Macroscopic and microscopic approaches toward bacterial adhesion
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A comparison of AFM interaction forces between bacteria and silicon nitride substrata for three commonly used immobilization methods

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3.1 INTRODUCTION
During the last years Atomic Force Microscopy (AFM) has been increasingly used in biosciences (Hamers, 1996; Bottomley et al., 1996). Theoretically, it combines the two most important aspects for studying structure-function relationships of biological specimens: high-resolution imaging with high signal-to-noise ratio in the molecular/sub-molecular scale and the ability to operate in aqueous environments,
allowing the observation of dynamic molecular events in real-time and under physiological conditions. The AFM is surprisingly simple in its concept. A sharp tip located at the free end of a flexible cantilever scans over a surface. Interaction forces between the tip and the sample surface subsequently cause the cantilever to deflect. The deflection signal is acquired and digitized to provide a three-dimensional image of the surface.

Several biological specimens have been imaged, achieving a lateral and vertical resolution in the nanometer and sub-nanometer scale, respectively (Bustamante & Rivetti, 1996; Shao et al., 1996; Engel et al., 1997). However, when imaging living microbial cell surfaces, the softness of the cell surface together with the high pressure over the contact area between the tip and the cell can prevent high resolution imaging. The image contrast is indeed influenced by the probe geometry, the imaging parameters, the surface topography, the visco-elastic and physico-chemical properties of the cell surface. Additional problems arise from friction and lateral displacement of the organism under study, which makes immobilization strategies critical.

Beyond being an imaging device, AFM has evolved as an instrument for measuring molecular interaction forces (Rief et al., 1997a; Rief et al., 1997b). Biological interactions that have been investigated include antibody-antigen recognition, protein-ligand binding and complementary DNA base pairing (Browning-Kelly et al., 1997; Boland & Ratner, 1995; Chilkoti et al., 1995; Florin et al., 1994; Lee et al., 1994). It was further shown that AFM can be applied to measure interaction forces between bacteria and a substratum surface, including the contribution of bacterial polysaccharides to bacterium-surface interactions (Razatos et al., 1998; Camesano & Logan, 2000). AFM has also been used to characterize under aqueous conditions the supra-molecular organization of bacterial extracellular polymeric substances (EPS) adsorbed onto solid substrata (Van der Aa & Dufrêne, 2002). Moreover, AFM allowed calculation of the Young’s modulus of the dried sheath of the archaeabacterium Methanospirillum hungatei. Measurements of the rigidity of the bacteria by AFM technique enabled the determination of the bacterial turgor pressure (Arnoldi et al., 2000).

Immobilization of the organisms is not only critical when using the AFM as an imaging device, but also when it is used as a force probe. In order to probe the structure, function, physico-chemical and mechanical properties of bacterial cell surfaces under physiological conditions, it is required that immobilization does not affect the chemical and structural integrity of the cell surface. Yet, organisms under study need to be firmly anchored in order to withstand the lateral forces of the scanning tip. Different approaches have been used for bacterial immobilization in AFM. For instance, poly-L-lysine or poly(ethyleneimide) (PEI) can be used to create positively charged glass surfaces promoting irreversible adhesion of bacteria (Bolshakova et al., 2001; Velegol & Logan, 2002). Glass slides have also been treated with aminosilanes to immobilize bacteria through cross-linking carboxyl groups on their surfaces with amine groups coupled to the glass (Camesano et al., 2000). Razatos et al. (1998) developed a procedure for coating the silicon nitride
AFM tip with a confluent layer of bacteria in which a drop of glutaraldehyde-treated bacterial suspension is placed on a PEI-coated tip (Razatos et al., 1998). Occasionally, organisms have been immobilized by mechanical trapping on membrane filters with a pore size chosen slightly smaller than the dimensions of the bacterium (Boonaert et al., 2002; Dufrêne, 2000; Van der Mei et al., 2000a). Rarely, minute glass beads functionalized with amino groups were coated with bacteria and linked to the silicon nitride cantilever using a small amount of epoxy resin (Lower et al., 2000). Other methods for the immobilization of bacteria on surfaces for AFM may exist, but it is considered beyond the scope of this paper to give a comprehensive list of sample preparation techniques. Different immobilization strategies, however, are likely to yield different results in AFM as not all methods equally well preserve the integrity of the immobilized cells.

