Analysis of Bacterial Detachment from Substratum Surfaces by the Passage of Air-Liquid Interfaces

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A theoretical analysis of the detachment of bacteria adhering to substratum surfaces upon the passage of an air-liquid interface is given, together with experimental results for bacterial detachment in the absence and presence of a conditioning film on different substratum surfaces. Bacteria (Streptococcus sobrinus HG1025, Streptococcus oralis J22, Actinomyces naeslundii T14V-J1, Bacteroides fragilis 793E, and Pseudomonas aeruginosa 974K) were first allowed to adhere to hydrophilic glass and hydrophobic dimethyldichlorosilane (DDS)-coated glass in a parallel-plate flow chamber until a density of 4 × 10^6 cells cm^-2 was reached. For S. sobrinus HG1025, S. oralis J22, and A. naeslundii T14V-J1, the conditioning film consisted of adsorbed salivary components, while for B. fragilis 793E and P. aeruginosa 974K, the film consisted of adsorbed human plasma components. Subsequently, air bubbles were passed through the flow chamber and the bacterial detachment percentages were measured. For some experimental conditions, like with P. aeruginosa 974K adhering to DDS-coated glass and an air bubble moving at high velocity (i.e., 13.6 mm s^-1), no bacteria detached upon passage of an air-liquid interface, while for others, detachment percentages between 80 and 90% were observed. The detachment percentage increased when the velocity of the passing air bubble decreased, regardless of the bacterial strain and substratum surface hydrophobicity involved. However, the variation in percentages of detachment by a passing air bubble depended greatly upon the strain and substratum surface involved. At low air bubble velocities the hydrophobicity of the substratum had no influence on the detachment, but at high air bubble velocities all bacterial strains were more efficiently detached from hydrophilic glass substrata. Furthermore, the presence of a conditioning film could either inhibit or stimulate detachment. The shape of the bacterial cell played a major role in detachment at high air bubble velocities, and spherical strains (i.e., streptococci) detached more efficiently than rod-shaped organisms. The present results demonstrate that methodologies to study bacterial adhesion which include contact with a moving air-liquid interface (i.e., rinsing and dipping) yield detachment of an unpredictable number of adhering microorganisms. Hence, results of studies based on such methodologies should be referred as “bacterial retention” rather than “bacterial adhesion.”

Bacterial adhesion forms the basis for several diverse problems in medicine (5, 10, 21, 38), industry (3, 15, 29, 32), and environmental areas (8, 33). The most detrimental effects are encountered in medicine, where failure of implanted devices may result from surface-associated bacterial infections. Different environmental, biological, and substratum-related factors have been proposed to influence bacterial adhesion to surfaces at the onset of the formation of a mature biofilm. Bacterial adhesion in natural and industrial environments is preceded by the formation of conditioning films, consisting of adsorbed organic components. These films may inhibit or promote bacterial adhesion (35, 37), and the final properties of the conditioning film have been suggested to be under the control of the physicochemical properties of the underlying substratum surface, such as its hydrophobicity and charge (40).

Adhesion of microorganisms to substratum surfaces is difficult to measure, and a variety of static and dynamic (25, 36) systems have been developed by different research groups, currently often with the aid of image analysis. Sometimes mass transport considerations are included in the design (9, 39), but frequently no distinction is made between kinetic and station-

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on rocks and ship hulls in the marine environment (8, 12). Adhering organisms, responsible for biodeterioration of monumental buildings, have to adhere strongly enough to withstand rain (41).

Several processes occur in rapid sequence when an air-liquid interface passes over an adhering micron-sized particle. These processes have only recently been fully understood and need to be introduced into microbiology in order to stimulate methodological advances in the field. Detachment of adhering polystyrene particles by the passage of air bubbles has been demonstrated to be more efficient when (i) the velocity of the passing air bubble decreases (19, 20), (ii) the air-liquid interfacial tension increases (19, 20), (iii) particle size decreases (17), (iv) the substratum surface is more hydrophobic (19), and (v) the substratum surface is negatively charged (18).

The aim of the present paper is to provide a theoretical background of the processes occurring during the passage of an air-liquid interface over bacteria adhering to a substratum surface and to report on the air bubble-induced detachment of different bacterial strains from hydrophilic and hydrophobic substratum surfaces in the absence and presence of a conditioning film.

