Combinatorial Phenotypic Signatures Distinguish Persistent from Resolving Methicillin-Resistant *Staphylococcus aureus* Bacteremia Isolates[▽]

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Persistent methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia (PB) (positive blood cultures after ≥7 days of therapy) represents a clinically challenging subset of invasive MRSA infections. In this investigation, we examined the potential correlation of specific virulence signatures with PB versus resolving MRSA bacteremia (RB) (negative blood cultures within 2 to 4 days of therapy) strains. Thirty-six MRSA isolates from patients enrolled in a recent multinational clinical trial were studied for (i) susceptibility to host defense cationic peptides (HDPs) (i.e., thrombin-induced platelet microbicidal proteins [tPMPs] and human neutrophil peptide 1 [hNP-1]); (ii) adherence to host endovascular ligands (fibronectin) and cells (endothelial cells); and (iii) biofilm formation. We found that PB isolates exhibited significantly reduced susceptibilities to tPMPs and hNP-1 ($P < 0.001$ and $P = 0.023$, respectively). There was no significant association between the PB outcome and fibronectin binding, endothelial cell binding, or biofilm formation ($P = 0.25$, 0.97 , and 0.064 versus RB strains, respectively). However, multiple logistic regression analysis revealed that the PB outcome was significantly associated with the combination of reduced susceptibilities to HDPs and extent of biofilm formation ($P < 0.0001$). Similar results were obtained in a second analysis using days of bacteremia as a continuous outcome, showing that reduced HDP susceptibilities and increased biofilm formation contributed to predict the duration of bacteremia. Our data indicate that PB isolates have specific pathogenic signatures independent of conventional antimicrobial susceptibility. These combinatorial mosaics can be defined and used to prospectively distinguish PB from RB strains in advance and potentially to predict ultimate clinical outcomes.

Staphylococcus aureus is a leading cause of bacteremia and infective endocarditis (IE) throughout the industrialized world (18, 22, 42, 54). A growing proportion of these bloodstream infections are due to methicillin-resistant *Staphylococcus aureus* (MRSA), which is associated with worse clinical outcome, longer hospitalization, and higher net cost than similar infections caused by methicillin-susceptible *S. aureus* (MSSA) (3, 6, 14, 15, 26, 49, 52, 60). Persistent-bacteremia (PB) outcomes comprise 20 to 30% of all episodes of MRSA bacteremia and are especially relevant to endovascular infections (13, 22, 23, 31). Why some MRSA bacteremia strains persist while others resolve (RB) despite similar baseline clinical and microbiologic characteristics and identical medical and surgical therapeutic strategies is poorly understood.

In two recent studies, we investigated *S. aureus* virulence factors that could potentially differentiate PB from RB strains in the context of endovascular infections (23, 56). We initially studied PB or RB isolates from the Duke University Medical Center and delineated several *in vitro* parameters that ap-

peared to distinguish these two strain cohorts, including enhanced matrix ligand and endothelial cell binding, and reduced susceptibility to polymorphonuclear leukocytes (PMN) and platelet-specific host defense peptides (HDPs) (23, 56). Several other recent investigations have also reported associations between putative pathogenic markers of MRSA and the PB outcome in patients with both IE and non-IE syndromes (24, 33, 43, 45). Taken together, these experimental and clinical data suggest that PB isolates may have specific pathogenic characteristics that distinguish them from RB isolates and that might potentially predict a worse clinical outcome. However, a decade of literature studying MRSA virulence factors has identified a recurring theme: no single virulence factor alone appears to be sufficient to describe MRSA virulence in endovascular infection. Rather, we hypothesize that combinations of virulence factors are necessarily or uniquely involved in PB.

