Nanoscopic vibrations by bacteria adhering to surfaces
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Chapter 3

Contribution of adsorbed protein films to nanoscopic vibrations exhibited by bacteria adhering through ligand-receptor bonds


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ABSTRACT

Bacteria adhering to surfaces exhibit nanoscopic vibrations that depend on the viscoelasticity of the bond. The quantification of the nanoscopic vibrations of bacteria adhering to surfaces provides new opportunities to better understand the properties of the bond through which bacteria adhere and the mechanisms by which they resist detachment. Often however, bacteria do not adhere to bare surfaces but to adsorbed protein films, on which adhesion involves highly specific ligand-receptor binding next to non-specific DLVO interaction forces. Here we determine the contribution of adsorbed salivary protein and fibronectin films to vibrations exhibited by adhering streptococci and staphylococci, respectively. The streptococcal strain used has the ability to adhere to adsorbed salivary proteins films through antigen I/II ligand-receptor binding, while the staphylococcal strain used adheres to adsorbed fibronectin films through a proteinaceous ligand-receptor bond. In absence of ligand-receptor binding, electrostatic interactions had a large impact on vibration amplitudes of adhering bacteria on glass. On an adsorbed salivary protein film, vibration amplitudes of adhering streptococci depended on the film softness as determined by QCM-D, and were reduced after film fixation using glutaraldehyde. On a relatively stiff fibronectin film, crosslinking the film in glutaraldehyde hardly reduced its softness and accordingly fibronectin film softness did not contribute to vibration amplitudes of adhering staphylococci. However, fixation of the staphylococcus-fibronectin bond further decreased vibration amplitudes, while fixation of the streptococcus bond hardly impacted vibration amplitudes. Summarizing, this study shows that both the softness of adsorbed protein films as well as the properties of the bond between an adhering bacterium and an adsorbed protein film play an important role in bacterial vibration amplitudes. These nanoscopic vibrations reflect the viscoelasticity of the bacterial bond with a substratum and play important roles in bacterial adhesion, detachment and susceptibility to antimicrobials.
INTRODUCTION

Bacterial adhesion to surfaces is ubiquitous in many industrial processes as well as in nature, including the human body. Control of bacterial adhesion not only provides a way to prevent human infections associated with bacterial adhesion, but also contributes to the undisturbed functioning and therewith the efficiency of industrial food and dairy processing. In addition, control of bacterial adhesion aids to increase the efficiency of bio-reactor systems. Bacterial adhesion to surfaces can be regarded as a colloidal phenomenon mediated by a non-specific interplay of attractive Lifshitz-Van der Waals forces operating over several tens of nanometers and electrostatic forces originating from the interaction between the diffuse electric double-layers at surfaces that operates over a distance ranging up to tens of nanometers, depending on the ionic strength of the medium. The sum interaction of both forces is described in the DLVO-theory of colloidal stability.

Recently it has been observed for both Gram-positive and Gram-negative bacterial strains, that bacteria adhering to glass surfaces exhibit nanoscopic vibrations, in essence due to Brownian motion. Amplitudes of these vibrations were highest under low ionic strength conditions and were governed by the viscoelastic properties of the bond between adhering bacteria and substratum surfaces. For streptococci, spring constants derived from bacterial vibration amplitudes decreased with increasing fibrillar density on the cell surface, while for staphylococci higher spring constants were obtained for strains having larger amounts of extra-cellular-polymeric substances. Possession of a viscoelastic bond aids adhering bacteria to withstand detachment forces and remain adhering.

Under many natural conditions, bacteria do not adhere to bare substratum surfaces, but to adsorbed macromolecular conditioning films. In the human body, these conditioning films are often comprised of adsorbed proteins, like salivary proteins on surfaces exposed to the oral cavity or fibronectin on surfaces exposed to human serum. Although bacteria adhere to proteinaceous conditioning films by the same fundamental forces as by which they adhere to homogeneous, bare substratum surfaces, the chemical groups mediating bacterial adhesion to proteinaceous conditioning films are often highly localized, yielding strong, highly specific so-called “ligand-receptor” bonds between bacteria and proteinaceous conditioning films. Streptococci for instance, may possess surface-associated antigen I/II with molecular masses ranging from 160 to 215 kDa that mediates binding to salivary conditioning films. Similarly, staphylococci may possess functional adhesins mediating their adhesion to adsorbed fibronectin films.

The aim of this paper is to determine the contribution of adsorbed protein films to the nanoscopic vibrations exhibited by adhering streptococci and staphylococci. Bacterial vibration spectroscopy is carried out for a streptococcal and a staphylococcal strain, possessing the ability of forming specific ligand-receptor bonds.
with salivary protein films or adsorbed fibronectin films, respectively. Bacterial vibrations are analyzed as a function of ionic strength for bacteria non-specifically adhering to bare glass surfaces. Next, vibrations are studied on adsorbed protein films, prior to and after fixation of the protein films using glutaraldehyde and after fixation of both the ligand-receptor bond and the adsorbed protein film. Vibrations are related to the film softness as determined using a quartz-crystal microbalance with dissipation.

