Correlation between Fundamental Binding Forces and Clinical Prognosis of *Staphylococcus aureus* Infections of Medical Implants

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Atomic force microscopy was used to "fish" for binding reactions between a fibronectin-coated probe (i.e., substrate simulating an implant device) and each of 15 different isolates of *Staphylococcus aureus* obtained from either patients with an infected cardiac prosthesis (invasive group) or healthy human subjects (control group). There is a strong distinction ($p = 0.01$) in the binding-force signature observed for the invasive versus control populations. This observation suggests that a microorganism's "force taxonomy" may provide a fundamental and practical indicator of the pathogen-related risk that infections pose to patients with implanted medical devices.

1. Introduction

Surgical implants (e.g., prosthetic heart valves or pacemakers) significantly improve the quality of life for many humans but paradoxically place these same patients at risk for life-threatening infection by bacteria. In the United States, for example, about half of the 2 million annual cases of nosocomial infection are associated with indwelling devices.[1] *Staphylococcus aureus* is one of the most frequently isolated microorganisms from infected medical implants.[1−3]

S. aureus is a common commensal microorganism of humans typically found in the anterior nares.[4−6] If it enters the bloodstream, *S. aureus* can be one of the most lethal human pathogens.[7] In the case of indwelling devices, *S. aureus* may adhere to the surface of an implant where it forms a biofilm that is difficult to combat with host defenses or antibiotics.[8−11]

S. aureus colonizes the surface of an implant by forming bonds with host ligands, such as fibronectin (Fn), which commonly coat a prosthetic device.[12,13] This type of bond is mediated by Fn-binding protein (FnBP), one of several microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) located on the cell wall of *S. aureus*.[12,14−20]

One way to prevent *S. aureus*-related device infections is to prohibit the initial binding reaction between an MSCRAMM on a bacterium and the surface of an implant. To this end, a few groups have used atomic force microscopy (AFM) or optical tweezers to probe the fundamental binding forces associated with a few type-strains or laboratory-derived strains of *S. aureus* (e.g., see refs 21−24). To the best of our knowledge, the work presented herein represents the first time that force measurements have been applied to a relatively large collection of *S. aureus* isolated from a real-world, clinical setting.

We used AFM to measure forces between an Fn-coated probe and each of 15 different clinical isolates of *S. aureus*, which were obtained from either patients with an infected device ($n = 7$) or healthy subjects ($n = 8$). A unique sawtooth-shaped force signature was observed when putative FnBPs on *S. aureus* formed a specific bond with the Fn-coated substrate. When grouped by the frequency of this force signature, the invasive and control isolates of *S. aureus* formed two statistically distinct populations. These results suggest that the activity or mechanism of binding may differ for different populations of *S. aureus*. Further, these results suggest a novel way to identify potentially harmful bacteria in clinical settings through the measurement of an intrinsic force attribute that goes to the heart of *S. aureus* biofilms on implanted medical devices.

2. Materials and Methods

2.1. Collection of Clinical *S. aureus* Isolates. Methicillin-susceptible *S. aureus* isolates were collected at Duke University Medical Center from subjects who were not injection drug users.

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Invasive isolates ($n = 7$) were obtained from the bloodstream of patients with confirmed infection of a cardiac prosthesis (e.g., permanent pacemaker, implantable cardioverter defibrillator, or prosthetic cardiac valve). The control isolates ($n = 8$) were isolated from the anterior nares of asymptomatic colonized subjects. To account for isolate variation, the control isolates were obtained from healthy individuals in the same geographic area as the disease-causing isolates.

2.2. Characterization and Growth of Clinical *S. aureus* Isolates. *S. aureus* speciation was confirmed by subculturing on sheep blood agar, Gram staining, and performing the Staphaurex test (Murex Diagnostics, Norcross, GA), a latex agglutination assay that detects *S. aureus*-specific proteins. Isolates were stored using the Protect Bacterial Preservers system (Key Scientific, Round Rock, TX) immediately after they were confirmed to be *S. aureus*. This preservative system uses a sterile vial containing chemically treated porous beads in a cryopreservative fluid consisting of tryptic soy broth (TSB) and glycerol with a hypertonic additive. The porous beads were inoculated with an isolate, briefly exposed to the cryopreservative, and then stored at −80 °C until culturing for AFM analysis (see below).

