Chapter 11

Binding Forces Associated with *Staphylococcus aureus* Biofilms on Medical Implants

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Staphylococcus aureus is one of the most frequently isolated bacteria from infected medical implants. *S. aureus* has the capacity to adhere to the surface of an implant where it forms a biofilm. We used atomic force microscopy to probe binding forces between a fibronectin-coated tip and isolates of *S. aureus*, which were obtained from either patients with infected prostheses or healthy humans. A unique force-signature was observed for binding events between the tip and the cells. There is a statistically significant difference in the binding force-signature observed for *S. aureus* isolated from the infected vs. healthy populations. This observation suggests a fundamental correlation between nanometer scale binding forces and the clinical outcome of patients with implanted medical devices.

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Introduction

Surgical implants significantly improve the quality of life for many humans but also place these same patients at risk for life-threatening infections by bacteria (*1*). *Staphylococcus aureus* is the most frequently isolated micro-organisms from such devices (*1, 2*). Mortality attributable to such *S. aureus* infections can be as high as 60% (*3, 4*).

S. aureus colonize the surface of an implant by forming bonds with host ligands, such as fibronectin (Fn), which commonly coat a prosthetic device (*5, 6*). This type of bond is mediated by MSCRAMMs (microbial surface components that recognize adhesive matrix molecules) located on the cell wall of *S. aureus* (*5, 7-12*). A predominant MSCRAMM on *S. aureus* is the fibronectin-binding protein (FnBP). Figure 1 illustrates the structures of Fn and FnBP, and highlights the several binding sites along each protein.

We and others have used atomic force microscopy (AFM) or optical tweezers to probe the fundamental binding forces associated with type-strains or laboratory-derived strains of *S. aureus* (e.g., *13-16*). These previous studies have been very useful, but they do not explore the binding mechanisms of the strains of *S. aureus* that actually cause complications in humans or "real world" clinical settings.

Therefore, we have used AFM to measure binding forces between a simulated prosthetic device and several clinical isolates of *S. aureus*, which were obtained from either patients with an infected device or healthy subjects. These data suggest an intriguing correlation between the nanometer-scale forces at the bacterium-material interface and the clinical outcome of patients with implanted medical devices. This chapter provides a concise description of this work. An extended version of this study can be found in Yongsunthon et al. (*17*).

Methods and Materials

Collection of *S. aureus* Isolates and Growth of Cells for AFM Experiments

Invasive isolates of *S. aureus* were collected at Duke University Medical Center from the bloodstream of patients with a confirmed infection of a prosthesis. The control isolates came from the anterior nares of healthy subjects. All isolates were obtained from individuals in the same geographic area to account for strain variation.

Growth cultures were started from cryogenically preserved samples. Each isolate was cultured to exponential stage at 37 °C in tryptic soy broth containing 0.25% dextrose (*18*). Approximately 1 mL of cell suspension was washed three

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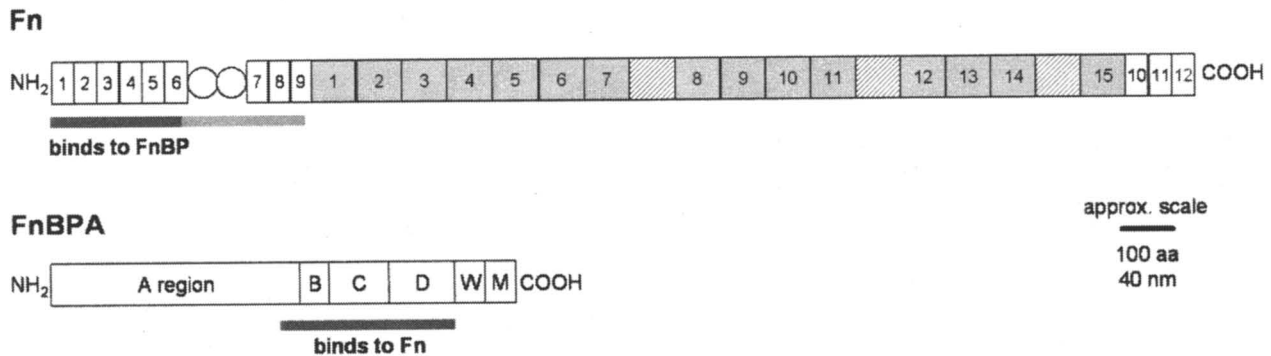

Figure 1. (Top) Structure of fibronectin (Fn), which is a human protein that commonly coats the surface of implanted devices. Fn is mainly composed of three domains FnI (white boxes; 45 amino acids, aa), FnII (circles; 60 aa), and FnIII (gray boxes; 90 aa). (Bottom) Structure of fibronectin binding protein A (FnBPA), which is exposed on the outer cell wall of S. aureus.

