DltABCD- and MprF-Mediated Cell Envelope Modifications of *Staphylococcus aureus* Confer Resistance to Platelet Microbicidal Proteins and Contribute to Virulence in a Rabbit Endocarditis Model

Christopher Weidenmaier,¹ Andreas Peschel,^{1*} Volkhard A. J. Kempf,¹ Natalie Lucindo,^{2,3} Michael R. Yeaman,^{2,3,4} and Arnold S. Bayer^{2,3,4}

Medical Microbiology and Hygiene Department, University of Tübingen, Tübingen, Germany¹; Department of Medicine, Division of Infectious Diseases, Harbor-UCLA Medical Center, Torrance, California 90509²; LA Biomedical Research Institute, St. John's Cardiovascular Research Center, Torrance, California 90502³; and Geffen School of Medicine at UCLA, Los Angeles, California 90024⁴

Received 2 June 2005/Returned for modification 27 June 2005/Accepted 23 August 2005

The DltABCD and MprF proteins contribute a net positive charge to the *Staphylococcus aureus* surface envelope by alanylation and lysinylation of teichoic acids and membrane phosphatidylglycerol, respectively. These surface charge modifications are associated with increased in vitro resistance profiles of *S. aureus* to a number of endogenous cationic antimicrobial peptides (CAPs), such as α -defensins. The current study investigated the effects of *dltA* and *mprF* mutations on the following host factors relevant to endovascular infections: (i) in vitro susceptibility to the CAP thrombin-induced platelet microbicidal protein 1 (tPMP-1), (ii) in vitro adherence to endothelial cells (EC) and matrix proteins, and (iii) in vivo virulence in an endovascular infection model (rabbit endocarditis) in which tPMP-1 is felt to play a role in limiting *S. aureus* pathogenesis. Both mutations resulted in substantial increases in the in vitro susceptibility to tPMP-1 compared to that of the parental strain. The *dltA* (but not the *mprF*) mutation resulted in a significantly reduced capacity to bind to EC in vitro, while neither mutation adversely impacted in vitro binding to fibronectin, fibrinogen, or platelets. In vivo, both mutations significantly attenuated virulence in terms of early colonization of sterile vegetations and subsequent proliferation at this site (versus the parental strain). However, only the *dltA* mutation significantly reduced metastatic infections in kidneys and spleens compared to those in animals infected with the parental strain. These data underscore the importance of resistance to distinct CAPs and of teichoic acid-dependent EC interactions in the context of endovascular infection pathogenesis.

Staphylococcus aureus is the most prevalent bacterial pathogen isolated from patients with endovascular infections (e.g., vascular catheter sepsis and infective endocarditis [IE]) (14, 24). At damaged endothelial sites, the establishment of endovascular infections is felt to involve a complex interaction between circulating bacteria and platelets, matrix ligands, and vascular endothelial cells (6, 37). Moreover, *S. aureus* has the capacity to undergo phenotypic switching (e.g., to small-colony variants [29]), which contributes to endovascular adaptation and persistence. The host utilizes a potent repertoire of host defense mechanisms to limit such infections. In this context, platelets are felt to play a key role in limiting the progression of endovascular infections through multiple mechanisms, but predominantly via the local secretion of endogenous cationic antimicrobial peptides (CAPs). These peptides, termed platelet microbicidal proteins (PMPs), are active against most common bloodstream pathogens (30). Infected endovascular foci (e.g., cardiac vegetations) are replete with platelets but relatively devoid of inflammatory cells, such as polymorphonuclear leukocytes (PMNs) (37). In contrast, endogenous CAPs within

PMNs, such as α -defensins and cathelicidins, are believed to limit the metastatic complications of endovascular infections (e.g., abscesses in target organs such as the spleen or kidneys). However, bacteria can exhibit resistance to such CAPs, with a resultant amplification of their virulence potential in vivo. For example, *S. aureus* strains which exhibit stable, low-level resistance to PMPs in vitro gain a distinct survival advantage in human and experimental endocarditis (IE) as well as in patients with persistent methicillin-resistant *S. aureus* bacteremia (1, 8, 11).

A number of distinct CAPs have been identified from thrombin-stimulated human platelets, including classical microbicidal chemokines ("kinocidins") such as platelet factor 4, CTAP-3, and RANTES, along with fibrinopeptide B and thymosin β -4 (4, 17, 31). Interestingly, such platelet kinocidins appear to kill target microorganisms, even at high inocula, while also recruiting PMNs and other leukocytes at low peptide concentrations (9). With regard to the above-mentioned outcomes for experimental IE in rabbits, Yeaman et al. and Yount et al. have shown that thrombin-stimulated rabbit platelets also secrete a similar cadre of CAPs, of which thrombin-induced PMP 1 (tPMP-1) is the most abundant (41, 43). tPMP-1 is a structural and functional orthologue of human platelet factor 4 and is a prototypical kinocidin, containing both an N-terminal chemokine domain and a C-terminal microbicidal domain separated by an interposing, γ -core-

* Corresponding author. Mailing address: Cellular and Molecular Microbiology Division, Medical Microbiology and Hygiene Department, University of Tübingen, 72076 Tübingen, Germany. Phone: 49-7071-2981515. Fax: 49-7071-293435. E-mail: andreas.peschel@uni-tuebingen.de.

containing domain which is common to kinocidins and broad classes of disulfide-stabilized CAPs (43). It is highly likely that both tPMPs and PMN CAPs contribute to the net host defense against endovascular infections. Both initially target the microbial cytoplasmic membranes (CM), although their microbicidal spectra and modes of action are distinct (38).

