP-1<sup>s</sup> or tPMP-1<sup>r</sup> phenotypes in vitro.

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TABLE 1. *S. aureus* strains used in this study

Strain	Description <sup>a</sup>	Reference
ISP479	Strain carrying pI258, which contains Tn551; tPMP-1 <sup>r</sup>	6
ISP479C	Plasmid-cured derivative of ISP479; tPMP-1 <sup>s</sup>	6
ISP479R	Tn551 mutant of ISP479; tPMP-1 <sup>r</sup>	6
19S	Clinical bloodstream isolate; tPMP-1 <sup>s</sup>	48
19R	Passage derivative of 19S; tPMP-1 <sup>r</sup>	48
SK982	Parental strain; tPMP-1 <sup>s</sup>	17, 19
SK2355	SK982 carrying pSK1; tPMP-1 <sup>r</sup>	17, 25, 32–34

<sup>a</sup> A strain is defined as tPMP-1<sup>r</sup> if  $\geq 40\%$  survival of a  $2 \times 10^3$  inoculum is determined after exposure in vitro to tPMP-1 (2  $\mu\text{g}/\text{ml}$ ) for 2 h (1).

(This study was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami Beach, Fla., May 1997 [abstr. 2539].)

#### MATERIALS AND METHODS

**Bacterial strains.** The *S. aureus* strains used in this study are described in Table 1. The detailed methods for the in vitro susceptibility testing of strains against tPMP-1 have been previously reported (6, 17, 48). ISP479 is a tPMP-1<sup>s</sup> strain that carries plasmid pI258, which encodes resistance to cadmium and ampicillin (6). This plasmid also contains the transposon, Tn551, which confers erythromycin resistance. ISP479C is a well-characterized, tPMP-1<sup>s</sup> strain that is the spontaneous, plasmid-cured variant of ISP479 (6). ISP479R is a transposon mutant of ISP479 that exhibits stable tPMP-1 resistance in vitro (6). This strain contains a single Tn551 chromosomal insert (6). Extensive phenotypic and genotypic analyses reveal no detectable differences between these two strains (6). Strain 19S, a clinical isolate, is tPMP-1<sup>s</sup> in vitro; strain 19R is a stable tPMP-1<sup>r</sup> variant of 19S selected after serial in vitro passage in the presence of tPMP-1 (48). Strains 19S and 19R are indistinguishable in colonial morphologies, biochemical and antibiogram comparisons, protein A or clumping factor expression, cell wall protein immunoblot profiles, coagulase or  $\beta$ -lactamase secretion, and genotypic characteristics (pulse-field gel electrophoretograms) (48). SK982 is a well-characterized tPMP-1<sup>s</sup> strain (17, 19). SK2355 contains the 28.1-kb multi-resistance plasmid, pSK1, that encodes *dfiA*-mediated trimethoprim resistance (34), *qacA*-mediated resistance to multiple organic cations (21, 25, 33), and *aacA-aphD*-directed aminoglycoside resistance (32). Additionally, recent studies have shown that the *qacA* determinant on pSK1 confers in vitro resistance to tPMP-1 (17). Thus, the above three strain pairs represent well-characterized, tPMP-1<sup>s</sup> parental strains and tPMP-1<sup>r</sup> counterpart strains derived by distinct strategies: transposon mutagenesis, in vitro passage in tPMP-1, and plasmid carriage.

**Assessment of hydrophobicity.** *S. aureus* surface hydrophobicities were determined using a dual-phase partitioning technique (38), employing the immiscible phases, polyethylene glycol (PEG) (hydrophobic phase), and dextran (hydrophilic phase). Previous studies have demonstrated a direct relationship between PEG affinity and cell hydrophobicity (38). One milliliter each of PEG (molecular weight, 6,000; 120 g/liter) and dextran (molecular weight, 37,000; 160 g/liter) (both from Sigma Co., St. Louis, Mo.) was added to glass cuvettes. Bacteria were grown overnight on brain-heart infusion agar (Difco, Detroit, Mich.), harvested from plates, washed, and adjusted in phosphate-buffered saline to a final inoculum of  $10^8$  CFU/ml. For each strain, 1 ml of the bacterial suspension was added to 1 ml of the biphasic system, and the tubes were vigorously vortexed and then held stationary at 22°C for 2 h for phase separation. Then, 100  $\mu\text{l}$  was carefully sampled from each phase and quantitatively cultured. The hydrophobicity index was expressed as the percentage of the total bacterial inoculum recovered from the PEG phase.