Therefore, this chapter compares the interaction forces obtained between Klebsiella terrigena and the silicon nitride tip of an AFM for three immobilization methods: a) mechanical trapping, b) adsorption to positively charged glass, c) fixation to the tip.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strain, growth conditions and harvesting
The Gram-negative strain Klebsiella terrigena ATCC33527, occurring commonly in soil, water, grain, fruits and vegetable, was used in this study. K. terrigena was grown aerobically in Nutrient broth (OXOID, Basingstoke, UK) at 37 ºC. For each experiment, the strain was inoculated from Nutrient agar in a batch culture. This culture was used to inoculate a second culture that was grown for 16 h prior harvesting. Bacteria were harvested by centrifugation (5 min at 10,000 g), washed twice with demineralized water and resuspended in water or in 0.25 mM potassium phosphate buffer at pH 6.8.

3.2.2 Sample preparation
Imobilization of K. terrigena was carried out by three different methods:
(a) Bacterial cells were suspended in water to a concentration of $10^5$ per ml after which 10 ml of this suspension was filtered through an Isopore polycarbonate membrane (Millipore) with pore size of 0.8 µm, i.e. slightly smaller that the bacterial dimensions, to immobilize the bacteria through mechanical trapping (Kasas & Ikai, 1995). Filtration was carried out by placing a filter on a vacuum filtration flask, after which bacteria were added to the top of the filter and a vacuum was applied for approximately 10s. After filtering, the filter was carefully fixed with double-sticky tape on a glass slide and transferred to the AFM.

(b) Bacteria were also attached through electrostatic interactions (physical adsorption) to a glass slide, made positively charged through adsorption of poly-L-lysine hydrobromide. In order to coat a glass surface with poly-L-lysine hydrobromide, glass was first cleaned by sonicating for 2 min in 2 % RBS35 surfactant solution in water (Omnilabo International BV, The Netherlands), rinsed thoroughly with tap water, dipped in methanol, and again rinsed with
demineralized water, after which a drop of 0.01 % (w/v) poly-L-lysine hydrobromide solution was added. After air-drying, the slide was rinsed with demineralized water and dipped into the water bacterial suspension. After 15 min, the bacteria-coated slide was rinsed with demineralized water to remove loosely attached bacteria and transferred to the AFM (Camesano et al., 2000).

(c) Finally, bacteria were immobilized through glutaraldehyde fixation onto the silicon nitride tip of the AFM. This method requires pretreatment of both, the K. terrigena cells and the AFM cantilevers (Park Scientific Instruments, Mountain View, CA). Bacteria were first treated with 2.5 % v/v glutaraldehyde solution (pH adjusted to 6.8) for 2.5 h at 4 °C. After glutaraldehyde fixation, bacteria were washed in 0.25 mM potassium phosphate solution and pelleted by centrifugation at 10,000 g for 5 min. To prepare AFM cantilevers a drop of 1% v/v poly(ethyleneimide) solution was adsorbed onto the cantilevers for 2.5 h. The cantilevers were subsequently rinsed in demineralized water and stored at 4°C. A bacterial pellet was manually transferred onto the PEI-coated silicon nitride tip employing a micromanipulator, while viewing the procedure under an optical microscope. The bacteria-covered tip was further treated with a drop of glutaraldehyde (2.5 % v/v) at 4 °C to strengthen and anchor the pellet onto the tip. After incubation for 1 to 2 h, the cantilevers were rinsed in demineralized water and transferred to the AFM (Razatos et al., 1998).