A polymerase chain reaction was used according to Peacock et al.[25] to provide independent confirmation of the presence of the gene coding for FnBP A (*fnbA*; accession number J04151) in all *S. aureus* isolates used in this study. Western blot analysis,[26] performed on two of the control isolates and two of the invasive isolates, confirmed that FnBP was localized to the cell wall of the *S. aureus*.

All growth cultures for AFM analysis were started from cryogenically preserved samples of the clinical isolates. Each isolate was cultured to early exponential stage ($OD_{600} = 0.51 \pm 0.01$; $OD_{550} = 0.54 \pm 0.01$) at 37 °C in TSB containing 0.25% dextrose.[27] Under such conditions, *S. aureus* is known to express MSCRAMMs such as FnBP.[8]

Approximately 1 mL of cell suspension was harvested using a centrifuge (5000*g* for 3 min). Cells were then washed three times in saline solution (∼0.1 M NaCl). A small volume of washed cells was dropped onto a glass cover slip and allowed to sit (without drying) for 5 min. Loose cells were washed off with phosphate-buffered saline (PBS; 0.01 M containing 0.85% NaCl at pH 7.2), which was also the solution used in the AFM experiments. This sample preparation may inadvertently select for a subpopulation of each isolate that "firmly" attaches to the glass cover slips. However, any potential bias is the same for all *S. aureus* isolates analyzed with AFM.

2.3. Force Measurements with the Atomic Force Microscope (AFM). An Fn-coated probe was used as a proxy for an implanted medical device. Silicon nitride AFM cantilevers were cleaned in piranha solution,[28] rinsed with MilliQ water (18.2 MΩ cm), immersed in a 100 μg/mL Fn (Sigma-Aldrich) PBS solution for 45 min, and then rinsed four times in PBS. A total of four Fn-coated probes were used in these experiments. The same tip was used across as many cell isolates as possible with intermittent testing to detect tip degradation (e.g., probing a clean glass slide with the Fn-coated tip).

Force measurements were performed with an AFM (Veeco/Digital Instruments Bioscope AFM and NanoScope IV controller) as described in the work of Yongsunthon and Lower.[23] Briefly, an inverted optical microscope (Axiovert 200M, Zeiss) was used to position an Fn-coated probe (nominal tip radius 20 nm; spring constant 0.02 nN nm^{-1}) over a single bacterium that was isolated on the coverslip, part of a binary fission pair, or part of a small cluster of 4−8 cells. The probe was brought into contact with a bacterium, pushed against the cell wall until the cantilever flexed 100 nm, and then pulled away from the bacterium. For two of the isolates (141 and 399), the relative trigger was also set to 50 nm. There was no statistically significant difference in the force curves for a relative trigger of 50 nm versus that of 100 nm. The vertical travel distance of the *z*-piezoelectric scanner was 2.7 μm. A single approach−retraction cycle took 1 s (i.e., 1 Hz scan rate).

Force measurements commenced within 33 ± 6 minutes of the initial harvesting of a particular isolate (i.e., when a cell culture reached early exponential stage). To mitigate the possible effects of cell senescence, we confined the data acquisition to a time window of a just over 1 h (75 ± 18 min) of the initial harvesting of cells. The general viability of cells was confirmed after force measurements by replacing the PBS medium with TSB and observing subsequent division of cells across a glass cover slip.