We have recently described two types of *S. aureus* mutations that confer an increased susceptibility to CAPs from human and porcine PMNs as well as to CAPs of other diverse origins (e.g., tachyplesins and gramicidins) (16, 18, 27, 28). The genetic systems inactivated in these mutants normally modify negatively charged cell envelope components with positively charged amino acids. These normal modifications are believed to enhance the net positive surface charge of the organism, potentially facilitating a repulsion of CAP molecules and leading to relative CAP resistance (26). One of these modifications involves D-alanylation of polyanionic cell walls and lipoteichoic acids by the gene products of the *dltABCD* operon (28). The other modification involves lysinylation of the anionic and major staphylococcal CM phospholipid, phosphatidylglycerol, by the membrane protein MprF, resulting in the synthesis of lysyl-phosphatidylglycerol (27). Mutants lacking these modifications exhibit (i) profound alterations of their cell envelope surface electrostatic properties, leading to an increased binding capacity of CAPs for such mutants compared to that for the parental strain; (ii) a concomitantly higher susceptibility to killing by CAPs; (iii) enhanced killing by α -defensin-containing PMNs, but not by monocytes lacking such CAPs; and (iv) attenuated virulence in murine models of infection due to *S. aureus* (5, 27). Recently, it was demonstrated that both of the above mutants are also more efficiently inactivated than the parental strain by group IIa phospholipase A₂, a major host defense factor in inflammatory fluids (16), suggesting a role for DltABCD- and MprF-mediated modifications beyond protection against α -defensins. In addition to their impacts on CAP-mediated host defenses, cell wall-anchored teichoic acid polymers (wall teichoic acid [WTA]) also play an important role in interactions with host endothelial cells (EC) (34) and epithelial cells (32, 34). We have recently shown that a WTA-deficient *S. aureus* mutant exhibited attenuation in causing visceral organ abscesses as well as in the induction and propagation of IE in a rabbit model (34).

In an attempt to further study the in vivo relevance of teichoic acid and phospholipid modifications with positively charged amino acids, we utilized an experimental *S. aureus* IE model which allows several relevant parameters to be simultaneously examined for a multisystem endovascular infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* Sa113 is a frequently used laboratory strain (15) which we have employed in prior in vitro and in vivo studies. The inactivation of the *dltA* and *mprF* genes in the Sa113 background by their replacement with spectinomycin and erythromycin resistance gene cassettes, respectively, has been described in detail before (27, 28). Plasmids pRBdlt1 and pRBmprF, used for complementation of the respective mutants, have also been described previously (27, 28). The growth kinetics of these study strains were virtually identical in a standard medium (trypticase soy broth) (data not shown). A fibronectin-binding protein mutant (*fmbA* and *fmbB* deficient), along with its parental strain in the *S. aureus* 8325-4 background (12), and a fibrinogen-binding protein mutant (*clfA* deficient), along with its parental strain in the *S. aureus* Newman strain background (35), were used as control strains in matrix protein-binding assays.

CAP susceptibility assays. We utilized tPMP-1 as a prototypic PMP. Susceptibility profiles of the study strains for tPMP-1 were assayed in vitro in a timed-kill assay using Eagle's minimum essential medium (MEM), as previously described (41), rather than with a traditional MIC assay (conventional nutrient medium interferes with the activity of this peptide). We modified our previously published assay system by using a final inoculum of 10^6 CFU at the stationary phase of growth (instead of 10^5 CFU at the logarithmic growth phase) in order to represent bacterial densities and growth phases usually observed in target tissues with the experimental IE model (see below). Moreover, pilot investigations suggested that this higher inoculum allowed for excellent discrimination between the susceptibility profiles of the various constructs studied. Results are expressed as mean percentages ($\pm$ standard deviations [SD]) of the initial inoculum surviving a 2-h exposure to tPMP-1 at 1 μ g/ml. Two independent experimental assays were performed on separate days. The preparation, purification, authentication, and bioactivity of tPMP-1 have been described in detail elsewhere (36). By convention, we utilized a breakpoint of $\geq 50\%$ survival to indicate low-level resistance to the microbicidal impacts of tPMP-1 against *S. aureus* (1).

