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Atomic force microscopic corroboration of bond aging for adhesion of *Streptococcus thermophilus* to solid substrata

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### 6.1 INTRODUCTION

Microbial adhesion is at the basis of several medical (Dankert _et al._, 1986; Gristina _et al._, 1988), industrial (Bouman _et al._, 1982) and environmental problems (Cooksey & Wigglesworth-Cooksey, 1995). The initial stages of microbial adhesion are for a major part determined by mass transport of microorganisms towards a substratum surface, the efficiency of collisions between organisms and the substratum surface and the reversibility of the adhesion process. Microbial deposition, adhesion and desorption can best be studied in controlled flow devices.

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(Adamczyk, 1989) with in situ observation and real-time image analysis enabling the measurement of the deposition and adhesion as well as accurate information on the spatial arrangement of the adhering microorganisms and their residence time-dependent desorption (Sjollema et al., 1990b; Dabros & Van de Ven, 1982). Desorption implies that microbial adhesion is reversible. This is true for the very initial stages of the adhesion process, but overtime the adhesive bond between microorganisms and a substratum surface may strengthen, causing a prohibitive high activation energy for desorption. Alternatively, the adhesive bond may weaken overtime, leading to increased desorption rates.

A parallel plate flow chamber is a suitable device for studying the kinetics of microbial adhesion to surfaces, while modern image analysis techniques have made it possible to enumerate adhesion and residence time-dependent desorption simultaneously in situ (Meinders et al., 1992; Sjollema et al., 1989). The measured desorption will therefore be indicative for the reversibility of the process, although it may include a contribution from collisions between flowing and adhering organisms as occurring in natural systems (Van de Ven, 1989). Dabros & Van de Ven, (1982) proposed that desorption rate coefficients for a particle adsorbed at time \( \tau \) and desorbing at time \( t \), i.e. after residing on the surface for a time \( (t-\tau) \), change exponentially from an initial value \( \beta_0 \) to a final value \( \beta_\infty \) during aging of the bond with a relaxation time \( 1/\delta \)

\[
\beta(t-\tau) = \beta_\infty - (\beta_\infty - \beta_0) e^{-\delta(t-\tau)} \quad (6.1)
\]

Depending on whether the initial desorption rate coefficient \( \beta_0 \) is larger or smaller than the final value \( \beta_\infty \), bonds are assumed to strengthen or weaken during aging.

Meinders et al. (1994) applied this model to analyze the residence time-dependent desorption of *Streptococcus thermophilus* B, a fouling organism encountered in dairy processing (Bouman et al., 1982), in a parallel plate flow chamber under moderate shear conditions of 50 s\(^{-1}\). Desorption rate coefficients decreased exponentially on glass with residence time \( (t-\tau) \) from an initially high value \( \beta_0 \) to an almost negligibly low value \( \beta_\infty \). Initial desorption rate coefficients were higher for experiments carried out at pH 2 (30\(\times\)10\(^{-4}\) s\(^{-1}\)) than for experiments at pH 7 (23\(\times\)10\(^{-4}\) s\(^{-1}\)). The initial desorption rate coefficients decreased rapidly to about 10\(\times\)10\(^{-6}\) s\(^{-1}\) with a relaxation time of about 48 and 59 s for pH 2 and 7, respectively. Consequently, it was concluded that the bond strength between *S. thermophilus* and glass substratum surfaces increased during aging, although this conclusion was never verified experimentally due to the absence of adequate instrumentation at that time.

Atomic force microscopy (AFM) provides a direct measure of the adhesive forces between microorganisms and surfaces. Utilizing a small tip mounted on a flexible cantilever, interaction forces between the tip and a bacterial surface can be recorded as the tip approaches, makes contact and retracts from the bacterial surface. Accordingly, AFM has been used extensively to study the initial interaction between bacteria and different substratum surfaces in aqueous solutions, including quantification of the contribution of surface polymers to bacterial
adhesion (Razatos et al., 1998; Camesano & Abu-Lail, 2002). Mechanical properties, such as turgor pressure and Young’s modulus of the bacterial cell surface, have also been calculated by means of AFM (Arnoldi et al., 2000; Velegol & Logan, 2002). In contrast, interfacial and adhesive forces originated during physical contact of the bacteria with the AFM tip are only sparsely found in literature (Abu-Lail & Camesano, 2003a; Lower et al., 2000).

