Fibronectin binding of persistent versus resolving methicillin-resistant *Staphylococcus aureus* bacteremia isolates

Honors Research Thesis

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Publications

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ABSTRACT

Staphylococcus aureus bacterial infection is a serious problem in medicine. The initial and necessary step in virtually all *S. aureus* infections is binding of a bacterium to surfaces within a host (e.g., tissue or indwelling device). One of the most important binding reactions is between human fibronectin (e.g., presented on endothelial cells or coatings on prosthetic heart values) and fibronectin-binding proteins produced on the surface of *S. aureus*. I propose that *S. aureus* isolates that exhibit a clinically complicated progression of infection will show stronger and more frequent binding to fibronectin-coated surfaces *in vitro*. This study used methicillin-resistant *S. aureus* (MRSA) isolates from two distinct clinical populations: patients whose bacterial infection resolved within 4 days of antibiotic treatment ($n = 7$; resolving bacteremia; RB), and patients who still had positive MRSA blood cultures after ≥7 days of receiving antibiotics ($n = 7$; persistent bacteremia; PB). Fibronectin binding was experimentally measured by using atomic force microscopy (AFM) to probe attractive forces between a fibronectin-coated AFM tip and putative fibronectin-binding receptors on *S. aureus* cells from each of the 14 isolates. The bacterial isolates that originated from patients that had PB showed statistically higher binding frequency ($p = 0.0218$), force of binding ($p < 0.0001$), and energy of binding ($p < 0.0001$) relative to RB isolates. These measurements suggest that binding to fibronectin is associated with increased risk of serious infection by *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is a Gram-positive inhabitant of the nasal passages and skin of humans, and can cause mild to severe infection if it enters the bloodstream (28). *S. aureus* is now recognized as the leading cause of bacteremia and infective endocarditis in the industrialized world (4). A significant number of bloodstream infections are due to methicillin-resistant *S. aureus* (MRSA), which tend to be associated with inferior clinical outcome and longer hospitalization than methicillin-susceptible *S. aureus* (23). Persistent bacteremia (PB) outcomes comprise 20-30% of all episodes of MRSA bacteremia (25). Why some MRSA bacteremia strains persist while others resolve (resolving bacteremia, RB) despite similar clinical and microbiologic characteristics, as well as similar surgical and therapeutic strategies is poorly understood.

One possible explanation is that organisms associated with persistent infections have a greater ability to endure, invade and proliferate at initial infection sites associated with endocarditis (25). Upon entering the bloodstream, *S. aureus* must avoid being cleared by host defense mechanisms. In addition to persisting in the bloodstream, the organism must adhere to damaged cardiac valve endothelium to initiate infective endocarditis. In order to persistently infect such sites, *S. aureus* must subsequently invade the cardiac endothelial cells (i.e., endocardium).

Here, I investigate the initial step in infective endocarditis, that is, adherence of *S. aureus* to a surface. Endothelial and endocardium cells present a number of ligand molecules to which *S. aureus* can bind. Fibronectin (Fn) is one of the most important host ligands as *S. aureus* express two important Fn-binding proteins (FnBPA and FnBPB) that help this organism adhere to surfaces (22). Animal studies have demonstrated that native valve endocarditis is associated

with secretion of fibronectin at the point of injury of endothelium in aortic valves (5). Similarly, *in vivo* studies of humans demonstrate that foreign materials (e.g., intravenous catheter, central venous catheter, ventricular assist device) become coated with plasma proteins like fibronectin, and *S. aureus* use primarily FnBPs for attachment and initial colonization of an implanted devices (1, 10, 26, 27). Other studies show that *S. aureus* use FnBP to bind to other host molecules such as human heat shock protein 60 as well as β_1 integrins through a fibronectin bridge (6).

In this study, atomic force microscopy (AFM) is used to directly probe the binding mechanism between a fibronectin-coated substrate and putative FnBPs expressed on the outer surface of individual *S. aureus* cells immersed in buffer solution. These experiments were conducted with 14 different isolates of *S. aureus* obtained from patients with persistent (n = 7) or resolving bacteremia (n = 7). I determined the activity or frequency of binding, the maximum binding force (measured in nanoNewtons), and the work or energy of binding (measured in attoJoules) for each of the 14 isolates. These data were analyzed to determine whether there were differences in fibronectin binding between the PB and RB groups. I also compared the AFM data by grouping the 14 isolates into their respective clonal complexes, as determined by multi-locus sequence typing (7).

By determining how known invasive and non-invasive isolates of *S. aureus* (i.e. PB vs. RB) bind to fibronectin-coated surfaces, a method may be developed to identify isolates that are likely to be pathogenic. Patients who naturally harbor such "invasive" isolates may be forewarned of the risks associated with certain future surgical procedures (e.g. heart valve replacement) and receive prophylactic antibiotic treatment before surgery.