**EXPERIMENTAL SECTION**

**Bacterial Strains and Culture Conditions**

Two bacterial strains with known specific binding sites and two isogenic mutants lacking these binding sites were selected for this study: *Streptococcus mutans* LT11 (expressing antigen I/II) and *S. mutans* IB03987 (lacking antigen I/II), and *Staphylococcus aureus* 8325-4 (possessing fibronectin binding proteins) and *S. aureus* DUS883 (lacking fibronectin binding protein). *S. mutans* LT11, like most oral streptococci, possesses antigen I/II that mediates its ligand-receptor binding to adsorbed salivary protein films and is a commensal bacterium in the human oral cavity, associated with dental caries. *S. aureus* 8325-4 is equipped with a fibronectin binding protein involved in its adhesion to adsorbed fibronectin films, such as on plasma-exposed biomaterial implant surfaces and tissue cells in the human body, and possesses a high potential to cause infection.

Streptococci and staphylococci were separately maintained at -80°C in brain heart infusion broth (BHI; OXOID, Basingstoke, United Kingdom) and tryptone soya broth (TSB; OXOID) containing 7% dimethylsulfoxide (Merck, Darmstadt, Germany). *S. mutans* LT11 was cultured on BHI agar plates and *S. mutans* IB03987 on BHI agar supplemented with 5 mg/mL kanamycine monosulfate and incubated in 5% CO₂ and *S. aureus* was cultured on TSB agar plates under aerobic conditions overnight at 37°C. Subsequently, *S. mutans* was pre-cultured in 10 mL BHI, while *S. aureus* was pre-cultured in TSB under constant shaking (150 rpm). After 24 h, each pre-culture was used for a main culture in 190 mL growth medium. For the *S. mutans*, the main culture was grown for another 16 h under identical conditions, while *S. aureus* was grown for 2 h which corresponds with peak expression of fibronectin binding proteins in *S. aureus* 8325-4. Bacteria were harvested by centrifugation (*S. mutans*, 5000 g, 5 min, 10°C; *S. aureus*, 6500 g, 5 min, 10°C) and washed twice with a low (0.57 mM) ionic strength buffer (0.5 mM potassium chloride, 0.02 mM potassium phosphate, 0.01 mM calcium chloride, pH 6.8). Finally, bacteria were suspended to a density of $3 \times 10^8$ bacteria per mL either the low ionic strength buffer or in a buffer with a hundred fold higher (57 mM) ionic strength (50 mM potassium chloride, 2 mM potassium phosphate, 1 mM calcium chloride, pH 6.8) and sonicated for $3 \times 10^8$ s at
30 W in an ice/water bath to break bacterial aggregates.

**Saliva Collection and Preparation**

Saliva was prepared from a stock of human whole saliva from at least 20 healthy volunteers of both genders, collected into ice-cooled beakers after stimulation by chewing Parafilm®, and subsequently pooled, centrifuged, dialyzed, and lyophilized for storage. Prior to lyophilization, phenylmethyl sulfonyl fluoride was added to a final concentration of 1 mM as a protease inhibitor in order to reduce protein breakdown. Freeze-dried saliva was dissolved in the high ionic strength buffer (1.5 mg/mL). All volunteers gave their informed consent to saliva donation, in agreement with the guidelines set out by the Medical Ethical Committee at University Medical Center Groningen, Groningen, The Netherlands (letter 06-02-2009).

**Salivary Protein and Fibronectin Adsorption**

Prior to each experiment, glass slides were cleaned in 2% RBS (Chemical Products R. Borghgraef S.A., Brussels, Belgium) in an ultrasonic bath and rinsed with methanol and water. The glass slide was immersed in a salivary protein solution for 18 h at 4°C (in order to maintain microbial growth and enzymatic activity as low as possible), to create a salivary conditioning film. Eighteen hours is known to be long enough to allow protein adsorption and displacement by higher molecular weight ones to yield a clinically “mature” adsorbed film. Then, the saliva-coated glass slide was rinsed in the low ionic strength buffer and used immediately for streptococcal adhesion and analysis of vibration amplitudes. For adsorption of a fibronectin film (human fibronectin; Sigma-Aldrich, Zwijndrecht, The Netherlands), the glass slide was drop-coated with 0.05 mL sterile fibronectin solution (25 μg/mL, 100 μg/mL and 250 μg/mL) in the high ionic strength buffer for 2 h at room temperature (commercially obtained fibronectin has no issues with enzymes or contaminating bacteria to the extent that saliva has) to create a more or less circular fibronectin-coated region with a diameter of 1 cm, followed by rinsing with the low ionic strength buffer. In addition, the glass slide was immersed in 1% bovine serum albumin (BSA) (Sigma-Aldrich, USA) in order to block the non-specific adhesion sites. Finally, the glass slide was rinsed in low ionic strength buffer and used immediately for staphylococcal adhesion and bacterial vibration spectroscopy.

