Analysis of bacterial adhesion forces
Chen, Yun

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Chapter 7

Viscous Nature of the Bond between Adhering Bacteria and Substratum Surfaces Probed by Atomic Force Microscopy

(Chen, Y.; Van der Mei, H. C.; Busscher, H. J.; Norde, W. to be submitted)
ABSTRACT

Here we report on the viscous nature of the bond between adhering bacteria and a substratum surface. A tailor-made script was written for an atomic force microscope, that enabled a constant loading force of 1 nN or 5 nN to act for 30 s upon a bacterium wrenched between a cantilever and a glass surface, while measuring its deformation. Time-dependent deformation was fitted to a one element Kelvin-Voigt analogue of the bond to yield a characteristic relaxation time and viscosity of the bond. Viscosities of streptococcal bonds were smaller (< 20 kPa·s) than of staphylococcal bonds (> 31 kPa·s). Since staphylococci are relatively rich in extracellular polymeric substances, it can be concluded that the presence of extracellular polymeric substances yields the major contribution to a viscous response. The viscous nature of the bond between adhering bacteria and substratum surfaces provides the bacteria with more time to respond and protect themselves against external stresses.
Bacteria adhere to virtually all natural and man-made surfaces. Bacteria adhering to substratum surfaces rapidly develop into “biofilms”, that consist of an initial layer of so-called “linking film” bacteria, adhering directly to a substratum surface, and bacteria adhering to each other (“aggregates”). In a biofilm mode of growth, bacteria embed themselves in a matrix of self-produced extracellular polymeric substances (EPS). Detachment of bacterial biofilms from substratum surfaces is a visco-elastic process\textsuperscript{1,2} and stress-relaxation analysis of deformed biofilms of different strains and species have identified the individual EPS components responsible for the visco-elastic properties of biofilms. However, it is likely that the visco-elastic properties of a biofilm not only depend on the composition of its EPS matrix, but also on the visco-elasticity of the bond between bacteria in aggregates and between bacteria and a substratum surface.\textsuperscript{3}

The use of atomic force microscopy (AFM) has extended far beyond being an imaging technique and AFM is frequently employed to determine the strength of ligand-receptor binding at the single molecule level, or the adhesion force between bacteria and a substratum surface. In most experiments involving bacteria, adhesion forces are measured between the AFM tip and a bacterial cell surface, which has as a major advantage that forces can be measured at specific locations of interest on the bacterial cell surface. As a drawback, the small contact area between the tip and the bacterial cell surface produces a large, highly localized pressure, under which the tip can penetrate the surface and potentially damage the cell wall. This drawback can be avoided by using bacterial probes instead of AFM tips, \textit{i.e.} an entire bacterium fixed to a tipless cantilever. Using bacterial probes, adhesion forces between bacteria as well as between bacteria and different substratum surfaces have been studied.\textsuperscript{4-6} Measurements of adhesion forces are mostly done in retract force-distance curves, after application of a specified loading force and retracting the tip or bacterial probe at a specified
Visco-elasticity can be described using analogues consisting of a combination of springs and dashpots, where the spring represents the elastic component and the dashpot the viscous one. For instance, the Maxwell model, represented by a spring and a dashpot in series, provides a good model for stress-relaxation at constant strain (“deformation”), while the Kelvin-Voigt model, in which spring and dashpot are placed parallel to each other, is better fitted for creeping processes (“increasing strain at constant stress”). More complex visco-elastic processes can be modeled by other combinations of springs and dashpots.

The elastic nature of the bond between a bacterial cell surface and a substratum surface has recently been derived from the relation between deformation and applied external loading force in AFM, assuming that contact is established through a cylinder with constant volume. Reduced Young’s moduli (8 - 47 kPa) and dimensions of the contact cylinders obtained could be interpreted on the basis of the bacterial cell surface features and cell wall characteristics, i.e. surfaces that were more rigid (either because of fewer fibrillar surface appendages, possession of less EPS or a higher degree of cross-linking of the peptidoglycan layer) had shorter contact cylinders and higher reduced Young’s moduli.

The viscous nature of the bond between a bacterial cell surface and a substratum surface has never been investigated using AFM with a bacterial probe, because such experiments imply either measuring the distance between a bacterium and a substratum surface as a function of time at a constant loading force, or the force required to maintain a certain distance over time. The recent introduction of AFM, equipped with the possibility to write a tailor-made script for the control of either force or distance, enables to study the visco-elastic response
Viscous Nature of the Bond between Bacteria and Surfaces Probed by AFM

The aim of this chapter is to determine the viscous nature of the bond between adhering bacteria and a substratum surface for six bacterial strains (see also Table 1) under two different loading forces that allow pair-wise comparisons to identify the effects on the viscous nature of the bond of bacteria, either to a fixed loading force or under a fixed deformation of a bacterium, wrenched between a substratum surface and a tipless AFM cantilever, therewith avoiding high, local pressures.