MATERIALS AND METHODS

Bacteria specimens and growth conditions.

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (n = 14) were kindly provided by Dr. Arnold Bayer from the University of California at Los Angeles Medical Center. All MRSA isolates used in this study, originated from a multinational *S. aureus* bacteremia clinical trial collection conducted between 2002 and 2005 (8). MRSA isolates came from patients who were well matched for epidemiologic, demographic, and clinical features and were grouped into persistent bacteremia (PB) and resolving bacteremia (RB) cohorts based on clinical blood culture data, as described below (25). All 14 patients (where MRSA isolates were obtained from) used in the present study had complicated MRSA bacteremia and/or infective endocarditis, and all received either daptomycin mono- therapy or dual-drug standard therapy consisting of vancomycin plus low-dose, short-course aminoglycosides (25).

Study isolates were grouped according to clinical blood culture data: PB (n = 7) was defined as ≥7 days of positive MRSA blood culture despite receiving antibiotics to which the isolate was susceptible; RB (n = 7) was defined as an initial positive MRSA blood culture with subsequent negative blood cultures 2 to 4 days after initiation of antibiotic therapy.

Genetic profiling using PCR was used to assign isolates to multilocus sequence typing (MLST)-defined clonal complexes (CCs). MLST has been used as a definite method for assigning MRSA and MSSA isolates to known clonal complex groups or to assign them as novel groups (7). This method of grouping *S. aureus* has been validated by showing that pairs of isolates with identical allelic profiles produced similar *SmaI* restriction fragment patterns by pulsed-field gel electrophoresis (7). CC5 (n = 5) and CC45 (n = 5) are represented among 10 of the MRSA isolates, where the other 4 isolates are either CC1 or CC8.

The *S. aureus* isolates used in atomic force microscopy (AFM) experiments were grown from cryogenically preserved samples. The *S. aureus* isolates were incubated at 37 °C in tryptic soy broth supplemented with 0.25% dextrose. *S. aureus* has shown expression of microbial surface components (such as FnBPA and FnBPB) recognizing adhesive matrix molecules when cultured under these conditions (16, 29).

Fibronectin binding as determined by atomic force microscopy (AFM)

Each isolate was harvested at mid-exponential phase (OD_{600} = 0.51 ± 0.02). One mL of cell suspension was centrifuged at 5000 x*g* for 3 min. The resulting pellet of cells was washed and resuspended in saline buffer three times. After washing, the cells were suspended in phosphate-buffered saline (PBS). One-hundred µL of the washed cell suspension was transferred onto fibronectin-coated slides (BD Biosciences) and allowed to adhere for 5 min. Unbound cells then were washed off by a gently flowing solution of ~10 mL of PBS solution.

Force measurements of *S. aureus* were collected using silicon nitride AFM cantilevers (nominal radius 20 nm, measured spring constant 0.1 ± 0.06 nN nm^{-1}). The cantilevers were cleaned by sequential washing with approximately 10 mL acetone, 10 mL 70% ethanol, and 10 mL distilled water. The cantilevers were coated with fibronectin by immersing in a 100 µg/mL fibronectin (Sigma-Aldrich) PBS solution for 45 min and rinsed with PBS solution (2).

Force measurements were performed in a PBS solution using an atomic force microscope (Veeco/Digital Instruments Bioscope AFM and NanoSCOPE IV controller) as previously described (29). Briefly, an inverted optical microscope (Axiovert 200M; Zeiss) was used to position a fibronectin-coated cantilever tip over an individual *S. aureus* cell or binary fission pair within the AFM (Figure 1). Forces were measured on cells immersed in PBS solution (pH 7.4) as previously described (20, 21). The tip was pressed against a cell until the cantilever was

flexed 100 nm. A single approach–retraction cycle took 1–2 s. The vertical travel of the z-piezoelectric scanner was 2.7 µm. Data acquisition was confined to a narrow window after harvesting (<30 min) to minimize data collection on quiescent bacteria.