Table 1. Description of *Staphylococcus aureus* Isolates and AFM Experiments

ID		*fnbA*[a]	# cultures[b]	# cells[c]	# spectra[d]
control isolates	C52	+	1	6	1033
	C53	+	2	12	1886
	C55	+	1	3	730
	C57	+	1	5	1042
	C58	+	1	4	793
	C59	+	1	4	668
	C67	+	1	3	446
	C68	+	1	3	881
invasive isolates	141	+	2	5	746
	221	+	2	7	1434
	306	+	1	6	901
	386	+	2	9	1396
	399	+	2	8	1744
	1066	+	1	2	541
	1790	+	1	3	865

[a] Presence (+) or absence (-) of the *fnbA* gene as determined by PCR analysis. [b] Number of growth cultures that were used in the AFM experiments. [c] Number of different bacteria on a cover slip that were probed with an Fn-coated tip (radius ∼20 nm). In most instances, the tip was positioned over on a single isolated cell or a pair of *S. aureus* cells. In some instances, a small cluster of 4−8 cells was observed in the vicinity of the AFM tip.[d] A total of 15106 force spectra (retraction curves) were analyzed for the presence or absence of a sawtooth-shaped force signature.

3. Results and Discussion

3.1. Using the AFM to "Fish" for Binding Reactions. The AFM was, in essence, used to "fish" for binding reactions between a probe that was baited with Fn, and putative FnBPs on *S. aureus*. Table 1 shows the number of bacterial cells for each isolate that were analyzed with the AFM, and the number of force curves collected for each of the eight control isolates and seven invasive isolates of *S. aureus*. A total of 7479 force profiles were analyzed for 40 different cells from the control population, and 7627 force profiles were analyzed for 40 different cells from the invasive population (see Table 1).

The approach curves (not shown) for all isolates exhibited repulsive forces consistent with electrostatic and/or steric forces between *S. aureus* and an Fn-coated substrate (see, for example, ref 23). The retraction curves, on the other hand, often showed a strong attractive force as an Fn-coated probe was pulled from contact with a bacterium's cell wall. This attraction manifested itself as one or more discrete sawtooth-shaped force signatures in the retraction profiles (Figure 1). The invasive isolates tended to yield multiple sawteeth in a single force trajectory, whereas the control isolates tended to exhibit only a single sawtooth binding event in a retraction curve (see Figure 1).

The sawtooth's nonlinear force−distance profile is distinctly different from the generalized, nonspecific adhesion that is often observed when a tip is pulled from contact with a surface (see Supporting Information). The trajectory of the sawtooth has been

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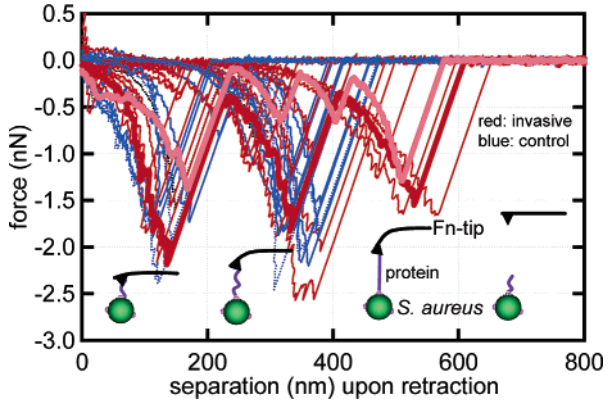

Figure 1. Retraction force profiles (i.e., force spectra) collected as an Fn-coated probe was pulled from contact with the cell wall of *S. aureus*. Shown are randomly selected curves from the eight control isolates (blue) and seven invasive isolates (red). The light red curve was collected on a mutant strain of *S. aureus* that overproduces FnBP on its cell wall. The phenotype of this mutant strain is described in Greene et al.[12]

shown to reflect a profile consistent with the unfolding of a protein and suggests a specific binding event.[29] For the measurements shown in Figure 1, this nonlinear force signature reflects a specific binding event between Fn on a substrate (i.e., the probe) and putative FnBPs on *S. aureus*. Indeed, these force signatures were noted in retraction profiles collected on a mutant strain of *S. aureus* that overproduces FnBP (see light red curve in Figure 1).