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times in saline solution, dropped onto a glass cover slip, and allowed to sit without drying for 5 min. Loose cells were rinsed off with phosphate buffered saline (PBS; 0.01 M with 0.85% NaCl, pH 7.2), which was also the solution used in AFM experiments. It is worth mentioning that the glass cover slips were used as supplied by the manufacturer (Corning, Inc.). We found that the bacteria did not adhere very well to clean cover slips, presumably because the cleaning process removed the manufacturer's proprietary coating.

AFM Force Measurements

Force measurements were conducted with a Bioscope AFM (Veeco) mounted on an Axiovert 200M inverted optical microscope (Zeiss). The AFM's *z*-piezoelectric scanner was calibrated with two NIST certified calibration gratings. The tips used in these experiments were coated with Fn using a slightly modified version of a method developed by Oberdorfer et al. (*19*). Briefly, silicon nitride tips were cleaned in a piranha solution (*20*), rinsed with MilliQ water (18.2 MΩ·cm), and then immersed in a 100 μg/mL Fn (Sigma-Aldrich) PBS solution for 45 minutes.

Force measurements were carried out as described in (*17*). Briefly, an inverted optical microscope was used to position an AFM tip over *S. aureus* cells that were sitting on a glass coverslip. A Fn-coated probe was brought into contact with a bacterium, pushed against the cell wall until the cantilever flexed 50-100 nm, and then pulled away from the bacterium. This approach-retraction cycle took 1 second (i.e., 1 Hz scan rate) and the vertical travel distance of the *z*-piezoelectric scanner was 2.7 μm. The AFM cantilevers had a spring constant of 0.02 nN nm^{-1} as determined by the hydrodynamic drag method (*21*). The theoretical loading rate (i.e., product of the spring constant and velocity of tip) was ~50 nN sec^{-1}. All force data were acquired within ~60-75 min of the initial harvesting of cells.

Results and Discussion

When collecting force measurements, it is a bit challenging to ensure that the AFM tip is positioned directly over a bacterium rather than the substrate that is supporting the cell. There are a couple of ways to locate a cell. A cell may be "found" by using the tip to collect an AFM image of the sample prior to collecting force curves. However, the imaging process may contaminant the tip.

Therefore, we used a different method to locate cells. An inverted optical microscope was used to position the tip over an *S. aureus* cell, which was usually a part of a binary fission pair. Figure 2 shows the positions of a Fn-coated tip and *S. aureus* cells on a glass cover slip in PBS solution. Because of the

resolution of an optical microscope, several practice runs were necessary before we were able to achieve competence with this method of alignment.

In our practice trials, the inverted optical microscope was used to position the tip over the glass substrate ~2 μm away from a cell. We collected force measurements on the glass substrate and then, with the aid of the inverted optical microscope, used the *x*- or *y*-piezos to move the sample stage under the tip at

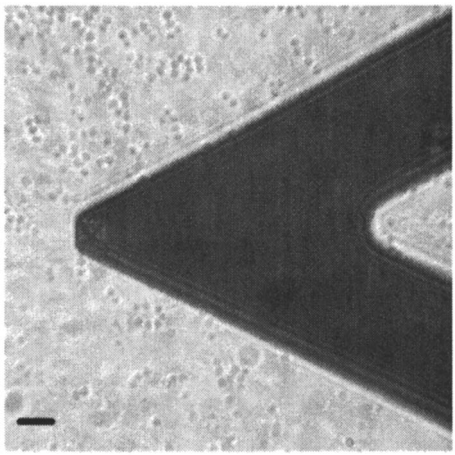

Figure 2. Optical micrograph that shows the positions of the AFM cantilever and tip, as well as coccus-shaped S. aureus cells on a glass cover slip. The cells are blurred because the plane of focus is on the AFM tip. The scale bar is ~4 μm.

intervals of a few nanometers. The resulting force curves, particularly the region of contact and retraction data, were significantly different for the cell versus the substrate. In this fashion, for this particular AFM and optical microscope, we were able to develop an effective strategy for coming down directly on top of a bacterium without the need to collect an AFM image of the cells beforehand.

Force profiles from all isolates of *S. aureus* demonstrated repulsion upon approach of the Fn-probe towards a bacterium. Figure 3 shows randomly selected approach curves collected on the 8 control and 7 invasive isolates of *S. aureus*. The magnitude and range of repulsion is consistent with electrosteric forces between *S. aureus* and a Fn-coated substrate (*15*). However, such repulsion is contrary to the common clinical observation of infectious *S. aureus*-biofilms on cardiac device implants. This inconsistency can be explained by examining the retraction force-profiles.