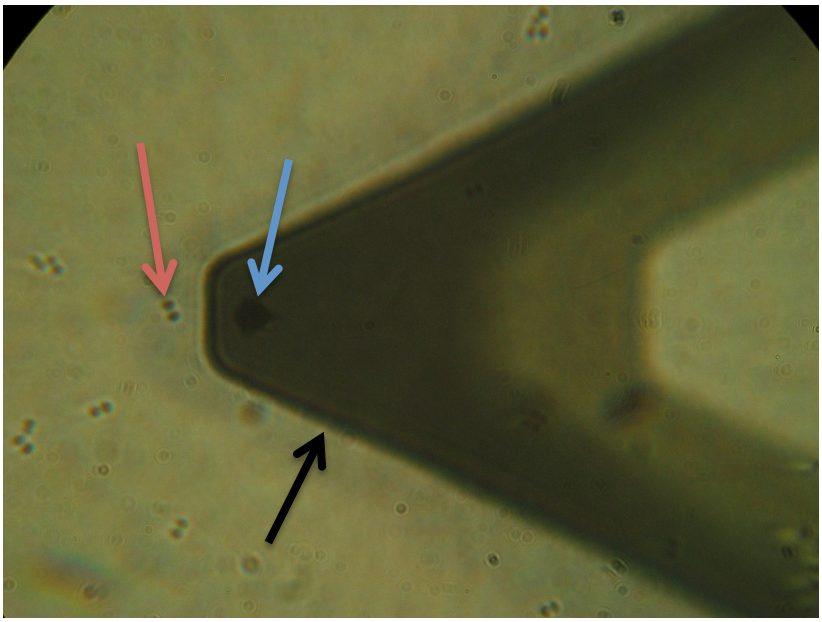


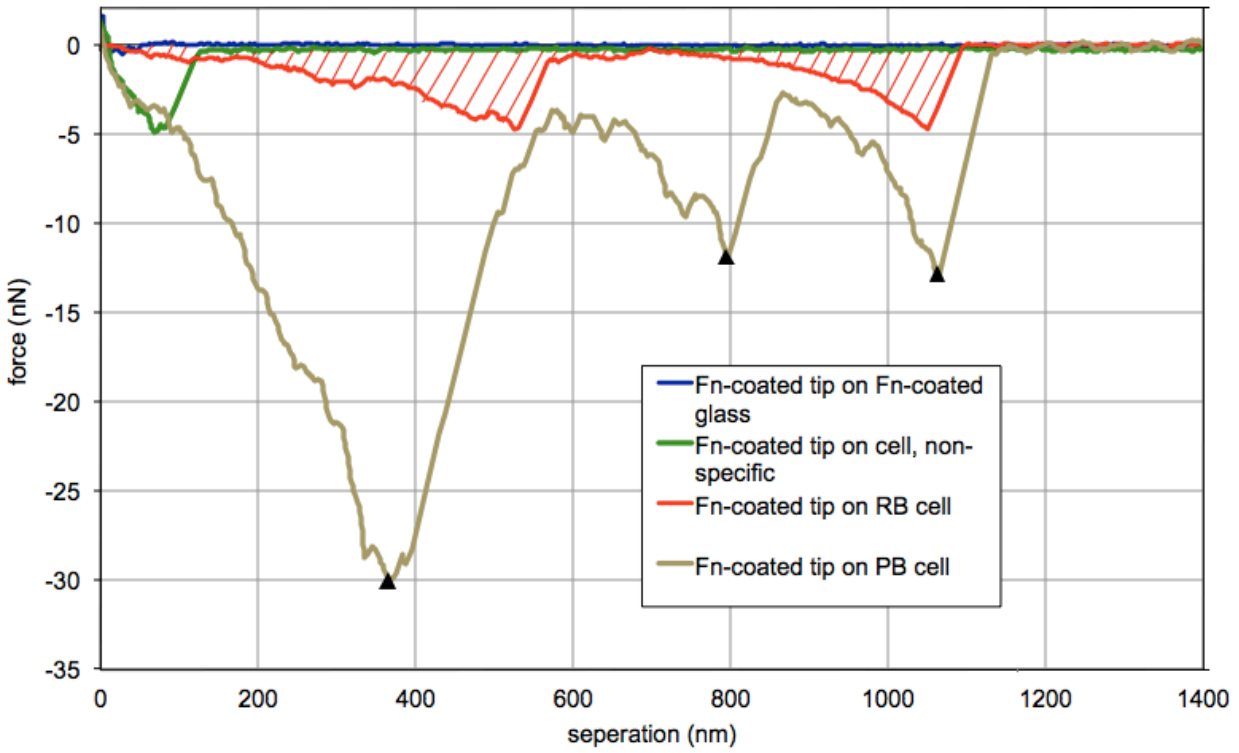
Figure 1. Binary fission pair of *S. aureus* cells (red arrow) about to be probed by a fibronectin-coated tip (blue arrow). The black arrow indicates the cantilever. The cells are supported on a fibronectin-coated glass slide. Image collected at 100X using a Zeiss confocal microscope.

RESULTS

Collection of data

AFM was performed with fibronectin coated AFM tips on *S. aureus* cells. Tips were positioned over individual cells or binary cell pairs (see Fig. 1). Fn-coated tips were pushed against and retracted from individual cells at a rate of 0.996 Hz, that is, approximately one approach-retraction cycle per second. This method allows one to directly measure the force of interaction between Fn on the AFM tip (i.e., proxy for a host cell displaying Fn or Fn-coated indwelling device) and Fn-binding proteins on *S. aureus*.