THEORETICAL BACKGROUND

Detachment of an adhering bacterium from substratum surfaces by a passing air bubble is controlled by a sequence of processes that includes the approach of the air bubble to the adhering bacterium; interception of the bacterium by the air bubble; deformation of the air-liquid interface, yielding a thin liquid film separating the bacterium and the air bubble; formation of a three-phase contact between an adhering bacterium, air, and liquid; and subsequent transport of the bacterium-bubble aggregate away from the substratum surface (19). Hence, the efficiency of the bacterial detachment ($E_b$) by a passing air-liquid interface can be expressed as $E_b = E_C \times E_A \times E_S$, where $E_C$ is the air bubble-bacterium collision efficiency, $E_A$ is the bubble-bacterium attachment efficiency, and $E_S$ is the stability of the bubble-bacterium aggregate.

The direction and velocity of the air bubble with respect to the adhering bacterium determine the collision efficiency ($E_C$) (31). In a parallel-plate flow chamber, for an air bubble fully spanning the width of the chamber, the collision efficiency is 1. However, an air bubble moving in a narrow channel is surrounded by a liquid film (4), the thickness of which increases with increasing air bubble velocity. As this liquid film thickness decreases, the collision efficiency increases, as outlined in Fig. 1.

The interaction between a passing air bubble and an adhering bacterium in an aqueous medium includes, apart from hydrodynamic forces, Lifshitz-van der Waals, electrostatic, and hydrophobic (acid-base) forces (22). Hence, the bubble-bacterium attachment efficiency will increase with bacterial cell surface hydrophobicity (14). Note that an effective three-phase contact between the bubble and an adhering bacterium (Fig. 1) can form only when the bacterium-bubble contact time is long enough to thin the liquid film and form a three-phase boundary (23). Hence, the bubble-bacterium attachment efficiency will also increase with decreasing air bubble velocity. During thinning of the liquid film, a viscous force opposes the attachment of the bacteria to the air bubble. This viscous drag force increases with the size of the bacterium and the air bubble velocity (17).

Once and only if an effective three-phase contact between the bubble and an adhering bacterium has formed, a balance
between the surface tension force ($F_s$), the bacterial adhesion force ($F_a$), and the viscous drag force determines whether detachment will occur. Evidently (Fig. 1), the surface tension force opposes the adhesion and viscous drag forces depending on the position of the three-phase contact and can be expressed by the equation $F_s = 2\pi R_B \gamma_s \sin[\phi(t)] \sin[\theta - \phi(t)]$, in which $R_B$ is the radius of the bacterium, $\gamma_s$ is the air-liquid interfacial tension, $\phi$ represents the angle determining the position of the bacterium with respect to the air-liquid interface, $t$ is time (in seconds), and $\theta$ is the contact angle between the surface of the bacterium and the air-liquid interface. Figure 2 presents the surface tension detachment force as a function of $\phi$ for different bacterial contact angles or cell surface hydrophobicities, $\theta$. As can be seen, for each bacterial cell surface hydrophobicity, the surface tension detachment force passes through a maximum value at a different degree of immersion of the adhering bacteria (see also Fig. 1).

The adhesion force can be calculated theoretically according to the extended DLVO (Derjaguin-Landau-Verney-Overbeek) approach as a combination of Lifshitz-van der Waals, electrostatic, and acid-base forces (42) as follows: $F_a(d) = F_{LV}(d) + F_{EL}(d) + F_{AB}(d)$, where $F_{LV}(d)$ and $F_{EL}(d)$ denote the decay with the distance ($d$) to the collector surface of the Lifshitz-van der Waals and electrostatic interaction forces, respectively, and $F_{AB}$ denotes acid-base interaction forces, but theoretical adhesion forces that match experimentally reported adhesion forces have not yet been calculated. The most reliable estimates of bacterial adhesion forces have become available from atomic-force microscopy and range from $7 \times 10^{-7}$ to $4 \times 10^{-9}$ N between sulfate-reducing bacteria and Si$_2$N$_4$ or mica surfaces (13). Such adhesion forces are orders of magnitude smaller than the surface tension detachment forces induced by a passing air-liquid interface, ranging up to $2 \times 10^{-7}$ N, as can be seen in Fig. 2.

Once a bacterium is detached from a substratum surface, the balance between the surface tension forces acting on the bacterium as opposed to external forces in the flow (i.e., gravitational force, buoyancy force, hydrostatic pressure, and viscous drag force opposing the separation of the bacteria from the collector surfaces) determines the bacterium-bubble aggregate stability (38). For the dimensions considered in the parallel-plate flow chamber (i.e., $d/R_B \ll 1$ and $R_B \ll 10 \mu$m), the surface tension force dominates (28).