Building on our prior investigations, the current study aimed to identify further potential mosaic (“combinatorial”) signatures associated with the PB outcome. To circumvent possible geographic, single-center, and/or clonality biases from previous studies, we utilized a PB and an RB strain cohort from 2002 to 2005, involving a well-characterized, multinational *S. aureus* bacteremia antibiotic trial (20). We profiled relevant genotypic and *in vitro* phenotypic characteristics, including susceptibility

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profiles to prototypic innate HDPs, endovascular host cell and ligand binding, and biofilm formation.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All MRSA strains used in this study originated from a multinational *S. aureus* bacteremia clinical trial collection conducted between 2002 and 2005 (20). Detailed descriptions of the study patients have been previously reported (20). In brief, the patients in this study were well matched for epidemiologic, demographic, and clinical features (e.g., underlying diseases). All 36 patients in our analysis had complicated MRSA bacteremia and/or infective endocarditis, and all received either daptomycin monotherapy or dual-drug standard therapy consisting of vancomycin plus low-dose, short-course aminoglycosides. As demonstrated by the data, patients in daptomycin and vancomycin treatment groups had similar clinical outcomes ("noninferior") (20). Clinical blood culture data were used to group our study isolates into PB and RB cohorts. PB ($n = 18$) was defined as ≥ 7 days of positive MRSA blood cultures despite receiving antibiotics to which the isolate was susceptible *in vitro* (37). RB ($n = 18$) was defined as an initial positive MRSA blood culture, with subsequent blood cultures being negative 2 to 4 days after therapy initiation. These definitions of PB and RB are the same as those used in our previous strain set investigations of this clinical outcome (23, 56). When not otherwise specified, bacteria were grown overnight in tryptic soy broth (TSB) at 37°C.

Determination of MICs and population analysis. The MICs of vancomycin were tested by broth microdilution, as recommended by the CLSI (4). Vancomycin population analyses were performed as previously described (33).

Genotypic profiles. The staphylococcal cassette chromosome *mec* (SCCmec) type was determined by previously published PCR methods (25, 29, 51). The *spa* typing was defined following analysis of the gene's tandem repeat polymorphisms as determined by nucleotide sequencing (32, 51). *spa* types were used to provisionally assign isolates to multilocus sequence typing-defined clonal complexes (CCs), as described previously (39). The presence or absence of 30 additional standard staphylococcal virulence genes and *agr* typing were determined by multiplex PCR (11).

Susceptibility to HDPs. Thrombin-induced platelet microbicidal proteins (tPMPs) were prepared from thrombin-stimulated platelets isolated from fresh rabbit blood as previously described (55). The bioactivity of the tPMPs was assessed by a *Bacillus subtilis* ATCC 6633 assay as detailed previously (57, 59). Human neutrophil peptide 1 (hNP-1) was purchased from Peptides International (Louisville, KY). The HDP susceptibilities were assayed by exposing 10^3 CFU/ml bacteria to tPMPs (1 μ g/ml equivalent) or 10^5 CFU/ml bacteria to hNP-1 (10 μ g/ml) for 2 h at 37°C as previously described (23, 56). These HDP concentrations were selected based on extensive pilot studies identifying peptide levels that did not rapidly kill control *S. aureus* strains over the 2-h period of exposure to HDPs (data not shown). The results are expressed as the percentage ($\pm$ standard deviation [SD]) of the initial inoculum that survived exposure to HDPs. A minimum of two independent assays were performed for each strain.

Adherence to fibronectin. To measure the ability of *S. aureus* to adhere to fibronectin, 96-well plates were coated with purified human fibronectin (10 μ g/ml; Sigma Chemicals) for 18 h at 4°C, treated with 3% bovine serum albumin (Sigma Chemicals) for 2 h to prevent nonspecific adhesion, and washed with phosphate-buffered saline (PBS) three times before organism seeding. Mid-logarithmic-phase bacterial cells (100 μ l) grown in TSB were added at a final inoculum of 10^9 CFU/ml (optical density at 600 nm [OD₆₀₀] = 1.0) to the plates and incubated for 2 h. Then, the wells were washed three times with PBS and fixed at 55°C for about 30 min. Bound bacteria were detected by staining them with crystal violet and measuring the OD₅₇₀ with an enzyme-linked immunosorbent assay (ELISA) plate reader (61). Before all isolates were tested, the optimal fibronectin concentration was determined by establishing a standard curve with different fibronectin concentrations (2.5 to 50 μ g/ml) using *S. aureus* strain NCTC 8325-4. The results ($\pm$ SD) shown are the averages of at least two independent experiments for each isolate.