**Lipid extraction.** Bacterial cells were grown to mid-log phase (optical density at 650 nm [OD<sub>650</sub>] = 0.6), centrifuged, washed twice in double-distilled water, and sonicated. Sonicated cells were then mixed with 15 to 20 volumes of chloroform-methanol (2:1, vol/vol), and the suspension was stirred for 2 h at 30°C. The resulting extract was filtered through Whatman filter paper (no. 1), and the extraction procedure was repeated with the residual cell material. The extracts were pooled and washed with 0.2 vol of 0.9% NaCl to remove nonlipid contaminants. The clear, lipid-containing lower phase was removed, evaporated to dryness under a nitrogen atmosphere at 45°C, and stored at –20°C until analysis.

**Resolution, identification, and quantitation of phospholipids.** Phospholipids were separated by two-dimensional thin-layer chromatography using Silica Gel G as an adsorbent matrix (0.44 mm thick) and the following solvent system: solvent I (chloroform-methanol–30% ammonium hydroxide [65:30:4, vol/vol/vol]) and solvent II (chloroform-methanol-acetic acid-water [170:25:25:4, vol/vol]). The resolved phospholipids were visualized by exposure to iodine vapor and identified by comparison with internal phospholipid standards.

Isolated phospholipids were individually excised from chromatographic gels, and digested at 180°C with 1 ml of 60% perchloric acid for 2 h. The digested samples were incubated with 10 ml of chromatic developer (10% ascorbic acid–2.5% ammonium molybdate–4% perchloric acid [1:1:8, vol/vol/vol]) at 30°C for 2 h. Color intensity was measured at OD<sub>660</sub> and used to quantify the amount of each phospholipid compared to a standard generated from known amounts of that specific phospholipid (37, 39). The phospholipid species analyzed were: phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and phosphatidic acid.

**Fatty acid extraction and characterization.** Fatty acid extractions, methyl ester derivatizations, and their separation were essentially done as previously described (20). In brief, washed bacterial cells were suspended in 10 ml of 1 M alkaline ethanol (KOH) (90% ethanol, vol/vol). Saponification of lipids was achieved by refluxing the suspension for 2 to 3 h at 90°C, after which it was filtered through Whatman filter paper (no. 1). The residue was then washed with ethanolic KOH (10 ml) and transferred to a conical flask containing 30 ml of water. The solution was acidified with 6 N HCl (3.5 ml), and the fatty acids were extracted by shaking with 2 ml of chloroform for 30 min. The chloroform extracts were recovered and evaporated to dryness under a nitrogen stream.

Fatty acid methyl esters were prepared using thionyl chloride. Extracted fatty acids were dissolved in 5 ml of chloroform-methanol (3:7, vol/vol); 0.2 to 0.3 ml of thionyl chloride was then added with constant stirring at 4°C. After thorough mixing, the reaction was continued for 2 h to ensure complete conversion of fatty acids to their respective methyl ester derivatives. Double-distilled water (5 ml) was added to extract hydrophilic components and provide phase separation. The hydrophobic phase was washed four to five times with distilled water to remove residual thionyl chloride. The chloroform extract was then dried under nitrogen, and samples were stored at –20°C until used.

Derivatized fatty acids were analyzed by gas-liquid chromatography on a 2 M (10%) diethylene glycol succinate column (supported on a 100/200-mesh diatomic at 180°C). A Shimadzu (Tokyo, Japan) GC9A instrument equipped with a flame ionization detector was used for these analyses. The injection port was maintained at 210°C, and the carrier gas (nitrogen) flow rate was maintained at 40 ml/min. Methyl esters of specific fatty acids were identified by comparing their retention times to those of authentic internal standards and were quantified by a Chromatopac CR2A automated integrator using area normalization methods.