For each of the 80 cells that were probed with an Fn tip, the frequency of observing a specific binding event was determined by counting the number of curves that exhibited the sawtooth force signature and normalizing by the total number of curves for that cell. As noted above, the retraction curves for invasive isolates tended to yield multiple sawteeth in a single profile. Such curves that exhibited two or more sawteeth were only counted as a single event to determine the occurrence of specific binding between the substrate and a cell (see Supporting Information). We then grouped the results for all cells from a given isolate (Table 1) to determine an average frequency of specific binding for each of the clinical isolates of *S. aureus* (Figure 2A).

While parameters such as loading force or contact area may have an impact on binding forces, the specific-binding frequencies for all 15 isolates are normalized relative to one another, by virtue of the consistent methodology and materials used throughout the experiment. Relative to the control isolates, the invasive isolates exhibited higher frequencies of specific binding to the Fn-coated substrate (see Figure 2A).

3.2. Correlation between Fundamental Binding Force and Population of *S. aureus*. Figure 2A shows the incidence of specific binding for each of the 15 isolates. However, we are more interested in comparing force spectra for populations of the invasive versus control *S. aureus*. Figure 2B shows the distribution of observing a specific binding event for *S. aureus* isolated from either the control or invasive populations. The mean values for specific binding (i.e., the presence of a sawtooth force signature) are 0.57 ± 0.05 and 0.29 ± 0.05 for the invasive and control populations, respectively. A Student's t-test shows that these two means are statistically different at the 99% confidence level ($t_{calculated} = 4.16$ relative to $t_{critical} = 3.01$ for $p = 0.01$).

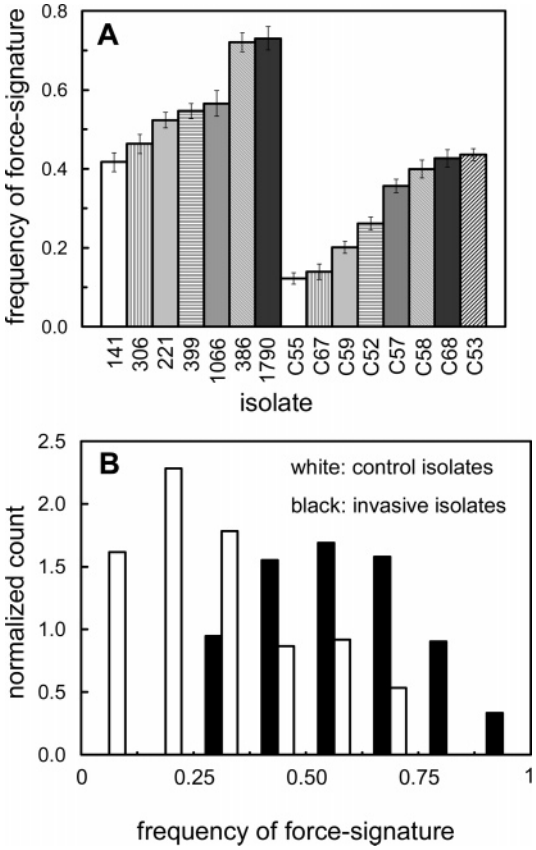

Figure 2. (A) Average incidence of observing a specific binding event (i.e., sawtooth force signature) for each isolate. Error bars represent Poisson uncertainty. The invasive isolates are labeled with a numeric identification. Control isolates are labeled with an alphanumeric identification beginning with the letter "C". (B) Distributions of specific binding events for the invasive (solid black) vs control (open white) populations. The distributions include force spectra for every cell probed and are normalized so that each of the 15 *S. aureus* isolates contributes a count of one. For example, summing the "normalized count" of each bar in the control distribution equals a value of 8. This corresponds to the number of isolates that were analyzed from the control group.