The aim of this chapter is to analyze microscopically the time dependence of the bond strength between \textit{S. thermophilus} B and the Si$_3$Ni$_4$ tip of an AFM, in order to present an independent verification of the conclusions drawn by Meinders et al. (1994) on aging of the bond between this organism and glass substratum surfaces.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strain and growth conditions
\textit{S. thermophilus} B was isolated from a heat exchanger plate in the downward section of a pasteurizer. Bacteria were grown overnight at 37 °C from a frozen stock in batch culture in M17 broth (OXOID), supplemented with 1 % saccharose. This culture was used to inoculate a second culture, which was grown for 20 h. After 20 h, cells were harvested by centrifugation at 5,000 g, washed twice in demineralized water and resuspended in 40 mM potassium phosphate solution with pH adjusted to 2 and 7 by addition of HCl or KOH.

6.2.2 Atomic force microscopy (AFM)
Bacteria were suspended in water to a concentration of $10^5$ per ml after which 10 ml of the suspension was filtered through an Isopore polycarbonate membrane (Millipore) with a pore size of 0.8 µm (Kasas & Ikai, 1995). The pore size was chosen slightly smaller than the streptococcal dimensions to immobilize the bacteria by mechanical trapping. After filtration, the filter was carefully fixed with double-sided sticky tape on a sample glass and transferred to the AFM. AFM measurements were made at room temperature under 40 mM potassium phosphate solution at pH 2 and 7, 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 $\sim$ 50 nm, according to manufacturer specifications, were used. Individual force curves with $z$-displacements of 100 – 200 nm were collected over the top of a trapped bacterium at randomly selected locations. Retraction of the tip from the bacterial surface was carried out after different contact times between the AFM tip and bacterial cell surface, ranging from 1 to 200 s. 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 data to force data was carried out as has been previously described by others (Dufrêne, 2000). The maximum adhesion peak $F_{adh, max}$ upon retraction was recorded as function of contact time between the AFM tip and the bacterial cell surface. For each bacterial cell, two force-distance curves were measured for each given time, and five cells were examined for a given pH.
6.3 RESULTS AND DISCUSSION

The initial stages of microbial adhesion are considered to be dominated by Lifshitz-Van der Waals and electrostatic interactions (Rutter & Vincent, 1980). Over a wide range of ionic strengths, the energy distance curve of the interaction between a bacterial cell and a surface of the same charge sign is characterized by the presence of a shallow, secondary minimum at separation distances of a few up to ten nm, an energy barrier closer to the surface and a deep, primary energy minimum at short separation distances (< 1 nm) (Norde & Lyklema, 1989). It is thought that after microorganisms are initially captured in a reversible fashion in the secondary energy minimum their extracellular polymeric substances (EPS) firmly anchor at the substratum surface thereby successfully surpassing the energy barrier (Busscher et al., 1992).

The adhesive forces between a hydrophilic, negatively charged AFM tip and S. thermophilus B cell surface are shown in Figure 6.1 for pH 2 after a contact time of 10 and 130 s. As the tip retracts the bacterial surface, several adhesion events are observed before reaching a maximum value $F_{\text{adh, max}}$. After having overcome $F_{\text{adh, max}}$, the tip eventually detaches stepwise reaching a null adhesion force between the tip and the bacterial cell surface at a relatively large separation distance (~ 800 nm). Note that this does not necessarily indicate that the organisms are surrounded by an 800 nm thick EPS coat, as EPS chains may extend during retraction of the AFM tip.

Figure 6.2 shows the maximum adhesion peak $F_{\text{adh, max}}$ observed upon retraction of the AFM tip from the bacterial cell surface as function of the contact time at pH 2 and pH 7. Bond strengthening occurs at both pH values yielding an increase in adhesive force over time. Although differences in initial and final desorption rate coefficients of S. thermophilus were reported for pH 2 and pH 7, these differences are not reflected in statistically significant way in the increases in adhesive force between the AFM tip and the cell surface (see Figure 6.2).