Interactions of *S. aureus* with HUVECs. *S. aureus* binding to human umbilical vein endothelial cells (HUVECs) was studied as described recently (34). In brief, HUVECs cultured in endothelial cell growth medium (Promocell) were used up to passage number six. Cells were seeded in 24-well culture plates at 1×10^5 /well and grown at 37°C under 5% CO₂ until confluent. Bacteria were prepared, labeled with fluorescein isothiocyanate as recently described (32), and resuspended in Iscove's modified Dulbecco's medium (IMDM; Gibco). Cell numbers were adjusted using a Neubauer chamber. Confluent HUVEC monolayers grown in 24-well plates were washed twice with IMDM and inoculated with fluorescein isothiocyanate-labeled bacteria. The inoculum dependency of bacterial adherence was confirmed by using bacterium-to-HUVEC ratios ranging from 5 to 100:1 in pilot studies. A ratio of 30:1 was found to be optimal and was used in the analyses below. After incubation for 1 h at 37°C under 5% CO₂, the wells were washed three times with IMDM and fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS). No morphological changes were observed in HUVECs after this procedure. The number of adherent bacteria/0.1 mm² was counted using a fluorescence microscope. Experiments were performed in duplicate, with 10 random fields counted in each well.

Interactions of *S. aureus* with platelets. The direct adhesion of *S. aureus* to rabbit platelets was measured in the absence of matrix ligands. Platelet-bacterium binding studies were performed at 20°C following mixing of the organism with platelets. Flow cytometry was used to detect and quantify the extent of bacterial binding to rabbit platelets, using fluorometrically distinct bacterial and platelet fluorophores and a FACScalibur cytometer (Beckman Instruments) as previously described (30). The percentage of bacteria bound to platelets was calculated by dividing the number of dually labeled particles by the total number of bacteria and multiplying by 100. Data are expressed as means $\pm$ SD for at least three independent experiments. Pilot studies indicated that a 1:1 bacterium-to-platelet ratio (10^8 cells) yielded optimal binding results and that maximal binding occurred within 5 min of incubation. These parameters were used for formal analyses (30).

Adherence to fibronectin or fibrinogen. *S. aureus* adherence to matrix proteins important in endovascular pathogenesis was studied as described recently (34). Briefly, the wells of a 96-well microtiter plate (Costar) were coated with 50 μ g fibronectin or fibrinogen (Sigma) in PBS for 15 h at 4°C. Subsequently, the wells were blocked with 2% bovine serum albumin in PBS for 1 h and washed twice with PBS. Bacteria were grown in IMDM to mid-logarithmic phase, washed twice with PBS, and adjusted to 1×10^9 cells/ml using a Neubauer chamber. One hundred microliters of bacterial suspension was added to each well. After 1 h of incubation at 37°C, the wells were washed three times with PBS and stained with safranin for 1 min, and the A_{600} was determined in a microtiter plate reader.

Rabbit model of IE. All animal experimentation was performed in accordance with the guidelines for animal health and welfare required by the LA Biomedical Research Institute at Harbor-UCLA. Female outbred New Zealand White rabbits (Irish Farms, Corona, CA) underwent carotid artery-to-left ventricle catheterization as previously described (39).

To examine any potential impacts of the *dltA* or *mprF* mutation on early valvular colonization, one group of animals underwent catheterization as described above to induce sterile vegetations on the aortic valve. Two animals each were then challenged at 24 h postcatheterization with an intravenous (i.v.) inoculum of 5×10^7 CFU of either a parental, mutant, or complemented strain. Animals were then sacrificed at 1 h postchallenge, their vegetations were removed and quantitatively cultured, and the number of bacteria adhering at this site was determined as previously described (8). Maximal valvular adhesion generally occurs within this initial 1-h-postchallenge period (8).

FIG. 1. Killing of *S. aureus* strains by tPMP-1. The graph shows the percentage of bacteria surviving a 2-hour exposure to tPMP-1. Data are given as means $\pm$ SD of three experimental runs. *, $P < 0.05$ versus wild-type SA113 (WT); compl., complemented.

To optimize the assessment of the early vegetation adhesion studies described above, we carried out an analysis of bacteremia clearances post-i.v. challenge in catheterized animals. Thus, five or six animals each received 1×10^8 CFU i.v. at 24 h postcatheterization. Blood samples were then obtained at 30 and 60 min postchallenge for quantitative cultures.

For comparative virulence assessments between strains, 6 to 10 rabbits each received an inoculum of 5×10^6 CFU of the parental strain, the *dltA* mutant, or the *mprF* mutant intravenously in the marginal ear vein at 24 h postcatheterization. At 48 h postinfection, all animals had developed IE. The animals were sacrificed, and heart vegetations, kidneys, and spleens (the major target organs involved in experimental IE) were aseptically removed. Vegetations and renal and splenic abscesses were then quantitatively cultured as previously described (39). Only animals with proper placement of the trans-aortic-valve catheter and macroscopic, culture-positive vegetations were considered to have active IE and were included in the final analyses.