**MATERIALS AND METHODS**

**Microorganisms and culture conditions.** Five bacterial strains were used in this study. *S. oralis* T14V-J1 was cultured in Schaedler’s broth supplemented with 0.01 g of hemin liter$^{-1}$ and *B. fragilis* 793E was cultured in chopped-meat medium (in one liter: 0.30% beef extract [Difco Laboratories, Detroit, Mich.], 30 g of peptone, 5 g of yeast extract, 5 g of K$_2$HPO$_4$, and 0.56 g of L-cysteine HCl · H$_2$O [pH 7.0]) both in an anaerobic cabinet (Don Whitley Scientific, West Yorkshire, United Kingdom) and in an atmosphere of 10% H$_2$, 85% N$_2$, and 5% CO$_2$ at 37°C. For each experiment, strains were inoculated from blood agar plates in batch culture for 24 h. This culture was used to inoculate a second culture, which was grown for 16 h prior to harvesting.

Bacteria were harvested in stationary phase by centrifugation (5 min at 5,500 × g) and washed twice with demineralized water, and to break bacterial chains and aggregates, cells were sonicated for 30 s at 30 W (Vibra Cell model 375; Sonics and Materials, Inc., Danbury, Conn.). Sonication was performed intermittently while cells were cooling in an ice-water bath. Finally, bacteria were suspended in a buffer (1 mM calcium chloride, 2 mM potassium phosphate, and 50 mM sodium phosphate, pH 7.5).
Experimental protocol. All experiments were performed at room temperature in triplicate with separate bacterial suspensions and collector surfaces. Before each experiment, all tubes, the flow chamber, and the syringe pump were filled with buffer and care was taken to remove all air bubbles from the system. The buffer solution was first perfused through the chamber for 30 min. At this stage and when appropriate, the flow was switched to adhesion buffer supplemented with 1.5 g of lyophilized saliva liter⁻¹ or with 10% pooled plasma for 2 h to create a conditioning film, after which the flow was switched for 20 min to buffer to remove all remnants of saliva or plasma from the tubing and the flow chamber. Thereafter, bacteria were allowed to adhere until a density of $4 \times 10^8$ cells ml⁻¹ was reached, as enumerated on 10 arbitrarily chosen areas of 0.017 mm² distributed over the length of the substratum surface. The volumetric flow was 0.025 ml s⁻¹ which yields a laminar flow (Reynolds number 0.6). Finally, the flow was switched again for 30 min to the buffer solution in order to remove all nonadhering bacteria from the flow chamber and the tubing system. In order to introduce air bubbles in the flow system, a syringe pump (model SC512; Terumo Tokyo, Japan) filled with the buffer solution was connected to the flow chamber by a three-way valve to control the velocity of the air bubble. Air bubbles (0.1 ± 0.02 ml) were introduced through injection in the tubing by a 1-ml syringe. The approximate projected dimensions of the air bubbles were 25 by 5 mm (length by width), i.e., they fully spanned the width of the flow chamber. Subsequently, the bacteria that remained adhering were enumerated, after the positions used before were retrieved with the aid of the cell finder.

Data analysis. Detachment data were analyzed by a one-way analysis of variance. Analysis of variance tests the ratios of the mean squares between groups and within groups, and provides an F ratio (i.e., Fisher distribution) and its corresponding P value. If the null hypothesis is true, the F ratio should be approximately 1 since both the mean squares between and within groups should be about the same. If the ratio is much larger than 1, the null hypothesis is false. The shape of the F distribution depends on the degrees of freedom within and between groups. The lower the degree of freedom, the larger the F statistic.

RESULTS

Table 1 presents the water contact angles on the different bacterial strains and substratum surfaces involved in this study, together with their zeta potentials in adhesion buffer.