To our knowledge, this paper is the first one reporting experimental proof of aging of the bond between bacteria and a substratum surface and relating this bond aging to resident time-dependent desorption of organisms from a surface. Although glass is admittedly not similar in surface properties as Si$_3$Ni$_4$, of which the AFM tip is made, both surfaces are hydrophilic and negatively charged. As an important qualitative association between macroscopic resident time-dependent desorption and bond aging as measured in AFM, it is noted that the relaxation times of the resident time-dependent desorption are of the same order of magnitude as the time scales on which the adhesive bond strengthens according to the AFM measurements.

Meinders et al. (1994) calculated a bond strength energy of 16 to 17 kT per cell for S. thermophilus B on glass, by applying

$$\beta_{\text{escape}} = (j_0 a / D_\infty c) (D_\infty / a \Delta h) \exp(\phi_m / kT)$$

(6.2)

(where $\beta_{\text{escape}}$ is the escape rate coefficient, $j_0$ the initial deposition rate, $a$ the particle radius, $D_\infty$ the Stokes-Einstein diffusion coefficient, $c$ the bacterial cell...
Figure 6.1. Retraction line of a force-distance curve for *S. thermophilus* B in 40 mM potassium phosphate solution at pH 2 after a contact time of 10 s (a) and 130 s (b) between the AFM tip and the bacterial cell surface.
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Figure 6.2. Maximum adhesion peak $F_{\text{adh, max}}$ upon retraction as function of the contact time between the AFM tip and bacterial cell surface in 40 mM potassium phosphate solution at pH 2 (♦) and pH 7 (●). Error bars denote the SD in $F_{\text{adh, max}}$.

concentration at the entrance of the flow chamber, $\Delta h$ the width of the energy minimum, $\phi_m$ depth of the energy minimum and $kT$ the energy of thermal motion), as proposed by Xia et al. (1989), and inserting measured values for the initial deposition rates and a desorption rate coefficient $\beta_{\text{escape}}$ measured in the absence of particle flow. Alternatively, integration of the area under the maximum adhesion peak after 130 s of contact in the force-distance curves yields a bond strength energy of $10^4 - 10^5$ kT between the tip and the organism. Comparing with the bond strength energy from Meinders et al. (1994), this would indicate that the bond between the tip and the bacterial cell surface is $10^3 - 10^4$ fold stronger than between a bacterium and a macroscopic substratum surface. The bond strength energy per organism estimated by Meinders et al. (1994) suggests that desorption of *S. thermophilus* from glass, under the experimental conditions of the parallel plate flow chamber, takes place out of the secondary minimum of the energy distance curve according to the DLVO theory for colloidal stability (Bos et al., 1999). The experimentally decreasing desorption rate with increasing residence time of the bacterial cell near the surface may reflect the rate by which EPS bridge the separation between the cell and the surface and anchor to the latter. Irreversible anchoring thus occur as a result of multiple attachments of EPS to the substratum surface during bond aging, that stepwise detach in the parallel plate flow chamber until an organism is weakly attached. The final bond strength energy to overcome prior to escape from the surface and calculated from macroscopic desorption amounts 16 – 17 kT. Like after bond aging in the flow chamber, there is close contact between the AFM tip and the bacterial cell surface. This contact is forced
by pushing the organism into the primary minimum, and may become more intimate through multiple contacts between EPS and the AFM tip than in the flow chamber. Detachment of the AFM tip from the bacterial cell surface after close contact is reached by the application of an external force to the tip. Therefore, opposite to detachment in the flow chamber, detachment from the AFM tip is forced after stepwise disruption of multiple EPS contacts which would account for the huge bond strength energy estimated from the retracting force-distance curves.

In conclusion, this chapter presents qualitative associations between resident time-dependent bacterial desorption from a macroscopic glass surface with strengthening of the microscopic bond of a bacterium to the tip of an AFM. Quantitative considerations of the bond strength energy from force-distance curves and those derived from resident time-dependent desorption under flow, indicate that macroscopic desorption of bacteria takes place from the secondary minimum of the energy distance curve, whereas bacterial detachment from the AFM tip develops stepwise from the corresponding primary minimum. Bond strengthening thus seems to be a general result of multiple attachments of EPS to a substratum surface.