We have previously shown that plasmid complementation of either the *dltA* or *mprF* mutation noted above restores parental-level susceptibility phenotypes for a number of CAPs (27, 28). However, extensive pilot studies in our laboratory showed that the plasmid-complemented construct for the *mprF* mutation was relatively unstable in vivo in the absence of antibiotics after 1 to 2 days of infection. For this reason, this construct was included in the early valvular colonization experiments but not in the extended virulence studies.

Statistical analyses. Comparisons of mean bloodstream and vegetation bacterial densities were performed by Kruskal-Wallis analysis of variance, with Tukey post hoc corrections for multiple group comparisons where indicated. P values of ≤ 0.05 were considered significant. Differences in binding to matrix proteins or HUVECs of parental versus mutant strains were assessed using two-tailed Student's t test.

RESULTS

In vitro data. (i) Susceptibility of $\Delta dltA$ and $\Delta mprF$ to tPMP. The *S. aureus* $\Delta dltA$ and $\Delta mprF$ mutants have been shown to be highly susceptible to several CAPs produced by PMNs. In order to study the potential role of the CAP resistance mechanisms encoded by the *dltABCD* and *mprF* genes in endovascular infections, the susceptibility profiles of the parental, mutant, and complemented mutant strains for tPMP-1 were determined (Fig. 1). Both mutants were considerably more susceptible to tPMP-1 than the parental strain, with the latter strain exhibiting in vitro resistance to this peptide. These data parallel our prior data showing these two mutants to be substantially more susceptible to hNP-1 and two porcine protegrins than the parental strain (27, 28). Plasmid complementation of either the *dltA* or *mprF* locus partially restored the susceptibility profile for tPMP-1.

FIG. 2. Binding of *S. aureus* strains to HUVECs. The means $\pm$ SD of three independent experiments are shown. ***, $P < 0.001$ versus wild-type SA113 (WT); compl., complemented.

(ii) Adherence to platelets. The bacterial ability to bind to platelets, either directly or via platelet-bound matrix proteins, contributes considerably to the virulence of endovascular pathogens (37). When the percentages of *S. aureus* parental, $\Delta dltA$, and $\Delta mprF$ bacteria bound to platelets were compared, no significant differences were detectable ($56\% \pm 10.4\%$, $53\% \pm 10.6\%$, and $53\% \pm 9.5\%$, respectively).

(iii) Adherence of $\Delta dltA$ and $\Delta mprF$ mutants to EC (HUVECs). Our previous studies have indicated an important role of *S. aureus* WTA in adherence to EC and in the course of endovascular infections. Therefore, we investigated a potentially altered capacity of the $\Delta dltA$ mutant (lacking the teichoic acid D-alanine residues) to adhere to HUVECs. The $\Delta dltA$ mutant was considerably defective in HUVEC adhesion compared to the parental strain (Fig. 2), which is in accord with recent data for a completely WTA-deficient *S. aureus* *tagO* mutant (34). Complementation of the $\Delta dltA$ mutation restored parental-level HUVEC adhesion. In contrast, the $\Delta mprF$ mutant (with unaltered wall teichoic acid) showed no significant difference in adherence to HUVECs compared to the parental strain.

(iv) Adherence to fibronectin and fibrinogen in vitro. The WTA of *Staphylococcus epidermidis* has been shown to interact with fibronectin (13), while our recent experiments did not support such an activity of *S. aureus* WTA (34). In order to elucidate whether the $\Delta dltA$ or $\Delta mprF$ mutation affect *S. aureus* binding to matrix proteins relevant to endovascular infections, the attachment of the two mutants to immobilized fibronectin and fibrinogen was analyzed. There were no significant alterations in the in vitro binding phenotypes of either mutant with fibronectin or fibrinogen. Control strains deficient in key fibronectin-binding proteins or fibrinogen-binding proteins exhibited reduced attachment to the corresponding matrix protein ligands (Fig. 3).

In vivo virulence of $\Delta dltA$ and $\Delta mprF$ mutants in a rabbit IE model. Early bacteremia clearance levels and early colonization of sterile cardiac vegetations by the parental and CAP-susceptible mutant strains were monitored over a 60-min post-i.v.-challenge period. Both the $\Delta dltA$ and $\Delta mprF$ mutants were cleared from the bloodstream to a significantly greater extent than the parental strain at both 30 and 60 min post-i.v. challenge (Table 1). In parallel, both mutants also colonized sterile vegetations at 60 min postchallenge to a significantly lower extent than that by the parental strain (38 ± 26 CFU/vegeta-