Figure 2 presents typical force retraction curves. The red-colored curve in Figure 2 shows the interaction when the fibronectin-coated tip was pulled from contact with one of the PB *S. aureus* cells. As the tip was retracted to ~500 nm there was one unbinding event followed by a second event at a separation of approximately 1000 nm. Both binding events exhibit a non-linear, force-distance response. These types of binding (or unbinding) events were observed for all 14 of the isolates used in this study. An average of 16.5 different cells were analyzed for each of the 14 isolates (range of 7 to 28 cells per isolate) (Table 1).



▲ indicates for maximum force of adhesion
/// shading indicates the area above the curve (energy)

Figure 2. Retraction force profiles collected for a fibronectin-coated tip on a glass substrate (blue) or *S. aureus* cells classified as persistent (PB; gold) or resolving (RB; red) bacteremia. The approach curves are not shown in this figure for clarity. Attractive forces (nanoNewtons; 10^{-9} N) are given a negative sign by convention. Triangles highlight force values used in data analysis. The red-colored region represents the energy (in attoJoules) of interaction.

Table 1. List of *S. aureus* isolates analyzed by AFM. PB isolates are represented in bold text, RB isolates are denoted with normal font. The clonal complex (CC) of each isolate is shown in parentheses. Shown is the total number of different cells analyzed for each isolate with AFM, the total number of binding curves collected for each isolate, and the number of force curves that exhibited an attractive interaction (including both non-specific and specific interactions).

isolate	*# of cells analyzed*	*total # of force curves*	*# with attractive interaction*
300-169 (45)	10	614	310
324-136 (45)	25	1510	1091
301-188 (45)	18	1002	428
300-087 (45)	9	891	33
300-103 (45)	7	497	52
300-246 (5)	8	549	521
088-180 (5)	9	694	55
077-107 (5)	24	1134	789
088-237 (5)	19	1119	853
010-016 (5)	8	598	162
031-038 (8)	24	1196	1040
300-111 (8)	22	1246	1046
027-017 (8)	24	1010	598
067-227 (1)	28	1691	541

Control experiments were performed to ensure that the unbinding events, mentioned above, were due to interactions that formed between fibronectin on the tip and fibronectin-binding molecules on *S. aureus*. The blue-colored curve in Figure 2 shows the interaction between a fibronectin-coated tip and the fibronectin-slides, which were used to support the *S. aureus* cells. As is clearly illustrated, there was a distinct difference in the force spectra for the Fn-Fn control vs. the fibronectin-cell experiments. This difference suggests that binding events observed on *S. aureus* (e.g., red and gold curves in Fig. 2) are due to fibronectin binding to Fn-binding receptors, FnBPA and/or FnBPB, on *S. aureus*. The control experiment (i.e., probing the Fn-tip against the Fn-coated slide) was also performed between analyses of different samples to ensure that the tip was free of contamination (e.g., ensure that cells were not sticking to the AFM tip).

Because I was interested in identifying binding events associated with FnBPs on *S. aureus*, I attempted to identify a binding-signature specific to protein interactions. Force profiles on *S. aureus* resulted in both linear and non-linear force-distance responses (see Fig. 2). By working with strains of *Lactococcus lactis* that produce only FnBP on their outer surface, others have demonstrated that non-linear force profiles indicate specific interactions involving fibronectin and/or FnBP (2). For the force curve analyses, I focused only on those nonlinear unbinding events that had a force greater than 0.5 nanoNewtons and a separation greater than 200 nm.

Frequency of binding to fibronectin

A protein-mediated binding event was defined as a retraction curve that exhibited a non-linear, sawtooth force-distance profile (Figure 2). Some binding profiles displayed more than one sawtooth waveform (two sawteeth in a single force-distance profile) but were counted as a single binding event as previously described (2). Binding frequencies were calculated by dividing all the non-linear interactions by the total number of curves collected on cells for the isolate being analyzed (Table 1 and 2). Figure 3 represents binding frequencies for all isolates when comparing CC45 (n = 5) to CC5 (n = 5) and PB (n = 7) to RB (n = 7).

Table 2. List of the binding frequency, maximum binding force (nanoNewton; 10^{-9} N), and work of adhesion (attoJoules; 10^{-18} J) for each of the 14 isolates. PB isolates are represented in bold text, RB isolates are denoted with normal font. The clonal complex (CC) of each isolate is shown in parentheses. The standard deviations for maximum force values and energy values are indicated after the value.