In order to determine the influence of the softness of the adsorbed films on the vibration amplitudes, we crosslinked the films with 2% glutaraldehyde in demineralized water followed by rinsing with low ionic strength buffer. Vibration experiments were also performed on these adsorbed salivary protein and fibronectin films. In these cases, bacteria were adhered to cross-linked protein films. In order to establish a proper time for crosslinking of the protein films, adsorbed salivary films were crosslinked in glutaraldehyde for 10 s, 60 s and 120 s. Crosslinking of the adsorbed protein films was confirmed by Fourier Transform Infrared Spectroscopy.
(see Supporting Information Figure S1). Softness of the films decreased progressively with crosslinking time, with no effects of crosslinking on the film thickness (see Supporting Information Figure S2). Consequently it was decided to carry out bacterial vibration spectroscopy on protein films crosslinked for 60 s (note that pilot experiments yielded the conclusion that fixation for 10, 60 or 120 s yielded the same reductions in bacterial vibration amplitude for the *S. mutans* LT11 on a salivary film (data not shown)). In addition, also adsorbed protein films with adhering bacteria were fixed with 2% glutaraldehyde for 1 min and rinsed with low ionic strength buffer, after which the vibration amplitude was measured representing the case in which both the adsorbed protein film and the ligand-receptor bond with adhering bacteria is fixed.

*Measurement of Vibration Amplitudes of Adhering Bacteria*

Bacterial vibrations were studied in a parallel plate flow chamber with channel dimensions 175 × 17 × 0.75 mm both on bare glass surfaces and on protein films adsorbed to a glass surface under conditions of a stagnant flow. The flow chamber has been described in detail previously, but was merely used as a convenient vehicle to allow bacteria to adhere. On bare glass, bacterial vibration spectroscopy was carried out in both the low (0.57 mM) and high (57 mM) ionic strength buffers in order to better reveal the influence of electrostatic interactions on bacterial vibrations, while in case of vibration spectroscopy on adsorbed protein films, experiments were only done in the low ionic strength buffer as this yields the largest vibration amplitudes. Prior to each experiment involving bare glass surfaces, all tubes and the flow chamber were filled with either low or high ionic strength buffer, while for experiments on adsorbed protein films the chamber was filled only with low ionic strength buffer. After removal of air bubbles by perfusion with buffer, a bacterial suspension was flowed through the system at a shear rate of 10 s⁻¹ for 1 h at room temperature. Subsequently, buffer was perfused for 15 min in order to remove all non-adhering bacteria. Then, flow was arrested for vibration amplitude measurements at the appropriate ionic strength. Vibrations of adhering bacteria were observed with a CCD camera (A101F, Basler AG, Ahrensburg, Germany) mounted on a phase-contrast microscope (BH2-RFCA, Olympus Optical Co., Tokyo, Japan). The camera was coupled with an image analysis program (Matlab, The MathWorks, Natick, MA), recording 60 consecutive images per second. Along the lines of equal pixel grey-values within the image of a single bacterium, a series of concentric elliptic contour lines were constructed, the center position of which was defined as the position of an adhering bacterium (Figures 1a and 1b). Different positions obtained at different time points over a 33 s time-interval were plotted as a distribution histogram along with the positions of a black marker on the glass slide representing a fixed position (Figure 1c). These distributions were fitted to a Gaussian distribution function and the vibration amplitudes were calculated from the half widths at half maximum of the Gaussian distributions. Next, in order to account for possible vibrations of the building or microscope, the vibration amplitude of the fixed
marker was subtracted from the uncorrected vibration amplitude calculated for adhering bacteria to yield the bacterial vibration amplitudes reported. Note from the example presented in Figure 1c, that the black marker is positioned at the same location for most images with only a small vibration amplitude relative to the bacterial vibration amplitude (10 nm for the example presented).

The vibration amplitudes presented, represent the averages over bacterial vibration amplitudes from three experiments with separate bacterial cultures. Each experiment involved the analysis of the vibration of 10 randomly selected bacteria.