FIG. 3. Binding of *S. aureus* strains to fibronectin (A) and fibrinogen (B). Bacterial adherence to fibronectin- or fibrinogen-coated microtiter plates is shown as percentages of wild-type (WT) binding. *S. aureus* 8325-4 and its isogenic fibronectin-binding protein mutant ($\Delta fnbA \Delta fnbB$) or *S. aureus* Newman and its isogenic fibrinogen-binding protein mutant ($\Delta clfA$) were used as control strains in panels A and B, respectively. The means $\pm$ SD of four independent experiments are shown. **, $P < 0.01$ versus wild type; ***, $P < 0.001$ versus wild type; compl., complemented.

tion and 33 ± 21 CFU/vegetation versus 113 ± 18 CFU/vegetation; the P value was <0.05 for both comparisons). Complementation of each mutation returned the capacity to colonize sterile vegetations to near parental levels (74 ± 20 CFU/vegetation and 62 ± 6 CFU/vegetation for the $\Delta dltA$ - and $\Delta mprF$ -complemented mutants, respectively).

In terms of later virulence in this model, at 48 h postinfection both the $\Delta dltA$ and $\Delta mprF$ mutants were profoundly impaired in the capacity to proliferate within cardiac vegetations

TABLE 1. Bacteremia clearance of *S. aureus* Sa113 wild type or $\Delta dltA$ or $\Delta mprF$ mutant

Bacterial strain	Bacterial count in blood (mean log ₁₀ CFU/ml $\pm$ SD)	
	30 min postchallenge	60 min postchallenge
Wild-type SA113	2.85 ± 0.32	2.85 ± 0.36
$\Delta dltA$ mutant	1.17 ± 1.08^a	1.51 ± 0.30^a
$\Delta mprF$ mutant	1.31 ± 0.55^a	0.90 ± 0.25^a

^a Significant difference versus wild-type value ($P \leq 0.05$).

TABLE 2. Bacterial counts in tissues of animals 48 h after i.v. challenge with *S. aureus* Sa113 wild type or $\Delta dltA$ or $\Delta mprF$ mutant

Bacterial strain	Bacterial count (mean log ₁₀ CFU/g tissue $\pm$ SD [no. of animals])		
	Cardiac vegetations	Kidneys	Spleen
Wild type	8.23 ± 1.57 (10)	6.03 ± 0.64 (10)	6.05 ± 0.57 (10)
$\Delta dltA$ mutant	4.27 ± 0.41^a (6)	3.69 ± 0.76^a (6)	3.76 ± 0.89^b (6)
Complemented $\Delta dltA$ mutant	7.11 ± 0.86^c (7)	4.90 ± 0.46^c (7)	4.54 ± 0.44^c (7)
$\Delta mprF$ mutant	6.36 ± 0.72^a (8)	5.99 ± 0.50^c (8)	5.58 ± 0.95^c (8)

^a Significant differences versus wild-type value ($P < 0.01$).

^b $P < 0.001$.

^c Not significant.

compared to the parental strain (Table 2), paralleling their substantially increased in vitro susceptibilities to tPMP-1. In order to study the abilities of the parental and mutant strains to leave the bloodstream and cause abscesses in distal organs, bacterial numbers in spleens and kidneys were determined. Only the $\Delta dltA$ mutant was significantly attenuated (versus the parental strain) in terms of bacterial numbers within renal and splenic abscesses. In contrast, the $\Delta mprF$ mutant was found in these organs at near parental levels. Complementation of the $\Delta dltA$ mutation resulted in substantial increases in intravegetation, renal, and splenic bacterial numbers to near parental levels (not significantly different from parental values).

DISCUSSION

Using *S. aureus* and other gram-positive organisms as prototypical pathogens, compelling data have been generated that support the notion that endogenous CAPs contribute substantially to the host defense response in diverse experimental models, including localized soft-tissue infections (19, 25), sepsis (5), and endovascular infections (e.g., IE) (7). These observations in such distinct anatomic niches likely reflect (i) the relative abundance of a particular CAP at that site, (ii) the capacity of the CAP of interest to function within that microenvironment, and (iii) the specific microbial pathogen involved. Common microbial pathogens have various susceptibilities to CAPs produced at distinct anatomic sites, including those from platelets (37) and PMNs (26). Collectively, these concepts have been integrated in the immunorelativity model of CAPs (42).

Even within a specific bacterial species, there appears to be a broad range of susceptibility profiles to individual CAPs (1, 23). The molecular mechanisms by which invasive pathogens such as *S. aureus* resist the microbicidal effects of CAPs remain incompletely understood. For example, *S. aureus* strains expressing the multi-cation efflux pump QacA exhibit relative tPMP-1 resistance in vitro (20). Notably, this mechanism is unrelated to tPMP-1 efflux (21). The inactivation of *snoD* (staphylococcal *nuoN*-like orthologue), encoding a putative component of a complex I NADH-oxidoreductase involved in proton motive force maintenance, also renders *S. aureus* strains relatively tPMP-1 resistant (2). Strains with an intact stress response sigma factor B are substantially more tPMP-1 resistant than isogenic strains with *sigB* deletions (2). It is partic-

ularly interesting that all of the above-mentioned tPMP-1-resistant mutants share a common phenotype, i.e., enhanced cell membrane (CM) fluidity (3). The *dltABCD* and *mprF* genes, on the other hand, lead to a relatively increased net positive charge of teichoic acids and phospholipids in the cell envelope and thus provide protection against killing by a broad range of cationic antimicrobial molecules (10, 26, 33), including tPMP-1, as shown in the present study.