Isolate[a]	*binding freq.*[b]	*max. force (nN)*	*energy (aJ)*
300-169 (45)	50.5	2.69 ± 1.43	46.4 ± 31.9
324-136 (45)	72.3	2.69 ± 1.42	80.9 ± 40.6
301-188 (45)	42.7	5.95 ± 3.92	337 ± 341
300-087 (45)	3.7	1.01 ± 0.723	11.8 ± 10.2
300-103 (45)	10.5	0.978 ± 0.883	14.0 ± 20.3
300-246 (5)	94.9	4.69 ± 2.98	472 ± 248
088-180 (5)	7.9	3.73 ± 2.83	147 ± 140
077-107 (5)	69.6	14.1 ± 8.93	1037 ± 997
088-237 (5)	76.2	7.59 ± 4.33	516 ± 408
010-016 (5)	27.1	0.981 ± 0.434	15.9 ± 10.3
031-038 (8)	87.0	13.6 ± 6.00	976 ± 568
300-111 (8)	83.9	13.5 ± 9.06	666 ± 511
027-017 (8)	59.2	3.32 ± 1.74	120 ± 85.4
067-227 (1)	32.0	4.99 ± 2.63	244 ± 222

[a] Clonal complex follows isolate number in parentheses
[b] Binding frequency is given as %

Comparison of PB and RB groups using Student's *t*-test shows statistically significant differences between the PB and RB isolate groups ($p < 0.05$; Table 3). In addition, CC45 isolates have a median binding frequency of 0.40 whereas isolates described by CC5 have a median binding frequency of 0.67. CC45 and CC5 were not quite significantly different ($p = 0.0682$) upon comparison of their binding frequencies. However, all five CC45 isolates were shown to be statistically significantly different from CC8/1 ($p = 0.0124$).

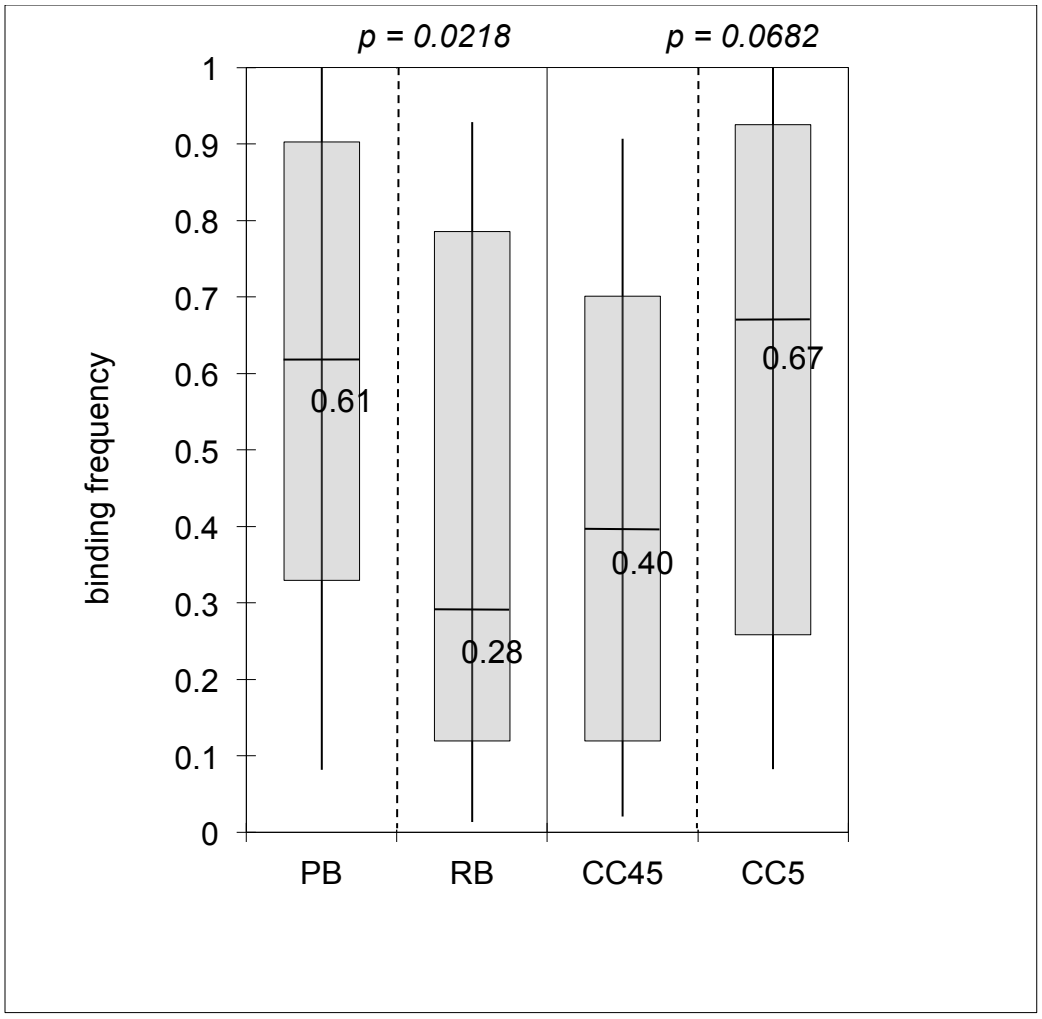


Figure 3. Box plots showing the frequency of binding of *S. aureus* to a fibronectin-coated surface. The upper portion each box represent the 75th percentile and the lower portion represent the 25th percentile. The upper part of each line represents the 91st percentile and the lower portion represents the 9th percentile. The median is represented by a horizontal line and numerically below the line. *P*-values represented the comparison of the (i) PB vs. RB and (ii) CC45 vs. CC5 groupings.