Endothelial cell binding. Endothelial cells harvested from umbilical cord veins were cultured as described elsewhere (17). Confluent endothelial cell monolayer 6-well plates were washed twice with Hanks balanced salt solution (HBSS) before organism seeding. Bacterial isolates were added to plates at a final inoculum of 5×10^3 CFU/ml and incubated for 1 h under static conditions in a

5% CO₂ incubator at 37°C. Unbound bacteria were removed by washing the plates with HBSS, and TSB agar was added. Adherence was expressed as the average percentage ($\pm$ SD) of the initial inoculum bound from at least two independent experiments for each isolate (56).

Biofilm formation under static conditions. MRSA cells from fresh blood culture plates were resuspended in physiological NaCl solution to a density of 0.5 McFarland standard and diluted 1:100 into brain heart infusion (BHI) supplemented with 0.5% glucose; 200 μ l of this suspension was transferred to wells of 96-well Nunc Delta tissue culture plates (Roskilde, Denmark) and incubated for 18 h at 37°C. After incubation, the wells were washed with PBS three times, air dried, stained with Safranin (0.1% in distilled water; Acros Organics) for 30 s, and washed three times with distilled water. After visual observation, the adhering dye was dissolved in 30% acetic acid, and absorption was measured at 490 nm (53). An A_{490} of >0.5 was considered biofilm. The results ($\pm$ SD) represent a minimum of three independent assays for each strain.

Biofilm composition. In order to determine if the net composition of PB-associated biofilms differed from that of RB-associated biofilms, we carried out biofilm stability assays in the presence of carbohydrate, protein, and DNA dispersal agents. For these investigations, we selected the five RB isolates with the strongest biofilm formation ($A_{490} > 1.0$) and, in parallel, selected five PB strains, also with strong biofilm formation ($A_{490} > 1.0$). The supernatants of 18-h-old biofilms were replaced by fresh medium supplemented with either 10 mM sodium metaperiodate (Alfa Aesar, Ward Hill, MA), 100 μ g/ml protease K (Acros Organics), or 140 U/ml RNase-free DNase I (Takara Bio Inc., Shiga, Japan) and incubated for 2 h at 37°C as previously described (30, 50, 53). Medium without supplement served as a negative control. After treatment, the biofilms were quantified as described above. Each experiment was performed independently at least twice on separate days, using triplicates for each run.

Statistical analysis. A two-sample test for binomial proportions was used to compare the genetic backgrounds of PB and RB isolates; P values of ≤ 0.05 were considered statistically significant. Means and standard deviations were calculated for all predictor variables (phenotypes). For analysis of the relationships of variables to PB versus RB, comparisons of individual variables were performed using simple logistic regression, and multiple logistic regression was used to assess the joint relationship of the predictors with the outcome. All variables with a P value of <0.2 were included in a stepwise regression procedure, and the criterion for remaining in the model was significance at an α of 0.05.

To address the somewhat arbitrary categorical definition used for grouping the strains into PB and RB cohorts, we performed a supplementary analysis using days of bacteremia as a continuous outcome. A log transformation was applied to make the outcome more normally distributed. Simple linear regression was conducted for all predictor variables, and a multivariable model was created using stepwise multiple linear regression procedure. Once the final model was identified, the regression coefficients and their confidence intervals were transformed back. Criteria for entry into and remaining in the model were the same as in the primary analysis.

RESULTS

Susceptibility testing. Vancomycin MIC ranges were 0.5 to 1.0 μ g/ml, and the median MICs did not differ between the PB and RB groups. Population analyses of the PB isolates revealed no evidence of vancomycin heteroresistant subpopulations (data not shown).