Our current studies were designed to test the hypotheses that (i) the *dltA* and/or *mprF* locus impact on in vitro susceptibility profiles to tPMP-1 alters EC binding and (ii) such in vitro phenotypes would influence the microbiologic outcomes of an experimental endovascular infection model in which tPMPs have been shown to be relevant (IE) (8, 22). Several interesting findings emerged from this investigation. Firstly, in terms of in vitro activity, the parental strain exhibited relative resistance to tPMP-1, concordant with prior data on this strain's resistance to other CAPs (27, 28). In contrast, both its *dltA* and *mprF* mutants were more susceptible to tPMP-1. Secondly, these in vitro results were roughly paralleled by our in vivo findings with an IE model. For both mutants, bacterial densities in mature cardiac vegetations were significantly reduced compared to those in animals infected with the parental strain. These mutants were equally susceptible to the microbicidal action of tPMP-1 in vitro (compared to the tPMP-1-resistant parental strain), and both were impaired in the capacity to colonize sterile cardiac vegetations. Thirdly, both mutants were cleared from the bloodstream to a significantly greater extent than the parental strain at early times postinfection. Collectively, these outcomes suggest that the local secretion of tPMPs at the sites of endovascular infection (i.e., vegetation) and enhanced bacteremic clearances each contribute to the mitigation of the early severity and later progression phases of IE.

In contrast, only the $\Delta dltA$ mutant was attenuated in virulence within extravascular target tissue organs such as the spleen and kidneys, which depends on the bacterial ability to interact with the endothelium and leave the bloodstream. The differential capacities of the $\Delta dltA$ and $\Delta mprF$ mutants to seed and proliferate within metastatic abscesses may reflect the differential susceptibilities of such mutants to antimicrobial molecules other than CAPs, such as PLA2 (16), which should be abundant within abscesses. On the other hand, only the $\Delta dltA$ mutant (with an altered teichoic acid structure) was impaired in its capacity to bind to EC in vitro, while the $\Delta mprF$ mutant adhered to EC and disseminated to kidneys and spleens equally well as the parental strain. These findings are reminiscent of our recent data on the role of the presence and intact structure of WTA in *S. aureus* binding to EC (34). In that study, a WTA-deficient *tagO* mutant generated in the same strain background as the *dltA* mutant also exhibited defective EC binding, while its susceptibility to CAPs was unaltered. Interestingly, the degree to which organ bacterial counts (e.g., vegetations) were reduced in the *tagO* mutant was substantially less than that observed with the *dltA* mutant. These data emphasize the notion that reduced virulence in the IE model represents a composite of increased susceptibility to CAPs, enhanced intravascular clearance mechanisms, and reduced EC binding. Experiments with WTA-coated latex beads have indicated a specific contribution of WTA to *S. aureus* binding

to EC (34), although WTA-binding molecules on EC have not yet been identified. Taken together, our studies indicate that teichoic acid structure and charge are critical in two key aspects of endovascular infections: (i) adherence to EC and subsequent dissemination to subendothelial tissues and (ii) resistance to platelet- and PMN-derived CAPs. Further studies are ongoing in our laboratories to investigate these concepts.

ACKNOWLEDGMENTS

We thank Friedrich Götz and Ingo Autenrieth (Tubingen, Germany) for continuing support and helpful discussions, Timothy Foster (Dublin, Ireland) and Christiane Wolz (Tubingen, Germany) for providing bacterial strains, and Yin Li and Gabriele Hornig for excellent technical assistance.

This research was supported by grants from the National Institutes of Health to A.S.B. (AI-39108) and M.R.Y. (AI-48031 and RR-13004) and from the German Research Council to A.P. (FOR 449, SPP 1130, and GRK 685).