Table 3. *P* values for analysis of force curve data. The *p* values were calculated using an unpaired Students t-test. Each comparison is between entire groups of clinical isolates (i.e. PB vs. RB).

groups compared	*binding freq*	*max. force*	*energy*
CC5 vs. CC45	$p = 0.0682$	$p = 0.0001^{***}$	$p = 0.0001^{***}$
CC45 vs. CC8/1	$p = 0.0124^{*}$	$p = 0.0001^{***}$	$p = 0.0001^{***}$
CC 5 vs. CC8/1	$p = 0.6714$	$p = 0.0007^{***}$	$p = 0.1016$
PB vs. RB	$p = 0.0218^{*}$	$p = 0.0001^{***}$	$p = 0.0001^{***}$

*P <0.05 and ***P <0.001

Maximum binding force toward fibronectin

Only force-distance profiles that exhibited a sawtooth curve (without other non-specific interactions) were analyzed for maximal force and energy values using SPIP™ software. These types of profiles were detected in 9% of the collected curves. Multiple force maximums were assigned to profiles that had more than one sawtooth (Fig. 2). Figure 4 are box-and-whisker plots of the force maximal values for CC45, CC5, PB, and RB groupings of isolates. Using Student's t-test, the PB group was shown to be statistically different from the RB group ($p <$ 0.0001). In addition, the CC45 group was shown to be statistically different the CC5 group ($p <$ 0.0001). Comparison of other CC groups is described in Table 3.