**Zeta Potential Determination of Bacteria and Substratum Surfaces**

Zeta potentials of the four bacterial strains were determined by particulate microelectrophoresis (Zetasizer nano-ZS; Malvern Instruments, Worcestershire, UK) in low and high ionic strength buffers at pH 6.8. To determine the zeta potentials of the bare glass surface, streaming potentials were measured in low and high ionic strength buffers at pH 6.8. Glass surfaces were mounted in a homemade parallel plate flow chamber, separated by a 0.1 mm Teflon spacer. A platinum electrode was placed at each side of the chamber. Streaming potentials were measured at 10 different pressures ranging from 50 to 400 mbar. Each pressure was applied for 10 s in both directions. Zeta potentials were calculated by linear least-squares fitting of the pressure-dependent streaming potentials.
Figure 1. (a) Snap-shot image obtained by phase contrast microscopy of *S. aureus* 8325-4 adhering on a glass substratum at low ionic strength.

(b) Concentric elliptic contour lines connecting points of equal greyness levels calculated from (a). The red point in the center of the concentric ellipses represents the position of an adhering bacterium. Different positions were obtained at different points in time over a 33 s time-interval and plotted in a distribution histogram.

(c) Distribution histogram of the positions of an adhering staphylococcus (black bars) and a fixed marker (blue bars) on a glass substratum over a 33 s time-interval, showing Gaussian distributions. Vibration amplitudes are taken as the full widths at half maximum of the distribution histograms. All bacterial vibration amplitudes reported further on in this paper are corrected for vibrations of the fixed marker by subtracting the marker vibration amplitudes from the uncorrected bacterial amplitudes.

(d) Vibration amplitudes, corrected for fixed marker vibrations, of *S. mutans* LT11 and *S. aureus* 8325-4 adhering on a bare glass substratum at low and high ionic strength. Error bars indicate standard deviations over three experiments with separately cultured bacteria. Each experiment involves a minimum of 10 randomly selected adhering bacteria. * indicates
significant differences (p < 0.05) between vibration amplitudes of both strains.

(e) DLVO interaction free energy for *S. aureus* 8325-4 as a function of distance to a glass surface.

(f) Vibration amplitudes as a function of the DLVO interaction free energy at the secondary minimum G_{SM} for *S. mutans* LT11 and *S. aureus* 8325-4 adhering on a glass substratum at low and high ionic strength. Lifshitz-Van der Waals interaction energies were calculated assuming identical Hamaker constants for both strains.\textsuperscript{35,36} Error bars indicate standard deviations over three experiments with separately cultured bacteria.

**Calculation of DLVO Interaction Energies**

Lifshitz-Van der Waals attractive interaction energies ($\Delta G_{adh}^{LW}(y)$) as a function of distance ($y$) were calculated using\textsuperscript{33,34}

$$\Delta G_{adh}^{LW}(y) = \frac{A_{sbl}r}{6y}$$

in which $A_{sbl}$ denotes the Hamaker constant (taken as $5 \times 10^{-21}$ J for both strains),\textsuperscript{35,36} and $r$ is the bacterial radius (taken as 0.5 µm). Distance dependent electrostatic interaction energies were calculated by

$$\Delta G_{adh}^{EL}(y) = \pi\varepsilon\varepsilon_0 r \left( \zeta_b^2 + \zeta_g^2 \right) \left\{ \frac{2\zeta_b\zeta_g}{\zeta_b^2 + \zeta_g^2} \star \ln \left[ \frac{1 + \exp(-\kappa y)}{1 - \exp(-\kappa y)} \right] + \ln\left[ 1 - \exp(-2\kappa y) \right] \right\}$$

in which $\varepsilon\varepsilon_0$ denotes the dielectric permittivity of the medium, $\zeta_b$ and $\zeta_g$ the zeta potentials of the bacterial cell surface and glass surface, respectively and $\kappa$ the reciprocal Debye length. The total DLVO interaction free energy is the sum of the Lifshitz-Van der Waals and electrostatic interactions.

**QCM-D Measurements of Adsorbed Protein Film Thickness and Softness**

A multiple-channel Quartz Crystal Microbalance with Dissipation (QCM-D; Q-sense E4, Q-sense, Gothenburg, Sweden) was used to measure the thickness and structural softness of adsorbed salivary protein and fibronectin films. A silicon dioxide-coated crystal was used as a substratum. Before each experiment, the crystals were rinsed in 2% (w/v) sodium dodecyl sulfate (SDS) for 15 min, followed by rinsing with ultra-pure water in a sonicating bath and finally treated with UV/ozone for 15 min to achieve a water contact angle of zero degrees. At the start of each experiment, the crystal was incubated in low ionic strength buffer under flow until stable base lines for both frequency and dissipation were obtained. Subsequently, solutions of salivary proteins or of fibronectin with different protein concentrations in high ionic strength
buffer were perfused through the QCM-D chamber at a flow rate of 50 μL/min, roughly corresponding with a shear rate of 3 s⁻¹. Adsorption of salivary proteins was pursued for 18 h. This adsorption phase was followed by rinsing for 30 min with low ionic strength buffer to remove un-adsorbed salivary proteins, after which the thickness and softness of the films were measured. For fibronectin adsorption, adsorption was pursued for 2 h, also followed by a rinsing with low ionic strength buffer for 30 min. Subsequently, the chamber was perfused with 1% bovine serum albumin for 1 min to block the non-specific adhesion sites followed by rinsing with low ionic strength buffer after which the thickness and softness of the fibronectin films were measured. In order to measure the thickness and softness of the protein films after fixation, 2% glutaraldehyde was flowed for 1 min and followed by rinsing with low ionic strength buffer before measurement.