3.2.3 Atomic force microscopy

AFM measurements were made at room temperature under 0.25 mM potassium phosphate solution at pH 6.8, using an optical level microscope (Nanoscope III Digital Instrument). “V”-shaped silicon nitride cantilevers from Park Scientific Instruments (Mountain View, CA) with a spring constant of 0.06 N m⁻¹ and a probe curvature of ~50 nm were used. Individual force curves were collected over the top of trapped and physically adsorbed bacteria on randomly selected locations with z-displacements of 100–200 nm at z-scan rates ≤1 Hz. Similarly, force curves were collected between the bacteria-coated AFM tip and silicon nitride sheets (Onstream, The Netherlands). The slope of the retraction force curves in the region where probe and sample are in contact were used to convert the voltage into cantilever deflection. The conversion of deflection into force was carried out as has been previously described by others (Dufrêne, 2000).

Approach curves were fitted to an exponential function, where the interaction force $F$ is described as $F = F_0 \exp (-d/\lambda)$, where $F_0$ is the force at zero separation distance between the interacting surfaces, $d$ the separation distance and $\lambda$ the decay length of the interaction force $F$. Retraction curves only showed a single adhesion peak in a number of cases. The percentage occurrence of an adhesion peak, its magnitude as well as the distance at which the adhesion peak appeared were recorded and averaged.

Results represent the average of at least 150 force-distance curves taken over 5 to 10 different organisms while measuring on 10 different locations per organism.
Figure 3.1. AFM deflection images of *K. terrigena* ATCC33527 trapped in an Isopore polycarbonate membrane (image size 2 µm×2 µm; z-range: 75 nm) (a) and physically adsorbed on poly-L-lysine coated glass (image size 3 µm×3 µm; z-range: 150 nm) (b) together with a scanning micrograph of an AFM tip coated with *K. terrigena* cells (c). Examples of force-distance curves between *K. terrigena* and silicon nitride for each immobilization method are presented below the corresponding images. Solid lines represent the approach curve, while the dashed lines indicate the retraction curve.
3.3 RESULTS
Figure 3.1 shows AFM deflection images of *K. terrigena* immobilized by mechanical trapping in an Isopore polycarbonate membrane (Figure 3.1a), attached through electrostatic interactions on a positively charged glass slide (Figure 3.1b) as well as scanning electron micrograph of bacteria immobilized onto the silicon nitride AFM tip (Figure 3.1c). Examples of force-distance curves performed over the top of a trapped and physical adsorbed bacterium as well as between the bacteria-coated tip and a silicon nitride sheet are presented below the corresponding images.

At first sight, similarities between force-distance curves associated with mechanically trapped and physically adsorbed bacteria (Figures 3.1a and 3.1b) can be observed. Upon approach, a long range (about 500 – 800 nm) repulsive force is encountered, while in the examples given no adhesion is recorded upon retraction of the tip from the bacterial cell surface. In contrast, as the bacteria-coated tip approached a silicon nitride sheet, repulsion began at much shorter separation distance (about 15 nm) and a single adhesion peak is always present upon retraction.

Quantitative features of the force-distance curves have been summarized in Table 3.1. It is remarkably that intimate contact between the interacting surfaces is achieved for an applied force $F_0$ that varies from 2.6 to 12 nN depending on the bacterial immobilization method used, whereas the decay length $\lambda$ of the repulsive force upon approach ranged from 2.0 to 111 nm. As the AFM tip was retracted, adhesion forces were found in 15 % to 13 % of all the cases with an average attractive force of -0.26 and -0.5 nN at separation distances of 60 and 102 nm for mechanically trapped and physically adsorbed bacteria, respectively. However, retraction of bacteria immobilized onto an AFM tip away from a silicon nitride sheet always showed adhesion with an average attractive force of -35 nN at 78 nm separation distance.