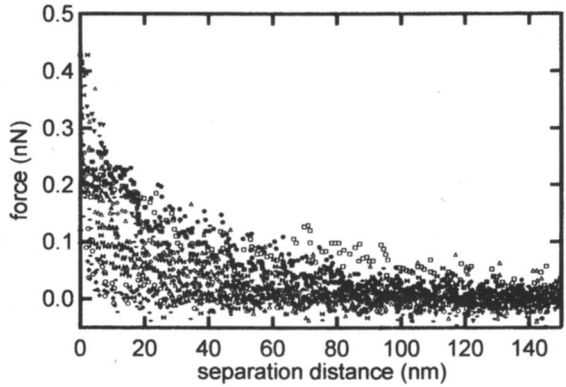

Figure 3. Force curves for a fibronectin-coated probe approaching the cell wall of the control strains of S. aureus (solid symbols) or the invasive isolates of S. aureus (open symbols). Positive force values indicate repulsion. All force data were collected in a PBS buffer solution.

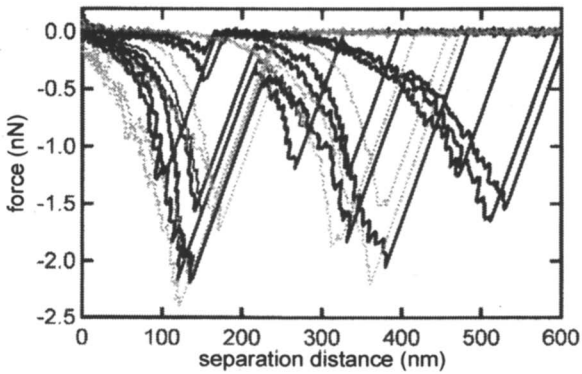

Figure 4. Force curves for a fibronectin-coated probe as it is pulled from contact with the cell wall of the control stains of S. aureus (dotted lines) or the invasive strains of S. aureus (solid lines). Negative force values indicate repulsion. All force data were collected in a PBS buffer solution. Modified after Yongsunthon et al. (17).

Retraction data often revealed an attractive bond as the Fn-coated probe was pulled away from a bacterium's cell wall. This attraction manifested itself as discrete sawtooth-shaped force signatures in the retraction profile (Figure 4). The sawtooth shape is consistent with the unfolding of a protein and suggests a specific binding event (*22*).

For each of the 15 *S. aureus* isolates, the frequency of specific binding events was determined by counting retraction curves that exhibited the sawtooth signature(s) and normalizing by the total number of curves for that isolate. The results are shown in Table I. Relative to the control isolates, the invasive isolates exhibited higher frequencies of adhesion and also tended to yield more force profiles with multiple sawtooth signatures (*17*).

Table I. Frequency of observing a specific binding event for each control isolate (label begins with "C") and invasive isolate (label begins with "I").

Isolate	*Frequency**
C55	0.12±0.01
C67	0.14±0.02
C59	0.20±0.02
C52	0.26±0.02
C57	0.36±0.02
C58	0.40±0.02
C68	0.42±0.02
C53	0.44±0.02
I141	0.42±0.02
I306	0.46±0.02
I221	0.52±0.02
I399	0.55±0.02
I1066	0.57±0.03
I386	0.72±0.03
I1790	0.73±0.03

*average ± Poisson standard deviation.

After determining the average adhesion frequency for each isolate (Table I), the results were grouped according to whether the *S. aureus* was from the invasive or control population. These two groupings are shown in Figure 5. Each isolate's data were normalized such that it contributed a total count of one. For example, the sum of the control bars shown in Figure 5 is eight, which equals the number of control isolates analyzed with AFM. In spite of the spread resulting from individual cell differences (Table I), isolates from the invasive and control

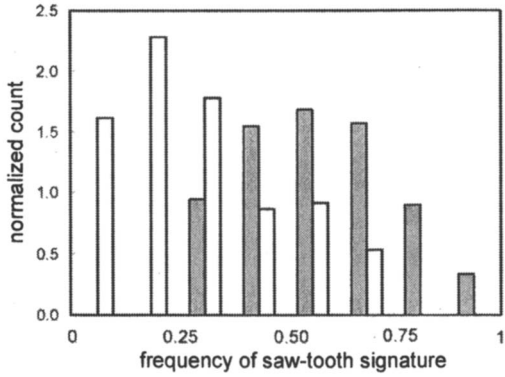

Figure 5. Histograms of the binding frequencies for the control (open bars) vs. the invasive (solid bars) strains. The distributions include retraction data for every cell probed and are normalized so that each of the S. aureus strains contributes a count of one. Modified after Yongsunthon et al (Modified from reference 17. Copyright 2007 American Chemical Society.)

populations form very distinct distributions. The frequency means of the invasive and control populations are 0.57±0.05 and 0.29±0.05, respectively. Using a Student T-test, we found that this difference is significant at the 99% confidence level.

Conclusion

In summary, this study presents an intriguing correlation that spans nine orders of magnitude from force measurements at the length scale of a bond to clinical studies of human patients with prosthetic implants. Our observations reveal that microorganisms of the exact same phylogeny (i.e., genus species classification) may be classified by the force-signatures of their binding proteins. This discovery could have a positive impact on health care as it bridges the disconnect between macroscopic, clinical investigations and nanometer-scale forces ultimately responsible for biofilms on implanted prosthetic devices.

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