**Figure 1.**
Left: Bacterium upon initial contact with a glass surface in absence of an external loading force. The contact volume is represented by a cylinder with an initial area $S_0$ and height $h_0$.

Right: Deformation of the cylindrical contact volume constituting the bond between a bacterium and a substratum surface under an external loading force $F_{ld}$.
1) the density and length of fibrillar surface appendages (two isogenic *Streptococcus salivarius* strains),\textsuperscript{12,13}

2) slime production (two *Staphylococcus epidermidis* strains)\textsuperscript{14} and

3) the degree of cross-linking in the peptidoglycan envelope (two isogenic *Staphylococcus aureus* strains)\textsuperscript{15}

Since scripting-mode AFM yields more precise control over force than deformation, we used a script measuring the time-dependent deformation $\Delta d$ of a bacterium under a constant applied force (see Fig. 1) of 1 nN or 5 nN over 30 s (Fig. 2a). As can be seen, deformation does not reach its final value immediately upon application of the force due to the viscous nature of the bond, but over a time-scale of 20 – 30 s (see Fig. 2b for an example). Importantly, repetitive measurements of the deformation of the bacterial cell surface under an applied force, yields highly reproducible results (Fig. 2c), attesting to the fact that the bacterial cell surface is not damaged by the measurements.

Deformations as a function of time under loading forces of 1 nN and 5 nN have been measured for the six strains considered in this chapter and fitted to a Kelvin-Voigt element consisting of an elastic component with Young’s modulus $E$ and a dashpot with viscosity $\eta$ (see Fig. 3a). Our previously published model to derive the Young’s modulus from the relation between deformation and applied loading force,\textsuperscript{10} not only yields a Young’s modulus but also the dimensions of an assumed cylindrical contact volume between an adhering bacterium and a substratum surface (see also Fig. 1). Since there is only a small (< 15%) deformation of the height of these contact cylinders under the small loading forces applied here, the change in the contact area of the cylinder is considered negligible.
Figure 2.
(a) Example of the script employed to determine the time-dependent deformation of bacteria under an applied loading force $F_{ld}$.
(b) Deformation $\Delta d$ for *S. epidermidis* ATCC 35984 as a function of time suspended in 10 mM potassium phosphate buffer (pH 7.0) for the script presented in Fig. 2a. The red line represents the best fit of the data to equation 6 ($R^2 = 0.98$).
(c) Repetitively measured staphylococcal cell surface deformation measured at $t = 30$ s after initiating loading at 1 nN, $\Delta d_{30s}$. For the example given, the average deformation amounts of 10 nm with a SD of 1 nm over 15 measurements at the same location.
Hence, constant loading force implies an approximately constant stress. According to

\[ \sigma = \frac{F_{ld}}{S_0} \]  

(1)

where \( \sigma \) is the applied stress, \( S_0 \) is the area of the contact cylinder and \( F_{ld} \) the applied loading force.\[^{10}\] Analogously, from the height of the contact cylinder \( h_0 \) and the deformation measured, the strain \( \varepsilon \) can be calculated

\[ \varepsilon = \frac{\Delta d}{h_0} \]  

(2)

According to a one element Kelvin-Voigt model (Fig. 3a), the strain \( \varepsilon \) increases exponentially over time \( t \) under an applied stress \( \sigma \) according to (see Fig. 3b)

\[ \varepsilon(t) = \varepsilon_{\max} (1 - e^{-\frac{t}{t^*}}) \]  

(3)

where

\[ t^* = \frac{\eta}{E} \]  

(4)

\[ \varepsilon_{\max} = \frac{\sigma}{E} \]  

(5)

and in which \( \varepsilon_{\max} \) represents the strain after infinite time.
Table 1 summarizes the $t^*$ values of the six bacterial strains involved in this study, including values for a staphylococcal strain with a disrupted integrity of its EPS layer, under a constant load of 1 nN and 5 nN.