**Characterization of membrane fluidity through unsaturation indices.** The unsaturation index for each tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pair was derived from the overall distributions of monounsaturated and polyunsaturated membrane fatty acids (as determined above), using the formula of Stubbs and Smith (39). The molar unsaturation index was defined as  $1(\% \text{ monoenes})/100 + 2(\% \text{ dienes})/100 + 3(\% \text{ trienes})/100$ , where % monoenes represents the percentage of monounsaturated fatty acid, % dienes represents the percentage of di-unsaturated fatty acids, and % trienes represents the percentage of tri-unsaturated fatty acids. Previous studies have proven that a higher unsaturation index directly relates to a more fluid membrane (39).

Membrane fluidity was also quantified by the relative distribution and three-dimensional orientation of fluorescent dyes within the lipid layers. In brief, a whole-cell suspension of each bacterial strain was prepared with a bacterial density of  $\sim 10^9$  CFU/ml. This suspension was pelleted by centrifugation ( $5,000 \times g$  for 15 min) and then resuspended in digestion buffer (20% [wt/vol] sucrose, 0.05 Tris-HCl, 0.145 M NaCl [pH 7.6]). The bacterial cell wall was then digested with lysostaphin (34  $\mu\text{g}/\text{ml}$ ; Applied Microbiology, Tarrytown, N.Y.) in the presence of DNase I (16  $\mu\text{g}/\text{ml}$ ; Boehringer Mannheim, San Diego, Calif.) for 1 h at 37°C (14). The sucrose-stabilized protoplasts were collected by centrifugation (10,000 rpm for 15 min) and resuspended in fresh digestion buffer. The adequacy of cell wall digestion was confirmed by Gram staining to ensure that the preparation consisted of gram-negative, spherical protoplasts rather than gram-positive cocci. All protoplast preparations were held at room temperature and used within 24 h of preparation. For these studies, stable, cell wall-free staphylococcal protoplasts (prepared as previously described [14]) were labeled with two distinct fluorescent dyes. Protoplasts were labeled with either 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-anilino-8-naphthalene sulfonate (ANS) (Sigma) (11, 37). DPH specifically labels and fluoresces within the hydrophobic regions of the lipid bilayer (11) but lacks fluorescence in aqueous environments. The steady-state fluorescence of the DPH probe predominantly reflects the structural order of membrane lipids, mainly as a result of preferential partitioning of the probe into the hydrocarbon phase of the lipid membrane bilayer when it is in a fluid state (11, 37). Thus, membrane fluidity may be considered the reciprocal of the structural order of membrane lipids. In contrast, ANS is a hydrophilic label that is used to monitor polar head regions. Thus, the two probes localize in distinct domains of the lipid bilayer, giving different absolute fluorescence polarization values related to polar head regions or hydrophobic cores.

For DPH labeling, a 2 mM solution of this dye was prepared in tetrahydrofuran, and 100  $\mu\text{l}$  was added to 50 ml of agitated 0.05 M Tris-HCl (pH 7.6). Excess tetrahydrofuran was removed by flushing with nitrogen. Protoplasts suspended in digestion buffer were then incubated in either DPH (final concentration = 2  $\mu\text{M}$ ) or ANS (final concentration = 33.3  $\mu\text{M}$ ) for 60 min at 30°C.

Fluorescence polarization was measured using a Kontron spectrofluorometer (Copenhagen, Denmark). Excitation of fluorescent probes was accomplished with vertically polarized monochromatic light at 360 nm for DPH or 350 nm for ANS. Emission intensity was quantified at 426 nm for DPH or 496 nm for ANS

using a detector oriented either parallel or perpendicular to the direction of the polarized excitation source. The degree of fluorescence polarization or polarization index ( $p$ ) was calculated from the following formula (11, 37):  $p = [I_V - I_H / (I_{HV} + I_{HH})] / [I_V + I_H / (I_{HV} + I_{HH})]$ . In this formula,  $I$  is the corrected fluorescence intensity, subscripts  $V$  and  $H$  indicate the values obtained with vertical or horizontal orientation of the analyzer, respectively. The corrected fluorescence was determined by subtraction of unlabeled control protoplast emissions from those of labeled protoplasts. The lower the fluorescence polarization value, the higher the degree of membrane fluidity.