To avoid bias, the pooled data shown in Figure 2B were normalized such that each invasive isolate ($n = 7$) and each control isolate ($n = 8$) contributed a single value to their respective population distribution. For example, force spectra were collected on six different cells of isolate C52 (see Table 1). We determined the incidence of specific binding for each of the six cells of C52. In calculating the overall distribution shown in Figure 2B, a value of 1/6 was used to represent each incidence in which a cell of C52 exhibited a particular frequency of specific binding. In this fashion, the results from all cells for all isolates were normalized in the construction of the distributions shown in Figure 2B.

A careful examination of Figure 2B reveals that the distribution of the invasive population does not trail off with very low frequency of specific binding (see sharp cutoff at 0.25). Perhaps this suggests that isolates that exhibit low specific-binding frequencies are unable to cause medical complications by attaching to implants and forming biofilms. On the other hand, a small portion of the control distribution exhibited a high frequency of specific binding (see ≥ 0.5 in Figure 2B). This overlap between the two populations suggests that some portion of the healthy human population is already colonized by potentially invasive bacteria.

Plans are currently underway to measure binding forces on a third group of *S. aureus* isolated from "uncomplicated" implant

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patients. That is, *S. aureus* isolated from patients who have prosthetic implants that are *not* infected with a biofilm. On the basis of the measurements shown in Figure 2B, we expect that the uncomplicated isolates will exhibit a specific-binding distribution that is skewed toward low specific-binding frequency and does not trail off to high specific-binding frequency.

3.3. Proof of Principle. Three double-blind tests were performed to further assess the accuracy and reproducibility of the AFM analyses. Briefly, one researcher cultured and prepared an unlabeled isolate of *S. aureus*. Another researcher performed an AFM analysis and assigned that particular isolate to either the invasive or control population, depending on the incidence of the sawtooth force signature (Figure 2B) and whether the retraction curves tended to exhibit a single sawtooth or multiple sawteeth. In all three tests, the "unknown" isolate (C53, C67, 386) was assigned to the appropriate population.

3.4. Implications for Health Care. In summary, this study presents an intriguing correlation between the phenomena observed at the length scale of a bond and the clinical outcome of patients with medical device implants. Our force data suggest that microorganisms of the exact same phylogeny (i.e., genus species classification) may be classified by the force signatures of their binding proteins. For bacteria that form biofilms, such as *S. aureus*, this is a potentially valuable means of classification because it reflects an intrinsic attribute (i.e., binding mechanism) at the heart of device-related infections.

S. aureus isolated from a particular patient, hospital, or type of infection could be classified according to its "force taxonomy". For example, patients whose personal *S. aureus* flora exhibit binding attributes consistent with the high end of the force-signature spectrum (see Figure 2B) could potentially benefit from preoperative nasal decolonization (e.g., with intranasal mupirocin[4,5]) and more rigorous postoperative monitoring. For patients with bacteremia caused by a high force-signature spectrum isolate, more aggressive diagnostic evaluations (e.g., transesophageal echocardiography) and treatment[30] might ultimately reduce rates of hematogenous seeding of prosthetic devices. In contrast, patients whose infecting or colonizing isolates exhibit low force-signature spectrum binding attributes may be less likely to develop an infected implant, reducing the clinician's index of suspicion for device involvement and potentially avoiding prolonged courses of antibiotic therapy. This too is important, as the overuse of antimicrobial drugs leads to antibiotic resistance in *S. aureus*.[31,32] For implant patients whose natural flora fall into the midrange of the "force taxonomy" spectra, host- as opposed to pathogen-related risk factors may play a more prominent role in determining the patient outcome.

It is important to stress that these are only suggestions based on the force data presented herein. Additional measurements and blind-tests with more isolates are clearly necessary before these suggestions should be put into practice. Nonetheless, this work indicates that fundamental force measurements may provide health care professionals with a probabilistic basis for assessing pathogen-related risk and caring for patients with implanted medical devices.

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Supporting Information Available: Supporting Information has been prepared to illustrate the difference between a specific binding event (i.e., sawtooth force signature) and the general, nonspecific type of adhesion that is often seen in AFM measurements. This Supporting Information also contains a more detailed discussion of certain aspects of the data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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