Genotypic characterization. A total of 6 CCs were represented among the 36 isolates (Table 1). The most common CC type observed was CC5 in both PB (44.44%) and RB (44.44%) isolates. RB isolates were significantly more likely to be CC1 than PB isolates (16.7% versus 0%; $P = 0.012$). The most common *spa* type was 2 in both PB (27.8%) and RB (33.3%) isolates. SCCmec IV was the most commonly observed type, with no differences between PB and RB strains (53% of total isolates). Collectively, 47% of all isolates were *agr* group I and 41% were *agr* group II. Taken together, there were no significant associations of either SCCmec type, *spa* type, or *agr* group and the PB or RB outcome. Carriage of the V8 protease gene (*sspA*) was significantly overrepresented among PB

TABLE 1. Genotype distribution between PB and RB isolates

Characteristic	No. (%) of MRSA isolates		<i>P</i> ^a
	PB group (<i>n</i> = 18)	RB group (<i>n</i> = 18)	
<i>agr</i> group			
I	9 (50.0)	6 (33.3)	0.31
II	8 (44.4)	9 (50.0)	0.74
III	1 (5.6)	3 (16.7)	0.27
SCC <i>mec</i> type			
I	1 (5.6)	0 (0.0)	0.15
II	7 (38.9)	9 (50.0)	0.50
IV	10 (55.5)	9 (50.0)	0.74
Predicted clonal complex ^b			
CC1	0 (0.0)	3 (16.7)	0.01
CC5	8 (44.4)	8 (44.4)	>0.99
CC8	6 (33.3)	3 (16.7)	0.24
CC30	1 (5.6)	0 (0.0)	0.15
CC45	2 (11.1)	3 (16.7)	0.63
CC59	1 (5.6)	0 (0.0)	0.15
ND ^c	0 (0.0)	1 (5.6)	0.15

^a *P* values comparing the values for the two groups were determined using a two-sample test for binomial proportions.

^b *spa* types were used to predict the clonal complex according to the method of McCalla et al. (39).

^c ND, not determined.

isolates versus RB isolates ($P = 0.042$). There were no other significant differences between the PB and RB strain sets with regard to the presence or absence of the other 29 virulence genes studied (data not shown).

Phenotypic characterization and statistical analysis. The phenotypic characteristics of PB and RB strains are summarized in Table 2; individual data are shown in Fig. 1. The PB isolates exhibited significantly reduced susceptibilities to *in vitro* killing by hNP-1 and tPMPs ($P = 0.02$ and $P < 0.001$, respectively, using simple logistic regression analyses). The association between the PB outcome and fibronectin binding, endothelial cell binding, or biofilm formation was not significant ($P = 0.25$, 0.97 , and 0.064 versus RB strains, respectively). Stepwise multiple logistic regression identified the combination of relative hNP-1 and tPMP resistance, together with biofilm formation, as the best composite model to predict the PB outcome ($P < 0.0001$; pseudo $R^2 = 0.486$) (Table 2). Model quality was assessed using the Hosmer-Lemeshow goodness-of-fit statistic, and it was determined that all models fit the data well.

Additionally, we performed a supplementary analysis using days of bacteremia as a continuous outcome. As in the primary analysis, higher relative resistances to *in vitro* killing by hNP-1 or tPMPs were significantly associated with longer duration of bacteremia in univariate analysis ($P = 0.014$ and $P < 0.001$, respectively). The associations were nonsignificant for fibronectin binding, endothelial cell binding, and biofilm formation ($P > 0.1$ for all comparisons). Multiple linear regression revealed that the combination of relative hNP-1 ($P = 0.007$) and tPMP ($P < 0.001$) resistance, together with biofilm formation ($P = 0.021$), was the best composite model to predict the duration of bacteremia ($P < 0.0001$; adjusted $R^2 = 0.492$) (Table 3). The appropriateness of the model was assessed using residual plots, and it was determined that all models fit the data well.