### Table 1. Water contact angles on the different bacterial strains and substratum surfaces involved in this study, together with their zeta potentials in adhesion buffer

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Substratum surface</th>
<th>$\theta_w$ (°)</th>
<th>$\zeta$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sobrinus HG1025</td>
<td>Glass</td>
<td>29</td>
<td>-14</td>
</tr>
<tr>
<td>S. oralis J22</td>
<td>Glass</td>
<td>24</td>
<td>-16</td>
</tr>
<tr>
<td>A. naeslundii T14V-J1</td>
<td>Glass</td>
<td>64</td>
<td>-12</td>
</tr>
<tr>
<td>B. fragilis 793E</td>
<td>Glass</td>
<td>55</td>
<td>-17</td>
</tr>
<tr>
<td>P. aeruginosa 974K</td>
<td>Glass</td>
<td>43</td>
<td>-26</td>
</tr>
<tr>
<td>Glass</td>
<td>DDS-coated glass</td>
<td>33</td>
<td>-17</td>
</tr>
</tbody>
</table>

* Adhesion buffer contained 1 mM calcium chloride, 2 mM potassium phosphate, and 50 mM potassium chloride (pH 6.8). All data are the mean results of three experiments with separately grown bacterial strains and substratum surfaces, yielding an average standard deviation of around 5 degrees for the contact angles ($\theta_w$) and 3 mV for the zeta potentials ($\zeta$).
from \(-12\) mV for *A. naeslundii* T14V-J1 to \(-26\) mV for *P. aeruginosa* 974K.

Table 2 shows the percentages of detachment of the different bacterial strains from hydrophilic glass and hydrophobic DDS-coated glass by a passing air bubble in the absence and presence of a conditioning film. Regardless of the bacterial strain involved and substratum surface hydrophobicity, the percentage of detachment was highest at the lowest velocity of the passing air bubble \((F_{4,2} = 43.2, P < 0.000007)\). However, the percentage of detachment by a passing air bubble depended greatly upon the strain and substratum surface involved, as well as on the presence or absence of a conditioning film on the substratum surface. At the lowest air bubble velocity \((i.e., 2.36\) mm s\(^{-1}\)) all bacterial strains presented similar detachment percentages when they adhered to hydrophilic glass substrata \((F_{3,48} = 1.2, P = 0.37)\) but differed significantly when they adhered to hydrophobic DDS-coated substrata \((F_{3,48} = 18.21, P = 0.0001)\). At the highest air bubble velocity \((i.e., 13.6\) mm s\(^{-1}\)), all bacterial strains detached more easily from hydrophilic glass than from hydrophobic DDS-coated glass substrata \((F_{3,20} = 22.0, P = 0.00006)\). Both streptococcal strains \((S. oralis J22 and S. sobrinus HG1025)\) were more effectively detached than rod-shaped organisms \((A. naeslundii T14V-J1, B. fragilis 793E, and P. aeruginosa 974K)\) when the air bubble moved at the highest air bubble velocity \((F_{4,96} = 19.3, P = 0.0009)\).

The presence of a conditioning film had various effects on detachment of adhering bacteria. While its presence did not affect the detachment of *B. fragilis* 793E, *A. naeslundii* T14V-J1, and *P. aeruginosa* 974K \((F_{4,36} = 0.3, P = 0.64)\), it stimulated the detachment of the streptococcal strains at the lowest air bubble velocity regardless of the hydrophobicities of the substrata \((F_{4,36} = 7.4, P = 0.012)\).

Redeposition of bacteria once they were detached or displacement of adhering bacteria by a passing air bubble was not observed, except with *P. aeruginosa* 974K after its detachment from hydrophobic DDS-coated glass in the absence of a conditioning film. While 90% of the *P. aeruginosa* 974K organisms were effectively detached without redeposition or displacement by air bubbles at a velocity of 2.36 mm s\(^{-1}\) \((F.3)\), no bacterial detachment was observed at an air bubble velocity of 13.6 mm s\(^{-1}\) \((F.4)\). Instead, areas on the substratum located near the sides of the flow chamber did not show any adhering bacteria after the passage of an air bubble \((F.4a)\) but areas near the center showed large bacterial agglomerates \((F.4b)\) from displacement and redeposition of adhering bacteria.

**DISCUSSION**

In this study a theoretical analysis of the detachment of bacteria adhering to substratum surfaces upon the passage of air-liquid interfaces is presented, together with experimental results for bacterial detachment in the presence of conditioning film on different substratum surfaces. Greater bacterial detachment was achieved by decreasing the air bubble velocity, regardless of the hydrophobicities of the substrata or bacterial strains or the presence of a conditioning film. These results are in line with previous observations on detachment of polystyrene particles from different substrata where a linear decrease in detachment was observed with an increasing air bubble velocity \((17–20)\). Presumably, at high air bubble velocities, the bacterium-bubble attachment efficiency decreases due to a lower degree of immersion of the bacterium in the air-liquid interface \((F.1)\) and, hence, the detachment force \(F_3 = 2\pi R_0\sin(\theta(t))\sin(\theta - \theta(t))\) induced by a passing air-liquid interface decreases \((19)\).