3.4 DISCUSSION
A proper interpretation of the force-distance curves between interacting surfaces in AFM requires bacterial immobilization that fully preserves the chemical and structural integrity of the cell surface. In this paper, the interaction forces between *K. terrigena* and silicon nitride are compared for three immobilization methods. Force-distance curves were different when bacteria were attached through fixation to the tip (Figure 3.1c) from those obtained for mechanically trapped or physically adsorbed bacteria (Figure 3.1a and 3.1b). For the latter two, qualitative similarities were found in force-distance curves, although for a bacterium immobilized through attachment to poly-L-lysine-treated glass stronger repulsive forces, occurring at larger separation distances, were measured upon approach (Table 3.1).

Mechanical trapping a single bacterium in a membrane filter with a pore size comparable with the dimensions of the cell does not require any chemical treatment or surface modification and the highest part of a trapped organism protrudes through the holes of the filter. Therewith, it can be easily probed with an AFM
Table 3.1. Characteristics of force-distance curves between *K. terrigena* ATCC33527 and silicon nitride for three different bacterial immobilization methods. $F_0$ is the repulsive force at zero separation distance and $\lambda$ the decay length of this repulsive force upon approach, while $F_{adh}$ is the average adhesion force recorded upon retraction, together with the separation distance $D_{adh}$ at which the adhesion force occurred. The percentage of force-distance curves for which adhesion upon retraction occurred is given, since not all force-distance curves showed adhesion upon retraction. All data are average values $\pm$ SD of 150 force-distance curves, taken over 5 to 10 different organisms while measuring on 10 different locations per organism.

<table>
<thead>
<tr>
<th></th>
<th>Mechanical trapping</th>
<th>Physical adsorption</th>
<th>Bacteria-coated AFM tip</th>
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<tbody>
<tr>
<td>$F_0$ (nN)</td>
<td>2.6 ± 1.7</td>
<td>12 ± 4</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>$\lambda$ (nm)</td>
<td>59 ± 52</td>
<td>111 ± 57</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>% adhesion</td>
<td>15</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>$F_{adh}$ (nN)</td>
<td>-0.26 ± 0.05</td>
<td>-0.5 ± 0.2</td>
<td>-35 ± 2</td>
</tr>
<tr>
<td>$D_{adh}$ (nm)</td>
<td>60 ± 8</td>
<td>102 ± 35</td>
<td>78 ± 13</td>
</tr>
</tbody>
</table>

under physiological conditions. In contrast, physical adsorption on a positively charged surface may stimulate the secretion of excess EPS by *K. terrigena*. The surface of *K. terrigena* adsorbed on a positively charged surface (Figure 3.1b) shows a similar morphology to the EPS substances previously scanned by Van der Aa & Dufrêne (2002). The surface presents stretchable coil-like structures which were depending on the scanning direction. A thicker negatively charged and highly hydrated EPS layer could account for the higher repulsion forces operating over larger distances observed upon approach of the AFM tip to such physically adsorbed bacteria. This is in line with observations by Razatos *et al.* (1998), reporting that an *Escherichia coli* mutant overproducing colanic acid in buffer, experienced greater repulsion upon approach of the AFM tip than the parent strain, which was attributed to the higher negative charge density of the capsular material produced.

The interaction force between a bacteria-coated AFM tip (Figure 3.1c) and silicon nitride sheets yields qualitatively and quantitatively distinct force-distance curves. Most notably upon approach, the distance over which repulsion is probed, is significantly reduced compared to both other methods. In addition, retraction of the bacteria-coated tip from the silicon nitride sheet always showed adhesion, whereas for the other two immobilization methods very weak adhesion forces (less than -0.5 nN) upon retraction was observed only in 13% to 15% of all force-distance curves recorded. The differences on adhesion forces could be readily attributed to the larger contact area probed by a bacteria-coated AFM tip. Assuming that five bacteria interact with the silicon nitride substratum an average adhesion force per bacterium of 7 nN can be calculated. The contact area for the other two immobilization method is
generally estimated based on the effective AFM tip radius thought to be \( \approx 250 \) nm. Therefore, average adhesion forces per bacterium of \( \approx 1 \) and \( 2 \) nN are found for mechanically trapped or physically adsorbed bacteria.