Biofilm composition. To investigate the net chemical composition of the extracellular matrix of PB and RB biofilms, we performed biofilm stability assays in the presence or absence of various dispersal agents. Sodium metaperiodate oxidation of polysaccharide intercellular adhesions (PIA) or polymeric *N*-acetyl-glucosamine (PNAG) led to an average reduction of 44% in the A_{490} across all 10 isolates. Reductions were significantly higher in RB versus PB strains, suggesting that RB isolates had a substantial carbohydrate content (58.4% versus 30.6%; $P = 0.04$) (Fig. 2). In addition, the biofilms of all 10 isolates were significantly reduced by treatment with proteinase K. Moreover, 8 of the 10 isolates (4 PB and 4 RB) were significantly reduced by treatment with DNase I. The average reductions in the A_{490} were 89% and 70%, due to proteinase K and DNase I, respectively, and the extents of dispersal were significantly higher than dispersal by sodium metaperiodate ($P < 0.001$ and $P < 0.05$ for proteinase K and DNase I versus sodium metaperiodate, respectively). The extents of dispersal due to proteinase K and DNase I were similar in the PB and RB groups (Fig. 2).

DISCUSSION

Conventional approaches to MRSA virulence assessments have predominantly focused on putative virulence determinants individually, using “molecular Koch’s postulates” (16). Such studies have provided valuable insights into the contributions of specific virulence factors to MRSA pathogenesis, yet an important theme that has emerged from these efforts is that no single virulence determinant alone is sufficient to enable full virulence of MRSA. In this study, we are, to our knowledge,

TABLE 2. Phenotypic characteristics and statistical analysis of PB and RB isolates

Phenotype	Mean		Simple logistic regression		Multiple logistic regression ^a	
	PB isolates (<i>n</i> = 18)	RB isolates (<i>n</i> = 18)	OR (95% CI) ^b	<i>P</i>	OR (95% CI)	<i>P</i>
hNP-1 (% survival)	40.9 ± 20.80	25.6 ± 15.62	2.25 (1.06–4.79)	0.023	3.51 (1.05–11.8)	0.042
tPMPs (% survival)	45.9 ± 17.95	23.1 ± 15.33	1.08 (1.03–1.14)	<0.001	1.09 (1.02–1.16)	0.004
Fibronectin binding (A_{570})	0.42 ± 0.13	0.47 ± 0.09	0.03 (0.00–15.83)	0.25		
Endothelial cell binding (% binding)	12.6 ± 4.50	12.5 ± 2.88	1.00 (0.84–1.19)	0.97		
Biofilm formation (A_{490})	1.07 ± 0.51	0.76 ± 0.46	3.55 (0.86–14.65)	0.064	17.04 (1.11–262)	0.042

^a Model $P < 0.0001$; pseudo $R^2 = 0.486$.

^b OR, odds ratio; CI, confidence interval.

FIG. 1. *In vitro* adherence of PB and RB isolates to fibronectin (A) and endothelial cells (B), susceptibilities to hNP-1 (C) and tPMPs (D), and biofilm formation (E). The bars and error bars indicate the averages and standard deviations for individual isolates (at least two independent assays per isolate). The average for each category is indicated by a horizontal dashed line.

TABLE 3. Simple and multiple linear regression of phenotypic characteristics of PB/RB isolates and duration of bacteremia

Phenotype	Simple linear regression		Multiple linear regression ^a	
	Regression coefficient (%) ^b (95% CI ^c)	<i>P</i>	Regression coefficient (%) ^b (95% CI)	<i>P</i>
hNP-1 (% survival)	158 (111–229)	0.01	148 (112–197)	0.007
tPMPs (% survival)	103 (102–105)	<0.001	103 (102–105)	<0.001
Fibronectin binding (<i>A</i> ₅₇₀)	11 (0–285)	0.17		
Endothelial cell binding (% binding)	102 (92–113)	0.69		
Biofilm formation (<i>A</i> ₄₉₀)	185 (88–389)	0.10	191 (111–329)	0.021

^a Model *P* value < 0.0001; adjusted *R*² = 0.492.

^b A log transformation was applied to make the outcome more normally distributed. Once the final model was identified, the regression coefficients and their confidence intervals were transformed back. Thus, the coefficients of the models correspond to a multiplicative factor (percentage change) in days of bacteremia associated with a 1-unit change in each predictor variable.