REFERENCES

1. Bayer, A. S., D. Cheng, M. R. Yeaman, G. R. Corey, R. S. McClelland, L. J. Harrel, and V. G. Fowler, Jr. 1998. In vitro resistance to thrombin-induced platelet microbicidal protein among clinical bacteremic isolates of *Staphylococcus aureus* correlates with an endovascular infectious source. *Antimicrob. Agents Chemother.* **42**:3169–3172.
2. Bayer, A. S., P. J. McNamara, R. A. Proctor, L. I. Kupferwasser, N. Lucindo, R. Prasad, T. Prasad, A. Peschel, and M. R. Yeaman. 2003. Disruption of the *mnhA-G* Na⁺/H⁺ antiporter operon is associated with resistance to the microbicidal activity of thrombin-induced platelet microbicidal protein-1 in *Staphylococcus aureus*, abstr. 2482. Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
3. Bayer, A. S., R. Prasad, J. Chandra, A. Koul, M. Smriti, A. Varma, R. A. Skurray, N. Firth, M. H. Brown, S.-P. Koo, and M. R. Yeaman. 2000. In vitro resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal proteins is associated with alterations in cytoplasmic membrane fluidity. *Infect. Immun.* **68**:3548–3553.
4. Cole, A. M., T. Ganz, A. M. Liese, M. D. Burdick, L. Liu, and R. M. Strieter. 2001. Cutting edge: IFN-inducible ELR-CXC chemokines display defensin-like antimicrobial activity. *J. Immunol.* **167**:623–627.
5. Collins, L. V., S. A. Kristian, C. Weidenmaier, M. Faigle, K. P. Van Kessel, J. A. Van Strijp, F. Götz, B. Neumeister, and A. Peschel. 2002. *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J. Infect. Dis.* **186**:214–219.
6. De Haas, C. J., K. E. Veldkamp, A. Peschel, F. Weerkamp, W. J. van Wamel, E. C. Heezius, M. J. Poppelier, K. P. Van Kessel, and J. A. Van Strijp. 2004. Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J. Exp. Med.* **199**:687–695.
7. Dhawan, V. K., A. S. Bayer, and M. R. Yeaman. 1998. In vitro resistance to thrombin-induced platelet microbicidal protein is associated with enhanced progression and hematogenous dissemination in experimental *Staphylococcus aureus* infective endocarditis. *Infect. Immun.* **66**:3476–3479.
8. Dhawan, V. K., M. R. Yeaman, A. L. Cheung, E. Kim, P. M. Sullam, and A. S. Bayer. 1997. Phenotypic resistance to thrombin-induced platelet microbicidal protein in vitro is correlated with enhanced virulence in experimental endocarditis due to *Staphylococcus aureus*. *Infect. Immun.* **65**:3293–3299.
9. Dürr, M., and A. Peschel. 2002. Chemokines meet defensins—the merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infect. Immun.* **70**:6515–6517.
10. Fedtke, I., F. Götz, and A. Peschel. 2004. Bacterial evasion of innate host defenses—the *Staphylococcus aureus* lesson. *Int. J. Med. Microbiol.* **294**:189–194.
11. Fowler, V. G., Jr., G. Sakoulas, L. M. McIntyre, V. G. Meka, R. D. Arbeit, C. H. Cabell, M. E. Stryjewski, G. M. Eliopoulos, L. B. Reller, G. R. Corey, T. Jones, N. Lucindo, M. R. Yeaman, and A. S. Bayer. 2004. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. *J. Infect. Dis.* **190**:1140–1149.
12. Greene, C., D. McDevitt, P. Francois, P. E. Vaudaux, D. P. Lew, and T. J. Foster. 1995. Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of *fmb* genes. *Mol. Microbiol.* **17**:1143–1152.
13. Hussain, M., C. Heilmann, G. Peters, and M. Herrmann. 2001. Teichoic acid enhances adhesion of *Staphylococcus epidermidis* to immobilized fibronectin. *Microb. Pathog.* **31**:261–270.
14. Ing, M. B., L. M. Baddour, and A. S. Bayer. 1997. Bacteremia and infective endocarditis: pathogenesis, diagnosis, and complications, p. 331–354. *In*