We envisage, that glutaraldehyde fixation of bacteria to a tip, stiffens the bacterium by cross-linking proteins and amino acids in the peptidoglycan layer with an impact on its adhesive properties. It has been found, for instance, that glutaraldehyde fixation caused yeast cells to become more hydrophobic (Bowen et al., 2001). However, Razatos et al. (1998) argued that glutaraldehyde treatment did not affect the adhesive properties of \( E. \ coli \) strains, because both contact angle and zeta potentials before and after glutaraldehyde treatment remained unchanged. In contrast, Burks et al. (2003) found that adhesion of these \( E. \ coli \) strains to glass was affected by glutaraldehyde treatment. Furthermore, AFM-based results showed that the addition of glutaraldehyde consistently increased the rigidity of the \( E. \ coli \) strains studied.

Table 3.2 summarizes the advantages and disadvantages of each AFM immobilization method evaluated in this study. In general, trapping bacterial cells in filters guarantees the physical and chemical integrity of the bacterial cell surface, whereas the applied vacuum needed to pull the cells into the holes could induce changes on mechanical cell surface properties. Also, adsorption of living cells onto positively charged surfaces may promote structural rearrangements in bacterial cell surface structure. Glutaraldehyde fixation of bacteria to the AFM tip clearly affects the chemical and structural integrity of the bacterial cell surface with a major impact on the interaction forces probed by AFM. Furthermore, complete coverage of the AFM tip by bacterial cells constitutes another problem. It is our experience that in three out of five cases the coverage is incomplete. See Figure 3.2.

In conclusion, the results from this study indicate that different immobilization methods of bacteria in AFM affect the qualitative and quantitative features of the force-distance curves between \( K. \ terrigena \) and silicon nitride. Mechanical trapping of single cells in a membrane filter is inferred to be the most suitable technique, as the two other methods evaluated change the chemical and structural integrity of the bacterial cell surface.

Figure 3.2. Electron micrographs of AFM silicon nitride tips after being coated with \( K. \ terrigena \) bacteria according to the procedure developed by Razatos et al. (1998). Bar marker represents 1 \( \mu \)m.
Table 3.2. Summary of advantages and disadvantages associated to bacterial immobilization method employed in this study, *i.e.* mechanical trapping of bacteria in membrane filters, physical adsorption of bacteria to a positively charged surface and cell fixation to the tip using glutaraldehyde.

<table>
<thead>
<tr>
<th></th>
<th><strong>Advantages</strong></th>
<th><strong>Disadvantages</strong></th>
</tr>
</thead>
</table>
| Mechanical trapping | ◆ Simple preparation  
◆ No chemical pre-treatment of either the tip or bacteria  
◆ Physico-chemical properties of bacterial cell remain unchanged  
◆ Exact positioning of the tip on the bacterial cell surface  
◆ Contact area can be estimated based on the dimensions of the tip | ◆ Rod-shaped bacteria are difficult to trap  
◆ Cells may be compressed as a result of applied vacuum  
◆ EPS may accumulate on the top of a trapped bacterium |
| Physical adsorption | ◆ Simple preparation  
◆ Bacteria of different shapes and dimensions can be studied  
◆ Exact positioning of the tip on the bacterial cell surface  
◆ Contact area can be estimated based on the dimensions of the tip | ◆ Chemical treatment of the substratum required  
◆ Physico-chemical properties of bacterial cell surface possibly affected by the surface modification  
◆ Immobilization not always adequate for different strains  
(Camesano *et al.*, 2000) |
| Bacteria-coated tip | ◆ Versatile choice of substratum | ◆ Long and difficult preparation procedure  
◆ Requires chemical treatment of both bacterial cell and substratum surface  
◆ Physico-chemical and mechanical properties of the bacterial cell surface changes  
◆ The number of cells interacting is unknown, as well as their spatial orientation when interacting with the substratum  
◆ Bacteria-coated tips need to be checked regularly for full coverage using electron microscopy |