The thickness and softness of the adsorbed protein films were determined using the Voigt model provided in the software QTools 3.0 (Q-sense, Gothenburg, Sweden). The softness of the films was indicated by the ratio of $\Delta D_3/\Delta f_3$ as measured by QCM-D. All measurements were performed in triplicate with separate protein solutions.

**Statistics**

All experiments were performed in triplicate with separately prepared bacterial cultures and adsorbed protein films. All data are presented as means ± standard deviations. A Student t-test was used to analyze the significance of differences between experimental groups. Differences between data sets were considered significant when the p-value was <0.05.

**RESULTS AND DISCUSSION**

First, nanoscopic vibrations exhibited by wild-type *S. mutans* LT11 and *S. aureus* 8325-4 were examined when adhering to bare glass surfaces. In absence of adsorbed protein films (Figure 1d), vibration amplitudes were higher under low ionic strength (0.57 mM) than under high ionic strength (57 mM) conditions, consistent with previous results using different bacterial strains of both a Gram-positive and Gram-negative nature.\(^9\) *S. mutans* LT11 generally exhibited smaller vibration amplitudes than *S. aureus* 8325-4. Considering the influence of ionic strength on the vibration amplitudes, we set out to measure the zeta potential of the strains as well as of the glass surface under low and high ionic strength conditions (see Table 1). All zeta potentials became less negative upon increasing ionic strength and glass had a more negative zeta potential than the bacterial strains. Wild-type *S. mutans* LT11 had a less negative zeta potential than its isogenic mutant IB03987 lacking antigen I/II, while wild-type *S. aureus* had a similarly negative zeta potential than its isogenic mutant DU5883 lacking fibronectin binding proteins. The influence of electrostatic
interactions on bacterial vibration amplitudes was further elaborated by calculating the depth and position of the secondary interaction minimum in the DLVO-theory, using the measured zeta potentials and assuming similar Hamaker constants ($5 \times 10^{-21}$ J) for both bacterial strains.\textsuperscript{35,36} Examples of the DLVO interaction free energy between \textit{S. aureus} 8325-4 and a glass surface as a function of the separation distance between the interacting surfaces is shown in Figure 1e for the two ionic strengths. Lifshitz-Van der Waals interaction is essentially invariant with ionic strength, but the electrical double layer compresses upon increasing the ionic strength therewith suppressing the electrostatic interaction at a given separation distance. Accordingly, the secondary interaction minimum ($G_{SM}$) at high ionic strength was found to be much deeper and more closely located to the glass surface (8 nm) than at low ionic strength (150 nm). Interestingly, vibration amplitudes increased with decreasing depth of the secondary interaction minimum. This suggests that the depth of the secondary interaction minimum plays an important role in bacterial vibration amplitudes on bare glass surfaces (Figure 1f). In the presence of specific ligand-receptor bonds mediating adhesion of the streptococci or staphylococci to adsorbed protein films, no relations between the depths of the secondary interaction minima, as calculated using the DLVO theory, and bacterial vibration amplitudes were found (data not shown), indicative of an entirely different binding mechanism.

### Table 1. Zeta potentials of the bacterial strains and glass surface involved in this study, measured at low and high ionic strengths. Bacterial zeta potentials were determined by particulate microelectrophoresis in low and high ionic strength buffers (0.57 mM and 57 mM) at pH 6.8.\textsuperscript{30} Zeta potentials of the glass surface were also determined in buffer using a home-made streaming potential instrument.\textsuperscript{31} All zeta potentials were determined in triplicate using separately prepared bacterial cultures or different glass surfaces. Data are presented as averages ± standard deviations.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>$\xi_{(0.57 \text{ mM})}$ [mV]</th>
<th>$\xi_{(57 \text{ mM})}$ [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. mutans} LT11</td>
<td>-27 ± 2</td>
<td>-4 ± 1*</td>
</tr>
<tr>
<td>\textit{S. mutans} IB03987</td>
<td>-42 ± 1</td>
<td>-9 ± 0*</td>
</tr>
<tr>
<td>\textit{S. aureus} 8325-4</td>
<td>-33 ± 1</td>
<td>-14 ± 1*</td>
</tr>
<tr>
<td>\textit{S. aureus} DU5883</td>
<td>-34 ± 1</td>
<td>-14 ± 1*</td>
</tr>
<tr>
<td>Substratum surface</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>-72 ± 4</td>
<td>-24 ± 4*</td>
</tr>
</tbody>
</table>