**Amino acid transport.** *S. aureus* strains were grown in brain heart infusion broth to mid-logarithmic phase of growth ( $OD_{650} = 0.6$ ), pelleted by centrifugation, washed, and resuspended as a 10% (wt/vol) cell suspension in ultrapure water (MilliQ water; Millipore). The bacterial cell suspension was incubated at 30°C for 10 min in 100 mM Tris-citrate (pH 4.5) containing chloramphenicol (200 µg/ml) to inhibit protein synthesis. Amino acid uptake studies were then initiated by the addition of one of the following:  $^{14}C$ -labeled amino acids (final concentration, 100 µM; Sigma) L-glutamic acid, L-lysine, or L-alanine (74 kBq/mol = 2 µCi/mol). Aliquots of 100 µl were sampled from this reaction mixture every 45 s up to 180 s and diluted into 3 ml of ice-cold wash buffer (10 mM Tris-citrate, pH 4.5) containing the respective unlabeled amino acid at the same concentrations as their labeled counterparts. These aliquots were rapidly passed through membrane filters (0.45-µm pore size; Millipore), and the filters then washed twice with buffer. The filters were dried at 65°C, and the radioactivity retained was counted in a Beckman LS 1801 liquid scintillation beta counter. The rates of amino acid uptake were calculated over the 180-s sampling period using linear regression analysis as described by Rao et al. (31), and are expressed as nanomoles per milligram (dry weight) of cell.

#### STATISTICAL ANALYSES

The fluorescence polarization indices were compared for each strain pair by Student's  $t$  test for unpaired samples, employing the statistical package within Sigma Plot (version 3.02).  $P$  values of  $\leq 0.05$  were considered significant.

## RESULTS

**Hydrophobicity studies.** Many endogenous cationic microbicidal peptides are amphipathic molecules exhibiting both charged regions that promote avidity for the electronegative characteristics of bacterial membranes and hydrophobic regions that may facilitate insertion into and span target membranes (8, 35). Therefore, it is conceivable that net bacterial surface hydrophobicity influences the overall microbicidal effects of tPMP-1. However, there were no significant differences in the hydrophobicity indices of the three tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs, with each member of a strain pair exhibiting a hydrophobicity index similar to its counterpart strain (data not shown).

**Fatty acid composition.** Compositions of cell membrane fatty acids for each strain studied are summarized in Table 2. Elevated levels of longer-chain, unsaturated fatty acid species (determined by molar ratios) were noted in all three tPMP-1<sup>r</sup> strains, in comparison to their tPMP-1<sup>s</sup> counterpart strains. Indeed, in the tPMP-1<sup>r</sup> strain SK2355 there appears to be a shift from smaller, saturated fatty acid species (exhibited by the tPMP-1<sup>s</sup> parental strain, SK982) to larger, more polyunsaturated species. Also, the membranes of the tPMP-1<sup>r</sup> strains contained from approximately two- to sixfold more of the triene, linolenic acid (an 18:3 fatty acid), than did the tPMP-1<sup>s</sup> counterpart strains. Similarly, the membranes of the tPMP-1<sup>r</sup> strain SK2355 (carrying pSK1), contained ~10-fold more of the diene lipid, linoleic acid (an 18:2 fatty acid), than did its plasmid-free, tPMP-1<sup>s</sup> counterpart, SK982. These findings are consistent with a correlation between tPMP-1 resistance and increases in fatty acid length and unsaturation.

**Phospholipid composition.** No substantive differences were observed in the proportional phospholipid compositions of the tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs (data not shown). Although there were differences in the proportions of specific phospholipids between individual tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs, these changes were not uniform across all of the strain pairs. For example, the proportion of phosphatidyl glycerol in the tPMP-1<sup>r</sup> strain ISP479R was substantially larger than that in its

TABLE 2. Percentage of fatty acid composition of tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> *S. aureus* strains