The characteristic relaxation times $t^*$ vary between 1.4 s and 6.2 s and appeared only slightly impacted by the loading force, i.e. 1 nN or 5 nN. Major differences were seen, however, within the different pairs of strains. From a comparison of the characteristic relaxation times of *S. epidermidis* ATCC 35984 with the ones of *S. epidermidis* ATCC 35983, it follows that the relative absence of EPS yields significantly ($p < 0.05$) shorter relaxation times. Furthermore, disrupting the EPS integrity in *S. epidermidis* ATCC 35984 caused a significant ($p < 0.05$) decrease in characteristic relaxation time under both loading forces.
Table 1. Structural features and visco-elastic properties of the different pairs of bacterial strains included in this study. ± signs indicate standard deviations in $t^*$ and $\eta$ over 10 separate cultures, taking three bacteria out of each culture.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Feature</th>
<th>$t^*$ (s)</th>
<th>$E$ (kPa)</th>
<th>$\eta$ (kPa∙s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 nN</td>
<td>5 nN</td>
<td>1 nN</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>ATCC 35983</td>
<td>Poor EPS producer</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ATCC 35984</td>
<td>Strong EPS producer</td>
<td>5.8 ± 0.5</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>ATCC 35984</td>
<td>Disrupted EPS integrity with DNase I treatment</td>
<td>3.6 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>ATCC 12600</td>
<td>EPS producer Wild-type</td>
<td>1.6 ± 0.3</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>ATCC 12600Δpbp4</td>
<td>Deficient in peptidoglycan cross-linking</td>
<td>4.4 ± 0.3</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td>HB-V51</td>
<td>Sparse fibrils of 63 nm length</td>
<td>1.8 ± 0.3</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>HB-7</td>
<td>Dense fibrils of 91 nm length</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

$^1$Reduced Young’s moduli were calculated according to the elastic deformation model introduced before.$^{10}$

$^2$Reduced Young’s moduli were taken from previously published data obtained using the elastic deformation model introduced before.$^{10}$

Interestingly, *S. aureus* ATCC 12600 possesses significantly ($p < 0.05$) smaller relaxation times than its isogenic mutant, *S. aureus* ATCC 12600Δpbp4 deficient in peptidoglycan cross-linking. This suggests that the weakened peptidoglycan layer participates in deformation. From a comparison of the relaxation times of both streptococcal strains, it can be concluded that increasing fibrillar density and length yields slightly longer relaxation times.

Young’s moduli of the different pairs of strains have been discussed before with the exception of DNase I treated *S. epidermidis* ATCC 35984 and the *S.
aureus variants, indicated in Table 1, and, in summary, the conclusions were that:

- the presence of increasing amounts of EPS decreases the Young’s modulus within S. epidermidis,
- deficiencies in peptidoglycan cross-linking within S. aureus decreases the Young’s modulus of the bacteria,
- increasing density and length of fibrillar surface appendages on S. salivarius decreases the Young’s modulus.

Viscosities $\eta$ vary between 15 kPa·s and 66 kPa·s and appear not to be significantly impacted by the loading force applied, with the exception of the viscosities for DNase I treated S. epidermidis ATCC 35984 with its disrupted EPS layer and S. aureus ATCC 12600Δpap4 with its weakened cell wall. Although differences exist within the viscosities for the staphylococcal strains included in this study, it is most striking that the two fibrillated streptococci show similar viscosities, that are significantly (one-sided, one-way ANOVA, $p = 0.031$) smaller (< 20 kPa·s) than of the staphylococcal strains (> 31 kPa·s). Since staphylococci are relatively rich in EPS, it is inferred that EPS yields the major contribution to the viscous response. Interestingly, deforming either Gram-negative or Gram-positive bacteria with pyramid-shaped AFM tips or colloidal probes, under constant loading forces of 2 nN up to 10 nN, yielded viscosities in the order of 1000 kPa·s, which is at least an order of magnitude higher than the values obtained here by wrenching whole bacteria between two surfaces (see Table 1). These high viscosities are likely due to the high, localized pressure on the cell wall resulting from the use of pyramid-shaped AFM tips or sub-micron colloidal probes, while in the present study the reported viscosities are restricted to the bond between adhering bacteria and substratum surfaces.
It is instructive to compare the visco-elastic response of such single bonds with the one of full grown biofilms. Using dynamic rheometry, relaxation times of staphylococcal biofilms were found to vary between 17 s and 19 s, for both S. *epidermidis* and *S. aureus* biofilms. Maxwell analysis of the stress-relaxation of different biofilms after an induced compression indicated that the stress-relaxation of biofilms can be described by three components, attributed to flow of water (characteristic relaxation times < 3 s), flow of matrix EPS through the biofilm (relaxation times 3 – 70 s) and re-arrangement of bacteria within a deformed biofilm (relaxation times > 70 s). Importantly, the relaxation times of the bond between bacteria and substratum surfaces found here (see Table 1) are much smaller than postulated for bacterial re-arrangements in a biofilm. In a biofilm however, bacteria are embedded in a matrix of EPS that is produced in addition to the cell-bound EPS involved in the bonds studied in this chapter. Matrix EPS clearly has a higher viscosity than the aqueous buffer in which we studied the visco-elastic response of bacterial bonds, which elongates the characteristic relaxation time. Moreover, in a biofilm, bacteria are surrounded by a large number of neighboring organisms and subject to multiple forces working in different and sometimes opposing directions, which constitutes a second mechanism through which relaxation times in a biofilm will be longer than of a single bond, experiencing only a single, uni-directional force.