*Significantly different from low ionic strength $p < 0.001$. 
Figure 2. (a) Vibration amplitudes of *S. mutans* LT11 adhering to a salivary protein film adsorbed from high ionic strength buffer on a glass surface and an adsorbed salivary film after fixation with 2% glutaraldehyde, as well as of streptococci adhering to a salivary film after fixation of both the film and the ligand-receptor bond between the bacteria and the film. All vibration amplitudes pertain to the low ionic strength buffer. Error bars indicate standard deviations over three experiments with separately cultured bacteria. Each experiment involves a minimum of 10 randomly selected adhering bacteria. * indicates significant differences (p < 0.05) between vibration amplitudes for different conditions of the film and bond.

(b) Schematics of the vibrations of *S. mutans* LT11 adhering on an adsorbed salivary protein film. Vibrations involve stretching of the bond as well as stretching of soft, adsorbed salivary proteins (see Table 2). Note that in this schematic presentation we have only drawn one ligand-receptor bond, while in reality more bonds exist.

(c) As in (b), now for a fixed salivary protein film. Vibrations only involve stretching of the bond due to cross-linking of the adsorbed proteins (compare Table 2) and are consequently smaller than in absence of film fixation.

(d) As in (b), now for a fixed salivary protein film and bond. Additional fixation of the ligand-receptor bond only yields a minor contribution to restricting bacterial vibrations.

Vibration amplitudes of *S. mutans* LT11 on a saliva-coated glass surface in the low ionic strength buffer were significantly higher than on bare glass (compare Figures 1d and Figure 2a). QCM-D analysis of adsorbed salivary proteins films on glass demonstrated that these films were relatively thick and soft compared to the fibronectin layer thickness and softness (see Table 2). A softness of $0.11 \times 10^{-6}$ for a film of different, adsorbed serum proteins on a SiO$_2$-coated crystals was measured.
by Tymchenko et al., which is smaller than of our adsorbed salivary protein films but in the same order of magnitude especially considering that both films are comprised of different proteins. Layer thickness and softness contribute to the degrees of motional freedom (stretching and wobbling) of adsorbed proteins, which suggests that the adsorbed salivary proteins contribute to higher vibration amplitudes of the adhering streptococci (Figure 2b). This suggestion is confirmed by the observation that adsorbed salivary protein films crosslinked by fixation with 2% glutaraldehyde to limit their degree of motional freedom leads to smaller streptococcal vibration amplitudes (from 79 nm to 11 nm; Figures 2a and 2c). Note that whereas the softness of adsorbed salivary films is significantly reduced upon crosslinking, the thickness of adsorbed salivary films is hardly affected (see also Table 2 and Supplementary information Figure S2). Finally, bacteria adhering to salivary films and the film itself were both fixed with glutaraldehyde, yielding a further, but small reduction in vibration amplitude from 11 nm to 8 nm (Figure 2a and 2d). This implies that vibration amplitudes of streptococci adhering to salivary films through specific ligand-receptor binding are governed by interplay of the softness of the adsorbed protein film and a minor contribution of the viscoelasticity of the bond itself to the vibration amplitudes. The relatively minor influence of the bond itself is confirmed by the observation that the vibration amplitude of the mutant strain IB03987 lacking antigen I/II, is less than of the wild-type strain. The wild-type strain binds through a viscoelastic bond with the protein film, that subsequently contributes to the vibration amplitude. The strain not able of ligand-receptor binding will sink into the soft salivary protein film restricting its vibrations (see Table 3). Antigen I/II plays an important role in specific adhesion and a 30-fold higher number of streptococci with antigen I/II were found adhering to a salivary coated glass surface than for a strain without antigen I/II. Based on adhesion force measurements, it was estimated that the density of antigen I/II on S. mutans LT11 is between $15 \times 10^3$ to $75 \times 10^3$ binding sites over an entire bacterial cell surface, arranged along structural surface features. Immuno-electron microscopy gold-labeled antigen I/II images confirm a high density of antigen I/II on the outer surface of the wild-type strain.