Strain	Fatty acid <sup>a</sup>												
	10:0	12:0	13:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3
19S	0.24 ± 0.08	1.13 ± 0.35	4.24 ± 1.23	39.9 ± 1.79	10.6 ± 1.0	17.2 ± 0.5	6.20 ± 1.5	1.94 ± 0.4 <sup>b</sup>					
19R	1.53 ± 0.28	0.72 ± 0.44	4.79 ± 0.74	43.97 ± 4.46	9.72 ± 1.30	15.73 ± 1.19	4.42 ± 1.30	6.0 ± 1.40					
ISP479C	0.72 ± 0.44	0.331 ± 0.20	2.48 ± 0.07	48.9 ± 3.1	12.98 ± 1.3	11.87 ± 1.07	8.71 ± 1.20	1.58 ± 0.20					
ISP479R	0.331 ± 0.20	50.8 ± 0.79	2.95 ± 0.08	44.7 ± 1.8	13.81 ± 0.50	13.84 ± 0.52	9.26 ± 0.70	5.78 ± 0.60					
SK982	<b>2.8 ± 0.65</b>	56.6 ± 0.56	<b>5.6 ± 1.7</b>		17.5 ± 1.43	12.63 ± 1.7	1.86 ± 0.67	0.67 ± 0.17					
SK2355	<b>0.36 ± 0.13</b>				16.3 ± 0.90	15.6 ± 2.2		<b>9.4 ± 0.78</b>					1.3 ± 0.12

<sup>a</sup> 10:0, capric acid; 12:0, lauric acid; 14:0, myristic acid; 14:1, myristoleic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 17:0, heptadecanoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. Data represent the means ± standard deviations of two independent runs.

<sup>b</sup> Data shown in boldface type are those where there are distinct differences (greater than threefold) in fatty acid composition between the tPMP-1<sup>r</sup> strain and its tPMP-1<sup>s</sup> counterpart strain.

TABLE 3. Membrane fluidity of tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> *S. aureus* strains

Strains	Fluorescence polarization <sup>a</sup>	Unsaturation index <sup>b</sup> (mole <sup>-1</sup> )
19S	0.022 ± 0.007	0.60 ± 0.09
19R	0.003 ± 0.001 <sup>c</sup>	0.75 ± 0.09
ISP479C	0.021 ± 0.006	0.620 ± 0.02
ISP479R	0.001 ± 0.01 <sup>d</sup>	0.632 ± 0.03
SK982	0.023 ± 0.01	0.17 ± 0.015
SK2355	0.007 ± 0.001 <sup>c</sup>	0.382 ± 0.002

<sup>a</sup> These data represent the means ± standard deviations of 10 independent runs with DPH (measured in fluorescence units).

<sup>b</sup> Unsaturation index = 1 (% monoene)/100 + 2 (% diene)/100 + 3 (% triene)/100 of two independent determinations (means ± standard deviations).

<sup>c</sup>  $P = 0.002$  (for comparisons between strains 19S and 19R and strains SK982 and SK2355).

<sup>d</sup>  $P = 0.00004$  (for comparison between strains ISP479C and ISP479R).

tPMP-1<sup>s</sup> counterpart strain, ISP479C. However, the reverse was true in the 19S-19R strain pair.

**Membrane fluidity.** Two methods were employed to ascertain whether differences in membrane fluidity were associated with tPMP-1<sup>s</sup> or tPMP-1<sup>r</sup> phenotypes in *S. aureus* strains: determination of unsaturation indices and fluorescence polarization. As noted in Table 3, by the DPH assay, the degree of membrane fluidity was uniformly and significantly higher for all three tPMP-1<sup>r</sup> *S. aureus* strains than for their tPMP-1<sup>s</sup> parental strains. Data generated with the other fluorescent membrane probe, ANS, also confirmed a higher degree of membrane fluidity in tPMP-1<sup>r</sup> strains than in tPMP-1<sup>s</sup> counterpart strains (data not shown). However, these differences did not reach statistical significance ( $0.05 < P < 0.10$ ).

There was a trend towards higher calculated lipid unsaturation indices for tPMP-1<sup>r</sup> strains compared to those calculated for their respective tPMP-1<sup>s</sup> counterparts; this potentially reflects the shift towards higher proportions of polyunsaturated fatty acids in the tPMP-1<sup>r</sup> strains (Table 2). However, this trend did not reach statistical significance.

**Amino acid transport.** The extent and rates of uptake of selected amino acids were assessed as additional comparative measures of cytoplasmic membrane function in tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs. A panel of amino acids including L-lysine (cationic), L-glutamic acid (anionic), and L-alanine (neutral, hydrophobic) was selected, since each amino acid has a distinct membrane transporter. Substantial differences in uptake profiles of these amino acids between the individual tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs were observed. However, there were no uniform variances in transport profiles among the tPMP-1<sup>s</sup> and tPMP-1<sup>r</sup> strain pairs studied (data not shown). For example, in tPMP-1<sup>r</sup> ISP479R, amino acid uptake was substantially lower than that seen in the corresponding tPMP-1<sup>s</sup> strain, ISP479C. In contrast, in strain pair 19S-19R, although the glutamic acid and lysine uptake profiles differed between the two strains, these differences were inversely related to those seen in the ISP479C-ISP479R strain pair (i.e., lowered rates of uptake by tPMP-1<sup>s</sup> strains). Thus, no amino acid transport characteristics were consistently associated with tPMP-1 resistance in vitro.