- K. B. Crossley and G. L. Archer (ed.), *The staphylococci in human disease*. Churchill Livingstone, New York, N.Y.
15. Iordanescu, S., and M. Surdeanu. 1976. Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. *J. Gen. Microbiol.* **96**:277–281.
 16. Koprivnjak, T., A. Peschel, M. H. Gelb, N. S. Liang, and J. P. Weiss. 2002. Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against *Staphylococcus aureus*. *J. Biol. Chem.* **277**:47636–47644.
 17. Krijgsveld, J., S. A. J. Zaai, J. Meeldijk, P. A. van Veelen, G. Fang, B. Poolman, E. Brandt, J. E. Ehler, A. J. Kuijpers, G. H. M. Engbers, J. Feijen, and J. Dankert. 2000. Thrombocidins, microbicidal proteins from human blood platelets, are C-terminal deletion products of CXC chemokines. *J. Biol. Chem.* **275**:20374–20381.
 18. Kristian, S. A., M. Dürr, J. A. Van Strijp, B. Neumeister, and A. Peschel. 2003. MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. *Infect. Immun.* **71**:546–549.
 19. Kristian, S. A., X. Lauth, V. Nizet, F. Götz, B. Neumeister, A. Peschel, and R. Landmann. 2003. Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. *J. Infect. Dis.* **188**:414–423.
 20. Kupferwasser, L. I., R. A. Skurray, M. H. Brown, N. Firth, M. R. Yeaman, and A. S. Bayer. 1999. Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the *qacA* locus. *Antimicrob. Agents Chemother.* **43**:2395–2399.
 21. Kupferwasser, L. I., R. A. Skurray, N. Firth, N. H. Brown, M. R. Yeaman, R. Prasad, M. Smriti, and A. S. Bayer. 2001. *Staphylococcus aureus* resistance to thrombin-induced platelet microbicidal protein-1 mediated by the *qacA* gene is specific and independent of multidrug efflux pump activity, abstr. 2288. Abstr. 101st Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.
 22. Kupferwasser, L. I., M. R. Yeaman, S. M. Shapiro, C. C. Nast, and A. S. Bayer. 2002. In vitro susceptibility to thrombin-induced platelet microbicidal protein is associated with reduced disease progression and complication rates in experimental *Staphylococcus aureus* endocarditis: microbiological, histopathologic, and echocardiographic analyses. *Circulation* **105**:746–752.
 23. Midorikawa, K., K. Ouhara, H. Komatsuzawa, T. Kawai, S. Yamada, T. Fujiwara, K. Yamazaki, K. Sayama, M. A. Taubman, H. Kurihara, K. Hashimoto, and M. Sugai. 2003. *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infect. Immun.* **71**:3730–3739.
 24. Moreillon, P., Y. A. Que, and A. S. Bayer. 2002. Pathogenesis of streptococcal and staphylococcal endocarditis. *Infect. Dis. Clin. N. Am.* **16**:297–318.
 25. Nizet, V., T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R. A. Dorschner, V. Pestonjamas, J. Piraino, K. Huttner, and R. L. Gallo. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **414**:454–457.
 26. Peschel, A. 2002. How do bacteria resist human antimicrobial peptides? *Trends Microbiol.* **10**:179–186.
 27. Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung, A. Tarkowski, K. P. Van Kessel, and J. A. Van Strijp. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J. Exp. Med.* **193**:1067–1076.
 28. Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Götz. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. *J. Biol. Chem.* **274**:8405–8410.
 29. Proctor, R. A., P. van Langevelde, M. Kristjansson, J. N. Maslow, and R. D. Arbeit. 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin. Infect. Dis.* **20**:95–102.
 30. Sullam, P. M., A. S. Bayer, W. M. Foss, and A. L. Cheung. 1996. Diminished platelet binding in vitro by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. *Infect. Immun.* **64**:4915–4921.
 31. Tang, Y.-Q., M. R. Yeaman, and M. E. Selsted. 2002. Antimicrobial peptides from human platelets. *Infect. Immun.* **70**:6524–6533.
 32. Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturia, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond, and A. Peschel. 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat. Med.* **10**:243–245.
 33. Weidenmaier, C., S. A. Kristian, and A. Peschel. 2003. Bacterial resistance to antimicrobial host defenses—an emerging target for novel anti-infective strategies? *Curr. Drug Targets* **4**:643–649.
 34. Weidenmaier, C., A. Peschel, Y. Q. Xiong, S. A. Kristian, K. Dietz, M. R. Yeaman, and A. S. Bayer. 2005. Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. *J. Infect. Dis.* **191**:1771–1777.
 35. Wolz, C., D. McDavitt, T. J. Foster, and A. L. Cheung. 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. *Infect. Immun.* **64**:3142–3147.
 36. Wu, T., M. R. Yeaman, and A. S. Bayer. 1994. In vitro resistance to platelet microbicidal protein correlates with endocarditis source among bacteremic staphylococcal and streptococcal isolates. *Antimicrob. Agents Chemother.* **38**:729–732.
 37. Yeaman, M. R., and A. S. Bayer. 2000. *Staphylococcus aureus*, platelets, and the heart. *Curr. Infect. Dis. Rep.* **2**:281–298.
 38. Yeaman, M. R., A. S. Bayer, S.-P. Koo, W. Foss, and P. M. Sullam. 1998. Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action. *J. Clin. Invest.* **101**:178–187.
 39. Yeaman, M. R., J. C. Lee, and A. S. Bayer. 1999. Experimental *Candida* endocarditis, p. 1709–1720. In M. A. Sande and O. Zak (ed.), *Handbook of animal model infections*. Academic Press, San Diego, Calif.
 40. Reference deleted.
 41. Yeaman, M. R., Y. Q. Tang, A. J. Shen, A. S. Bayer, and M. E. Selsted. 1997. Purification and in vitro activities of rabbit platelet microbicidal proteins. *Infect. Immun.* **65**:1023–1031.
 42. Yeaman, M. R., and N. Y. Yount. 2005. Code among chaos: the immunorelativity and the AEGIS model of antimicrobial peptides. *ASM News* **71**:21–27.
 43. Yount, N. Y., K. D. Gank, Y. Q. Xiong, A. S. Bayer, T. Pender, W. H. Welch, and M. R. Yeaman. 2004. Platelet microbicidal protein 1: structural themes of a multifunctional antimicrobial peptide. *Antimicrob. Agents Chemother.* **48**:4395–4404.