Similar experiments in the low ionic strength buffer were subsequently carried out with S. aureus 8325-4 adhering to adsorbed fibronectin films in order to determine whether the above conclusion on the role of film thickness, softness and viscoelasticity of the bond also pertains to an entirely different ligand-receptor system. Fibronectin was adsorbed from solutions of different concentrations (25 μg/mL, 100 μg/mL and 250 μg/mL). Fibronectin is a tubular protein with a length of approximately 15.5 nm and diameter of 8.8 nm, yielding an axial ratio of around 2. This axial ratio of around 2 coincides with the ratio between adsorbed fibronectin film thicknesses observed in QCM-D measurements at different fibronectin concentrations (Table 2): a film thickness of 5 nm (comparable with the diameter of the protein) was found at the lowest protein concentration in solution (25 μg/mL).
likely representative for side-on adsorption, while the film thickness doubled in accordance with the axial ratio at the higher fibronectin concentrations (100 and 250 µg/mL). This strongly suggests the development of a layer of end-on oriented adsorbed fibronectin molecules at the higher concentrations. Importantly, adsorbed fibronectin films were all relatively stiff to the extent that crosslinking by fixation in glutaraldehyde hardly reduced their softness (see also Table 2). The fibronectin layer is relatively stiff compared to the salivary protein layer due to a well-structured monolayer yielding little degree of motional freedom. A similarly small softness of $0.06 \times 10^{-6}$ for a fibronectin film adsorbed from a 10 µg/ml fibronectin solution in phosphate buffered saline on a SiO$_2$-coated crystals was measured by Tymchenko et al.$^{38}$

**Table 2.** The thicknesses and softnesses of adsorbed films of salivary proteins and fibronectin on silicon dioxide QCM-D crystals prior to and after fixation with 2% glutaraldehyde during 60 s. Salivary proteins were adsorbed from a high ionic strength buffer (57 mM, pH 6.8) containing 1.5 mg/mL of freeze-dried salivary proteins, approximately the protein content of natural saliva.$^{37}$ Fibronectin was also adsorbed from a high ionic strength buffer with different fibronectin concentrations. Data were derived from dissipation and frequency shifts measured at multiple overtones in a low ionic strength buffer (0.57 mM) in which also bacterial vibration spectroscopy was carried out. Data represent averages ± standard deviations over three independent experiments.

<table>
<thead>
<tr>
<th>Adsorbed film type</th>
<th>Thickness [nm]</th>
<th>Thickness after fixation [nm]</th>
<th>Softness $\Delta D_3/\Delta f_3$ [10$^{-6}$]</th>
<th>Softness $\Delta D_3/\Delta f_3$ after fixation [10$^{-6}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary proteins (1.5 mg/mL)</td>
<td>41 ± 17</td>
<td>40 ± 16</td>
<td>0.38 ± 0.04</td>
<td>0.33 ± 0.03**</td>
</tr>
<tr>
<td>Fibronectin (25 µg/mL)</td>
<td>5 ± 3</td>
<td>4 ± 2</td>
<td>0.02 ± 0.02</td>
<td>0.00*</td>
</tr>
<tr>
<td>Fibronectin (100 µg/mL)</td>
<td>9 ± 1</td>
<td>8 ± 0**</td>
<td>0.02 ± 0.02</td>
<td>0.00*</td>
</tr>
<tr>
<td>Fibronectin (250 µg/mL)</td>
<td>10 ± 2</td>
<td>8 ± 1</td>
<td>0.02 ± 0.03</td>
<td>0.00*</td>
</tr>
</tbody>
</table>

* Negative dissipation values were taken as zero.

** Significantly different from before fixation at p < 0.05.
Figure 3. (a) Vibration amplitudes of *S. aureus* 8325-4 adhering to a fibronectin film adsorbed on a glass surface and an adsorbed fibronectin film after fixation with 2% glutaraldehyde, as well as of staphylococci adhering to a fibronectin film after fixation of both the film and the ligand-receptor bond between the bacteria and the film. Fibronectin was adsorbed from solutions of different protein concentrations in high ionic strength buffer. All vibration amplitudes pertain to the low ionic strength buffer. Error bars indicate standard deviations over three experiments with separately cultured bacteria. Each experiment involves a minimum of 10 randomly selected adhering bacteria. * indicates significant differences (p < 0.05) between vibration amplitudes for different conditions of the film and bond.

(b) Schematics of vibrations of *S. aureus* 8325-4 adhering on an adsorbed fibronectin film (side-on configuration for low concentration fibronectin solution; end-on configuration for high concentration solution). Vibrations involve predominantly stretching of the bond and not of the relatively stiff, adsorbed fibronectin film (see Table 2). Note that in this schematic presentation we have only drawn one ligand-receptor bond, while in reality more bonds exist.

(c) As in (b), now for a fixed adsorbed fibronectin film. Vibrations are not affected by fixation of the fibronectin film as adsorbed fibronectin films are relatively stiff, regardless of fixation (compare Table 2).