## DISCUSSION

Cationic antimicrobial peptides are believed to target the cell membrane of microbial pathogens to initiate their lethal effects (2, 8, 22, 35). We have recently demonstrated that the cytoplasmic membrane of *S. aureus* is, indeed, a principal target for the microbicidal actions of tPMP-1. This conclusion

emanates from multiple lines of evidence, including (i) electrophysiological studies using artificial planar lipid bilayers (13, 14), (ii) transmission electron microscopic investigations of the ultrastructural effects of tPMP-1 upon whole *S. aureus* cells and cell wall-free protoplasts (14; Wu et al., Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996), and (iii) flow cytometry studies of tPMP-1-induced cytoplasmic membrane permeabilization (45).

Using planar lipid bilayers to model bacterial membranes in vitro, we have recently shown that tPMP-1 permeabilizes and disrupts such membranes in a manner that is influenced by peptide concentration and orientation of transmembrane voltage orientation (13). The membrane permeabilization effects of tPMP-1 were consistently enhanced in the *trans*-negative orientation, mirroring the voltage polarity of bacterial cytoplasmic membranes (13, 14). These data paralleled in vitro microbicidal data in which *S. aureus* cells defective in the generation of a threshold *trans*-negative membrane electrical gradient (gradient < -100 mV) were relatively resistant to the lethal actions of tPMP-1 (12; A. S. Bayer, M. R. Yeaman, H.-G. Sahl, D. Brar, and R. A. Proctor Abstr. 97th Gen. Meet. Am. Soc. Microbiol. abstr. A-106, 1997). Ultrastructurally, tPMP-1 induces rapid perturbations of the cytoplasmic membranes of whole *S. aureus* cells in tPMP-1<sup>s</sup> strains, which precedes cell lysis or death (45). Additionally, protoplasts derived from tPMP-1<sup>s</sup> strains (but not from tPMP-1<sup>r</sup> strains) are rapidly lysed in the presence of tPMP-1, coincident with ultrastructural disruption of the cytoplasmic membrane as demonstrated by transmission electron microscopy (14). Furthermore, flow cytometry analyses have shown that exposure of tPMP-1<sup>s</sup> (but not tPMP-1<sup>r</sup>) whole *S. aureus* cells to tPMP-1 leads to rapid uptake of a small (2 nm) fluorescent probe (propidium iodide) without depolarization of the cytoplasmic membrane (45). Thus, phenotypic resistance to tPMP-1 in vitro correlates with relative refractoriness to peptide-induced cytoplasmic membrane permeabilization (14, 45). Collectively, this body of data underscores the likely importance of the bacterial cytoplasmic membrane as a proximate target of the microbicidal actions of tPMP-1.

The current study sought to identify membrane characteristics in *S. aureus* that may account for susceptibility or resistance to the in vitro microbicidal actions of tPMP-1. To maximize the power of our studies, we employed three distinct *S. aureus* strain pairs of differing genetic lineages in which tPMP-1 resistance was induced via different mechanisms: transposon mutagenesis (6), serial passage (48), or plasmid carriage (17, 21, 41). Despite these differing modes of induction of tPMP-1 resistance in vitro, several themes emerged from these studies relative to membrane alterations in tPMP-1<sup>s</sup> vs tPMP-1<sup>r</sup> strains.