Bacterial adhesion to a substratum surface is accompanied by deformation of the bacterial cell surface and it has been recently suggested that adhesion-induced deformation of the bacterial cell surface triggers so-called stress-deactivation, which makes adhering bacteria more susceptible to antibiotics. The viscous nature of the bond between an adhering bacterium and a substratum surface slows down the impact of external stresses and may provide the bacterium with more time to respond and protect itself against such stresses.
MATERIALS AND METHODS

Bacterial strains and culture conditions

Staphylococci were pre-cultured from blood agar plates in 10 ml Tryptone Soya Broth (OXOID, Basingstoke, England), while streptococci were pre-cultured from blood agar plates in 10 ml Todd Hewitt Broth (OXOID). All pre-cultures were grown for 24 h at 37°C. After 24 h, 0.5 ml of a pre-culture was transferred into 10 ml fresh medium and the main culture was grown for 16 h at 37°C. Bacteria were harvested by centrifugation at 5000×g for 5 min, washed twice with 10 mM potassium phosphate buffer, pH 7.0 and finally suspended in the same buffer. When bacterial aggregates or chains were observed microscopically, 10 s sonication at 30 Watt (Vibra Cell model 375, Sonics and Materials Inc., Danbury, Connecticut, USA) was carried out intermittently for three times, while the suspension was cooled in a water/ice bath.

To disrupt the EPS integrity on the bacterial cell surface of *S. epidermidis* ATCC 35984, the bacterial suspension was diluted to a concentration of $3 \times 10^8$ bacteria/ml with 10 mM potassium phosphate buffer (pH 7.0) and treated with DNase I (Thermo Scientific, Waltham, MA) at a concentration of 1 unit/µl in the presence of 2.5 mM MgCl$_2$ for 45 min at 37°C and subsequently washed twice with the same buffer.$^{21,22}$ Experiments have indicated that the harvesting procedure does not affect hydrodynamic radii and deformabilities of the bacterial cells.$^{23}$

AFM force spectroscopy

Bacterial probes were prepared by immobilizing a bacterium to a NP-O10 tipless cantilever (Bruker, Camarillo, California, USA). Cantilevers were first calibrated by the thermal tuning method and spring constants were always within the range given by the manufacturer (0.03 - 0.12 N/m). Next, a cantilever was mounted to the end of a micromanipulator and, under microscopic observation, and the tip of
the cantilever was dipped into a droplet of 0.01% \( \alpha \)-poly-L-lysine of MW 70,000-150,000 (SIGMA-ALDRICH, St. Louis, Missouri, USA) for 1 min to create a positively charged coating. After 2 min of air-drying, the tip of the cantilever was carefully dipped into a bacterial suspension droplet for 1 min to allow bacterial attachment through electrostatic attraction and dried in air for 2 min. Bacterial probes were always used immediately after preparation. Experiments have indicated that the attachment protocol does not disturb the surface structure of bacterial cells.\textsuperscript{23}

**The AFM script**

All AFM measurements were performed in 10 mM potassium phosphate buffer (pH 7.0) at room temperature on a BioScope Catalyst AFM (Bruker). A bacterium was brought into contact with a glass microscope slide cleaned to a zero degrees water contact angle (Gerhard Menzel GmbH, Braunschweig, Germany) at a constant velocity of 100 nm/s. According to the script applied, a bacterium was kept wrenched between the cantilever and the glass surface under a constant loading force of 1 nN or 5 nN for 30 s by auto-adjustment of the instrument to maintain a constant loading force. The loading force \( F_{ld} \) (Fig. 2a) and the displacement of the piezo transducer were recorded as a function of time \( t \) and the displacement of the transducer used to calculate the deformation of the bacterial cell surface, \( \Delta d \) (Fig. 2b). Measurements were repeated multiple times on the same spot to conclude whether the bacterial cell surface had suffered damage from the measurements by raising the piezo transducers above the cell surface to release the loading and allowing the bacterium to recover for 120 s (Fig. 2c) before the next measurement.
REFERENCES