(d) As in (b), now for a fixed adsorbed fibronectin film and bond. Fixation of the ligand-receptor bond severely restricts staphylococcal vibrations.
Table 3. Summary of vibration amplitudes (nm) of the streptococcal and staphylococcal strains included in this study on adsorbed salivary protein and fibronectin films in absence of crosslinking. Experiments were only done for bacterial strains viz a viz the protein film for which they possess specific ligands.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Saliva</th>
<th>Fn (25 µg/mL)</th>
<th>Fn (100 µg/mL)</th>
<th>Fn (250 µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em> LT11</td>
<td>79±70</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. mutans</em> IB03987</td>
<td>30±30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em> 8325-4</td>
<td>-</td>
<td>21 ± 32</td>
<td>26 ± 27</td>
<td>44 ± 59</td>
</tr>
<tr>
<td><em>S. aureus</em> DU5883</td>
<td>-</td>
<td>38 ± 48</td>
<td>60 ± 59</td>
<td>80 ± 96</td>
</tr>
</tbody>
</table>

Staphylococcal vibration amplitudes increased with fibronectin concentration in solution, probably as a result of the change from side-on to end-on adsorption of fibronectin upon increasing its concentration (Figures 3a and 3b). Since the adsorbed fibronectin films are relatively stiff already in their native state, with little impact of crosslinking, no change in vibration amplitudes was observed upon crosslinking of the films (Figures 3a and 3c). As a major difference with the ligand-receptor binding system of streptococci adhering on salivary protein films, fixation of adhering staphylococci on fibronectin films yielded a major decrease in vibration amplitude (Figure 3a) due to crosslinking of the fibronectin binding proteins on the staphylococcal cell surfaces constituting the ligand-receptor bond with the relatively stiff adsorbed fibronectin film (Figure 3d). The lack of any contribution of the hard film towards the staphylococcal vibration in absence of ligand-receptor binding is confirmed by the observation that isogenic mutant DU5883 lacking fibronectin binding proteins had a higher vibration amplitude than the wild-type strain (see Table 3).

CONCLUSIONS

In conclusion, nanoscopic vibrations exhibited by bacteria adhering to adsorbed proteinaceous conditioning films involve both the viscoelasticity of the bond as well as the softness of the adsorbed protein film. Bacterial bonds with a substratum have been demonstrated important in the interaction of bacteria adhering on substratum with their susceptibility for antimicrobial peptides. Salivary films are relatively soft.
and have a dominant influence on the vibration amplitudes of adhering streptococci compared to the viscoelasticity of the antigen I/II ligand-receptor bond through which the streptococci adhere. Adsorbed fibronectin films are relatively stiff and do not contribute to vibration amplitudes of adhering staphylococci, that are governed by the viscoelasticity of the fibronectin binding proteins on the staphylococcal cell surface mediating their adhesion to fibronectin films. These findings constitute the first results to reveal the effects of Brownian motion forces of bacteria adhering to adsorbed protein films and help to understand their role in the viscoelastic process of bacterial attachment and detachment\textsuperscript{10} and the kinetics and mechanism of action of antimicrobial peptides on bacteria.\textsuperscript{42}

**SUPPORTING INFORMATION**

Differential infrared spectrum of a glutaraldehyde crosslinked \textit{versus} an untreated salivary protein film and softnesses and thicknesses of adsorbed salivary protein films crosslinked for different time periods measured with QCM-D.
REFERENCES


Supplementary Figure S1. Differential infrared spectrum of a fixed versus an unfixed salivary protein film obtained using attenuated total reflectance Fourier transform infrared spectroscopy. The infrared spectrum shows the difference between the spectrum of adsorbed salivary proteins on a Germanium prism prior to crosslinking and after 60 s crosslinking with 2% glutaraldehyde. The absorption band at 1076 cm\(^{-1}\) indicates the presence of glycosylated proteins and phosphorus-containing compounds. The absorption band at 1660 cm\(^{-1}\) and 1550 cm\(^{-1}\) are usually identified in proteins and characterize C=O (amide I) stretching and NH (amide II) bending vibrations.\(^1\) The negative values indicate tighter binding for the molecule groups indicated due to crosslinking.

Supplementary Figure S2. The softnesses and thicknesses of adsorbed salivary protein films on silicon dioxide QCM-D crystals prior to and after crosslinking with 2% glutaraldehyde for 10 s, 60 s and 120 s, followed by rinsing with low ionic strength buffer. Salivary proteins were adsorbed from a high ionic strength buffer (57 mM, pH 6.8) containing 1.5 mg/mL of freeze-dried salivary proteins. Data were derived from dissipation and frequency shifts measured at multiple overtones in a low ionic strength buffer (0.57 mM) in which also bacterial vibration spectroscopy was carried out. Data represent averages ± standard deviations over three independent experiments.