Membranes composed predominantly of saturated fatty acids tend to be relatively rigid. In contrast, polyunsaturated fatty acids possess multiple, restrictive double bonds which promote lipid disorder, translating into increased membrane fluidity (29, 42). In each of the three tPMP-1<sup>r</sup> strains studied, there was a substantial shift in fatty acid content toward a predominance of polyunsaturated species. Of interest, the presence of specific polyunsaturated fatty acids in tPMP-1<sup>r</sup> strains differed among the strain pairs as determined by unsaturation indices (11, 37, 39). Furthermore, fluorescence polarization demonstrated that the cytoplasmic membranes of the tPMP-1<sup>r</sup> strains were more fluid than their tPMP-1<sup>s</sup> counterpart strains. These data may provide important insights for future investigations into defining the mechanistic basis of tPMP-1 resistance in *S. aureus*, vis-à-vis the relationship of tPMP-1 resistance to membrane fluidity.

Analysis of the transport characteristics of either cationic,

anionic, or neutrally charged amino acids within individual strain pairs disclosed that substantial differences in uptake profiles are present between the tPMP-1<sup>r</sup> and tPMP<sup>s</sup> strains. However, there were no consistent defects in the uptake of individual amino acids observed for all tPMP-1<sup>r</sup> strains. Each amino acid tested in this study utilizes a specific membrane transport system (28, 31). Therefore, it is likely that the differences observed in uptake profiles of tPMP<sup>s</sup> and tPMP<sup>r</sup> strains were related to global alterations in the cytoplasmic membranes of resistant strains rather than to alterations of any one specific uptake system. In this regard, Lunbaek et al. (18), using both planar lipid bilayers and tissue culture monolayers, showed that membrane protein function is markedly affected by artificially varying the degrees of membrane rigidity.

The precise relationships between increased membrane fluidity and tPMP-1 resistance are under investigation. Of interest, fluconazole-resistant *Candida albicans* strains exhibit alterations in membrane lipid composition, membrane fluidity, and amino acid uptake similar to those which we have demonstrated for tPMP-1<sup>r</sup> *S. aureus* strains (R. Prasad, J. Chandra, A. Koul, P. Belanger, H. Sanati, and M. Ghannoum, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996, abstr. F-74, 1996). Such *Candida* strains have been shown to possess an energy-dependent drug efflux mechanism, putatively analogous to the multidrug resistance efflux pumps of mammalian tumor cells (15, 16, 24, 30). Likewise, there are at least two examples of energy-dependent systems conferring a cationic peptide-resistant phenotype in bacterial species. Peschel et al. and Otto et al. (23, 26) have studied the lantibiotics epidermin and gallidermin, which are lanthionine-containing peptides isolated from *Staphylococcus epidermidis*. Strains possessing the lantibiotic resistance gene complex, *epiFEG*, appear to extrude these peptides from the target cytoplasmic membrane after initial attachment but prior to transmembrane insertion, spanning and ensuing microbicidal effects. Although this mechanism is energy dependent and mediated by the ATP-binding cassette transporter, EpiFEG, it does not appear to be a typical efflux system. Additionally, Shafer et al. (36) have recently characterized the gonococcal proton-motive force-dependent efflux pump, Mtr, which confers resistance to a variety of structurally diverse, hydrophobic antimicrobial agents. Interestingly, this mechanism also confers gonococcal resistance to protegrin-1, a cysteine-rich, cationic antimicrobial peptide from porcine leukocytes.

In the context of the above observations on antimicrobial peptide extrusion and efflux systems (23, 26, 36), it is tempting to speculate that the *qacA*-encoded, proton motive force-dependent cation efflux pump carried by plasmid pSK1 (21, 25) confers tPMP-1 resistance (17) via efflux of the peptide. While this may prove to be the case, the collective results for all three tPMP-1<sup>r</sup> strains presented here, including that carrying *qacA*, raise the possibility that resistance to this peptide can arise via mechanisms that ultimately contribute to cytoplasmic membrane disorder.

#### ACKNOWLEDGMENTS

We thank Iri Kupferwasser and Cong Li for excellent technical assistance in the hydrophobicity studies. We also thank Pranab Mukerjee for his excellent help with the computer graphics.

These studies were supported in part by research grants from the National Institutes of Health to A.S.B. (AI39108) and to M.R.Y. (AI39001; AI39108). Work in the laboratory of R.A.S. was supported by a Project Grant from the National Health and Medical Research Council (Australia). In addition, R.P. was supported in part by the Department of Biotechnology (BT/R&D/15/02/94) and the Department of Science and Technology (SP/SO/D57/97), Jawaharlal Nehru University, and the India and European Commission (TS3-CT 94-0279).

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Editor: A. D. O'Brien