^c CI, confidence interval.

the first to employ a combinatorial strategy to assess the impact of phenotypic mosaics that potentially enable an MRSA PB outcome in the context of endovascular infections. The use of clinical MRSA isolates from a recent multinational clinical trial allowed us to exclude possible geographic, single-center, and/or clonality biases that may have substantially limited previous investigations.

A number of interesting observations emerged from the present investigation. First, an important factor in the pathogenesis of *S. aureus* infection is the ability of the microbe to circumvent clearance by platelet-mediated mechanisms, principally via resistance to killing by platelet HDPs (e.g., tPMPs) secreted in response to agonists generated at endovascular infection sites (58). Reduced killing by tPMPs *in vitro* has been correlated with enhanced *in vivo* virulence and less efficacy of antibiotic therapy (including vancomycin) in a rabbit endocarditis (IE) model in several previous studies from our laboratory (9, 10, 40). In addition, *S. aureus* bloodstream strains isolated from endovascular infections were significantly less susceptible to killing by tPMPs *in vitro* than bacteremia strains from other clinical syndromes (e.g., soft tissue abscesses), demonstrating the potential importance of relative tPMP resistance in clinical settings (1, 55). Of note, in both these prior experimental models and clinical investigations, a “biologic breakpoint” utilizing the same 2-h killing assay appeared to emerge. Thus, a survival profile in the range of >40% in this assay correlated well with (i) an endovascular source for bacteremia, (ii) more virulent infection in IE models, and (iii) reduced responsiveness to antimicrobial prophylaxis and therapy in these same models. Interestingly, in the present study, we observed an impressive statistical relationship between reduced tPMP-induced killing *in vitro* and the PB outcome, with the mean *in vitro* survival profile also greater than 40% for such strains. Finally, two recent clinical studies demonstrated a similar correlation between relative tPMP resistance and PB-RB outcomes in strains from both IE and non-IE infections (23, 44). Similarly, reduced *in vitro* susceptibilities to hNP-1 correlated with the PB outcome in our study, in accordance with our

previous findings (56). We have postulated that relative resistances to such PMN HDPs may foster PB by favoring MRSA survival within PMN-rich abscesses (e.g., within the kidneys or spleen). Taken together, these data indicate a potential role for relative resistance to tPMPs and hNP-1 as a surrogate biological marker for the PB outcome.

Second, the ability of bacteria to bind to matrix ligands and certain host cells is felt to be an important virulence factor in *S. aureus* endovascular infections (56). In the present study, we were surprised to find no significant differences in fibronectin-binding profiles between the PB and RB strain sets. This is in contrast to our previous study, in which the PB outcome was associated with better fibronectin binding (56). Interestingly, a recent study showed that *S. aureus* isolates at either extreme of fibronectin-binding capacity were equally capable of establishing IE in an experimental model (61). Even though the ability to bind fibronectin is crucial for inducing endocarditis (48), the extent of the binding seems to play a minor role in IE induction and in persistence. In parallel to fibronectin binding, no significant difference in endothelial cell binding profiles was noted when the PB and RB cohorts were compared. The fibronectin-binding phenotype and/or expression profiles of genes involved in fibronectin binding may differ substantially between the *in vitro* conditions we tested and *in vivo* infection conditions. In addition, the ability to invade and survive inside endothelial cells might be of more importance for persistence in the host than adhesion. Also, the extent of the proinflammatory response induced by the bacteria might play a role. Studies are ongoing in our laboratory to address these questions.