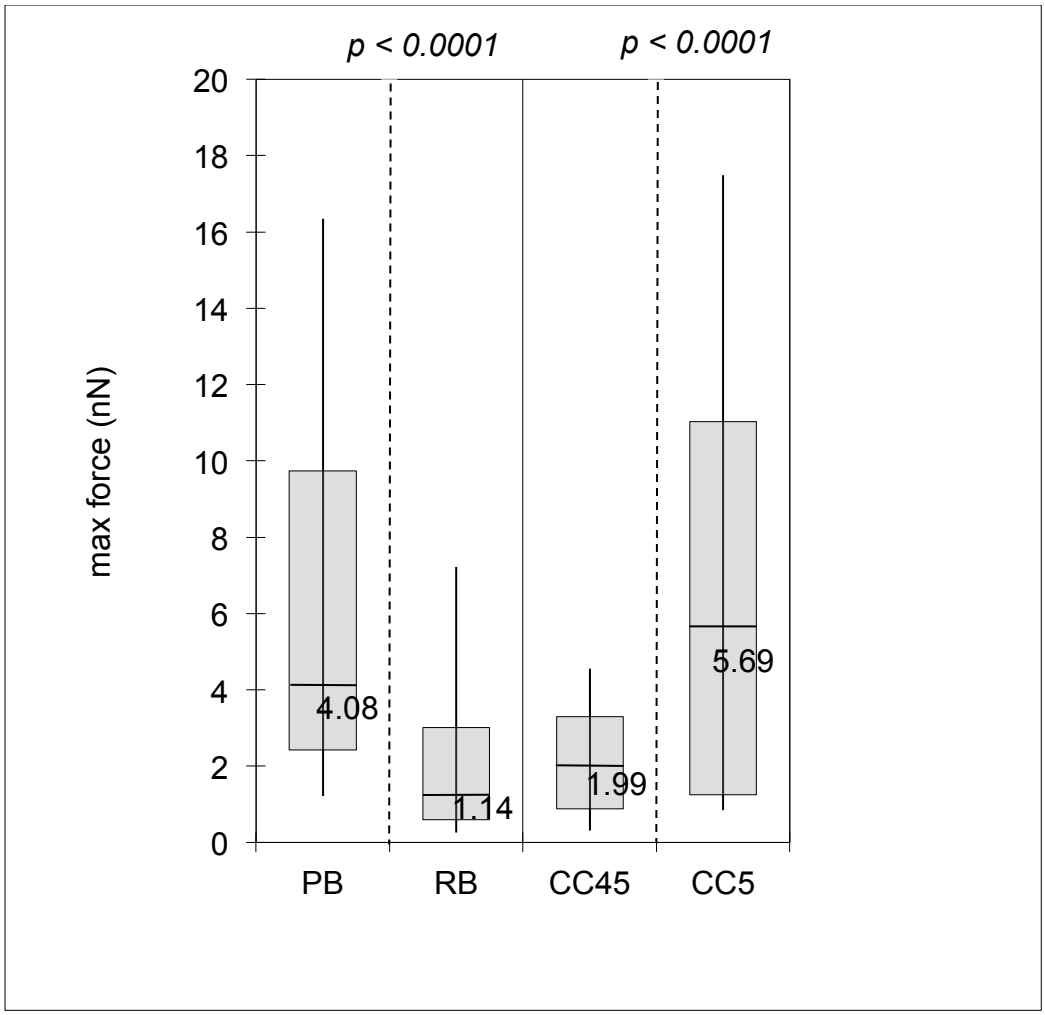


Figure 4. Box plots showing the maximum binding force for *S. aureus* to a fibronectin-coated surface. The upper portion each box represent the 75th percentile and the lower portion represent the 25th percentile. The upper part of each line represents the 91st percentile and the lower portion represents the 9th percentile. The median is represented by a horizontal line and numerically below the line. *P*-values represented the comparison of the (i) PB vs. RB and (ii) CC45 vs. CC5 groupings.

Work or energy of binding to fibronectin

The maximum force value is a useful measure of adhesion but it is only one point on a retraction curve. Therefore, I also calculated the energy of adhesion by integrating force with respect distance (see red-shaded region in Fig. 2). This integration protocol was carried out on approximately 5% of the 7,519 force-distance profiles analyzed that exhibited a binding event. As with the maximum force of adhesion, only those profiles exhibiting distinct sawtooth

curvature were included in this analysis. The PB group had a median energy/work of binding of 107 aJ. This value was found to be statistically different from the RB group (average energy= 14 aJ) ($p < 0.0001$). In addition, the CC45 group, with a median energy of 28 aJ, was found to be statistically different from the CC5 group, which had an average energy of 126 aJ, as described in Figure 5 ($p < 0.0001$).