Last, the extensive literature correlating indwelling endovascular devices, biofilm formation on such devices, and *S. aureus* bacteremia led us to examine the ability of the PB versus RB cohorts to form biofilms *in vitro* (12, 19, 21, 28, 34). Importantly, multiple linear and logistic regression data analysis showed that combining the extent of biofilm formation with reduced susceptibility to HDPs provided a phenotypic mosaic that was significantly correlated with both the PB outcome and increased days of bacteremia. Since biofilm formation restricts the access of many conventional antimicrobial agents (e.g., vancomycin) to sessile bacterial communities (12), it is reasonable to postulate that biofilm also mitigates the exposure of such colonies to HDPs generated at endovascular infection sites. Another interesting finding of our study is the overrepresentation of the *sspA* gene, encoding the V8 protease, in the PB strain cohort (100%) compared with the RB strain cohort (89%). V8 protease has been reported to be required for biofilm dispersal mechanisms, which in turn can play a role in colonizing new sites and favor recalcitrant infections (2).

Different factors, such as the production of PIA/PNAG by *icaADBC* operon-encoded enzymes (7); extracellular DNA (eDNA) (30, 38, 50); and several surface proteins, including Bap, SasG, FnBPs, and Spa (5, 8, 41, 46), have all been shown to be involved in *S. aureus* biofilm formation. In accordance with previous findings (35, 36, 47), we found that biofilm formation in our tested MRSA isolates depended more on the protein content than on the PIA/PNAG content. Biofilms of most of the isolates tested also contained eDNA, a recently described and important *S. aureus* biofilm component (30, 38,

FIG. 2. Biofilm stability assays of 5 PB and 5 RB isolates. Preformed biofilms were treated for 2 h with either sodium metaperiodate (10 mM), proteinase K (100 μ g/ml), or DNase I (140 U/ml), and then

50). Interestingly, our RB isolate biofilm matrices seemed to contain more PIA/PNAG than those of the PB isolates tested. Thus, the biofilm “component type” might be another useful marker for distinguishing PB from RB isolates. Further investigations are ongoing in our laboratory to investigate this notion.

We recognize that there are some important limitations in the present study. First, we assessed a relatively small number of PB-RB strains and phenotypes, affording a substantial chance for statistical bias. We also realize that *in vitro* testing of HDPs in sublethal concentrations much lower than would be encountered *in vivo* may also present limitations. We are currently examining larger PB-RB strain cohorts and additional phenotypic biomarkers to circumvent some of these issues. To complement our *in vitro* findings, *in vivo* studies are ongoing in our laboratories using an experimental IE model and selected isolates from our current MRSA strain cohort. These investigations are designed to address the issues of whether “PB” isolates are intrinsically more virulent than “RB” MRSA isolates or whether PB outcomes are relevant only during antimicrobial therapy. The outcomes from our previous experimental IE studies in other PB-RB strain collections would predict that the latter concept is more valid than the former concept (56).

We recognize that there is no clearly defined days of bacteremia “breakpoint” to distinguish PB and RB outcomes. As in our prior studies of PB and RB outcomes in other strain cohorts, we utilized the ≥ 7 days definition of PB for several reasons. First, our decision to define “persistent bacteremia” as ≥ 7 days is based on well-accepted definitions in the peer-reviewed literature (23, 27, 31, 37). Second, the definition is consistent with our own clinical experiences. The clinical signs and symptoms of patients with bacteremia lasting ≥ 7 days are typically quite different from those of patients with shorter bacteremic durations, even those with complicated infection (e.g., febrile morbidity). Thus, while far from perfect, our definition of PB is well accepted and allows investigators a reasonable yardstick for studying this fascinating clinical entity.

Collectively, our data strongly support our overall hypothesis that PB isolates have specific and combinatorial pathogenic “signatures” that are independent of conventional antimicrobial susceptibility. Importantly, the signatures that are predominant in one PB strain cohort (e.g., fibronectin binding [56]) may not be contributory in others, such as in our current strain set. Overall, defining common and consensus PB signatures among large well-defined strain collections might eventually be useful to identify patients at higher risk for PB to optimize anti-MRSA therapeutic strategies.

the biofilms were quantified (A_{490}) as described in the text. The error bars indicate the standard deviations of the mean A_{490} . * and **, $P < 0.05$ and $P < 0.01$, respectively, for treated biofilms versus untreated control. Representative results of treated biofilms in 96-well plates are shown below bar graphs.

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