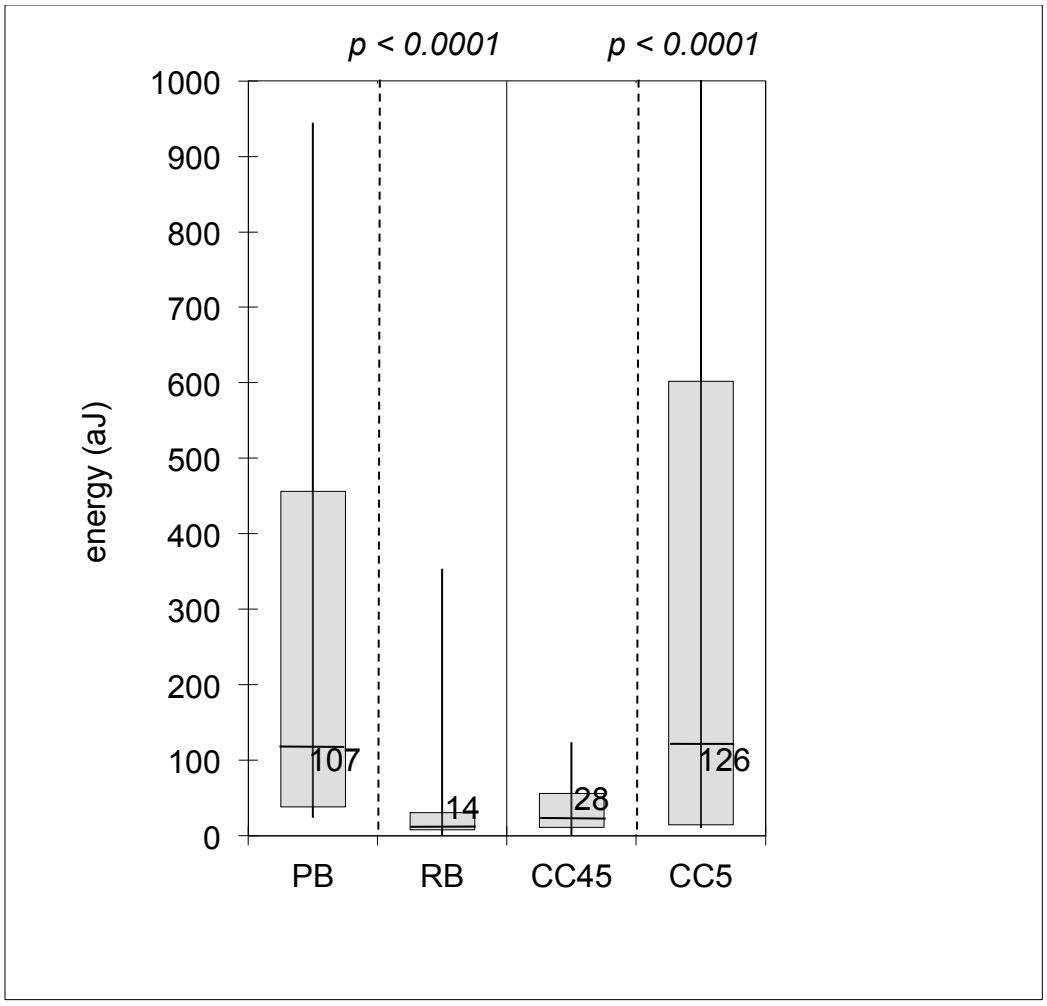


Figure 5. Box plots showing the binding energy (attoJoules; 10^{-18} J) for *S. aureus* to a fibronectin-coated surface. The upper portion each box represent the 75th percentile and the lower portion represent the 25th percentile. The upper part of each line represents the 91st percentile and the lower portion represents the 9th percentile. The median is represented by a horizontal line and numerically below the line. *P*-values represented the comparison of the (i) PB vs. RB and (ii) CC45 vs. CC5 groupings.

DISCUSSION

Recently it has been shown that community-associated MRSA strains (a distinct group from hospital-associated MRSA strains) are increasingly more prevalent in hospitals (8). Thus, describing the infectivity elements of such MRSA bacteria is of continuing importance. Many parameters have been used to describe or classify clinically relevant *S. aureus* isolates, and in particular, MRSA isolates. For instance, genetic descriptions such as CCs or clinical descriptions such as PB vs. RB are both used to classify *S. aureus* isolates.

Fourteen clinical MRSA isolates, originating from a multinational *S. aureus* bacteremia clinical trial collection, were analyzed using AFM with a fibronectin-baited tip. The resulting force-profiles were analyzed for frequency of binding (percentage), maximal force of binding (nN), and energy of binding (aJ).

The clinical comparison of persistent bacteria (PB) to resident bacteria (RB) is based on the *S. aureus* isolate's ability to establish bacteremia. This classification system is based strictly on clinical observation of patients infected with MRSA. The statistical analysis of the AFM data show that PB isolates bind more frequently, with stronger binding force, and greater binding energy then the RB isolates (Table 3). These results are consistent with the clinical outcome. PB isolates are able to establish positive blood cultures after >7 days of therapy..

There is also evidence that relates CC to the clinical patient outcome. Isolates of CC5 and CC30 have been found associated with increased levels of hematogenous complications (9). However, for the AFM data, only CC5 and CC8/1 groups were shown to be statistically different under the binding frequency parameter. All other comparisons under other parameters were shown to be significantly different (Table 3). It appears that CC5 and 8 are capable of stronger binding forces and increased binding energies. However, none of these clonal complexes have

been shown to be more virulent except CC1 and CC5 (25). In addition, although both CC groupings and clinical (PR/RB) groupings individually show differences among isolates, no CC group clearly fell into clinical PB or RB groups. The clinical PB group contained 2 CC45, 3 CC5, and 2 CC8 isolates whereas the clinical RB group contained 3 CC45, 2 CC5, 1 CC8, and 1 CC1 isolate. Classifying isolates based on specific genetic markers has advantages such as ease of identifying a *S. aureus* isolates obtained from a patient as particularly invasive. However, CC does not seem to correlate to the specific invasiveness of *S. aureus* isolates obtained from clinical patients. In addition, it must be remembered that whatever the classification used for *S. aureus,* binding to fibronectin is the first step for a successful infection.

In addition, the classification of MRSA clinically as a PB or RB isolate seems sufficient. Not only is there a clear difference between these two groups of isolates, with PB isolates showing a specifically increased binding frequency, energy of binding, and maximal force of binding, but there also is an established clinical difference between the two isolate groupings as described (25).

In conclusion, this study helps to establish key differences between PB and RB isolates. These differences validate the use of the described PB/ RB clinical classification system used to describe and treat infections caused by *S. aureus.* As some infections with methicillin-resistant *S. aureus* (MRSA) have been shown to be fatal and others to cause necrotizing fasciitis, necrotizing pneumonia, purpura fulminans, pyomyositis and sepsis, validation of such a clinical classification system is important (11).

While this study demonstrates a correlation between clinical outcome (PB vs. RB) and binding of *S. aureus* to fibronectin-substrates, it does not provide a possible explanation for the observed difference. There are at least three possible explanations. One explanation is that the

PB isolates could produce more FnBPs then the RB isolates. Another explanation could be that the PB isolates produce additional receptors that bind to fibronectin. A third explanation could be that there are differences in the amino acid sequence of FnBPs from the PB vs. RB isolates. A recent study suggests that this last explanation may be the most plausible (15). However, future studies will be necessary to explore these issues.

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