Nevertheless, bacterial cell and substratum hydrophobicity play a role in the detachment process. At low air bubble velocities, the detachment efficiencies were similar for all bacterial strains when they adhered to a hydrophilic substratum but differed when they adhered to a hydrophobic substratum. Presumably, bacterial surface hydrophobicity plays a more important role when bacteria adhere to a hydrophobic substratum, as the detachment of hydrophobic polystyrene particles \((i.e., water contact angle of 90 degrees)\) stimulated by a passing air bubble was not influenced by substratum hydrophobicity at low air bubble velocities \((19)\). At high air bubble velocities, strains detached more efficiently from a hydrophilic substratum surface. In this respect it must be realized that at low air bubble velocities, the detachment force is about 2 orders of magnitude greater than the bacterium-substratum adhesion force \((F.2)\) due to a high degree of immersion of the bacterium \((F.1c)\). Hence, substratum properties are not decisive in the detachment process. Conversely, at high velocities, the detachment force induced by the passing air bubble is lower \((i.e., due to a lower degree of immersion \((F.1b)\)) and becomes comparable.

**Table 2. Percentages of bacterial detachment by a passing air bubble moving at different velocities from glass and DDS-coated glass in the presence and absence of a conditioning film**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Glass</th>
<th>DDS-coated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. sobrinus HG1025(^a)</td>
<td>52 ± 15</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>S. oralis 322(^a)</td>
<td>72 ± 15</td>
<td>52 ± 20</td>
</tr>
<tr>
<td>A. naeslundii T14V-J1(^a)</td>
<td>68 ± 18</td>
<td>41 ± 22</td>
</tr>
<tr>
<td>B. fragilis 793E(^b)</td>
<td>57 ± 14</td>
<td>60 ± 21</td>
</tr>
<tr>
<td>P. aeruginosa 974K(^b)</td>
<td>58 ± 22</td>
<td>90 ± 7</td>
</tr>
</tbody>
</table>

\(^a\) Conditioning film consisted of saliva.

\(^b\) Conditioning film consisted of adsorbed plasma proteins.

Note that prior to the passage of an air bubble, the number of adhering bacteria was set at \(4 \times 10^6\) cm\(^{-2}\), as enumerated by the image analysis system. All data are means ± standard deviations based on the results of three experiments with separately grown bacterial strains and substratum surfaces.
to the bacterium-substratum adhesion force, making substratum properties decisive in bacterial detachment.

The present study reveals two new aspects of air-liquid interface passage-stimulated detachment of bacteria which have not been observed for polystyrene particles. For *P. aeruginosa* 974K detaching from DDS-coated glass, the detachment efficiency was almost 0 and redeposition and displacement were seen. These findings likely indicate that a combination of lateral capillary forces between bacterial cells and adhesion forces counteracts the surface tension detachment force to form aggregates, as was also suggested for proteins and latex spheres (11, 26). In addition, the present study reveals an effect of particle shape on detachment, since it appears that coccal cells detach more readily upon the passage of an air-liquid interface than rod-shaped organisms, like *P. aeruginosa* 974K, *B. fragilis* 793E, and *A. naeslundii* T14V-J1.

Conditioning films have been described to promote or hinder bacterial adhesion (4, 37), and in this study conditioning...
films of adsorbed salivary components or plasma proteins are described to have various effects on detachment of different strains after the passage of an air-liquid interface. In an earlier inventory it was suggested that the presence of a conditioning film yielded higher detachment than when bacteria adhered to a bare substratum surface (2). However, in the present study the number of adhering bacteria prior to the passage of an air-liquid interface was set for all combinations of strains and substratum surfaces (2). However, in the present study the number of adhering bacteria prior to the passage of an air-liquid interface was set for all combinations of strains and substratum surfaces to $4 \times 10^6$ cm$^{-2}$, whereas in an inventory by Bos et al. (2), the number of adhering bacteria varied strongly prior to detachment.

This study yields several important conclusions with respect to the study of bacterial adhesion to surfaces. First, since an unknown number of adhering bacteria is detached by passages of an air-liquid interface, as during slight rinsing and dipping, in situ enumeration methods should be preferred for the study of bacterial adhesion. In case in situ enumeration methods are not available, the terminology should be adjusted to emphasize that bacterial retention was measured rather than adhesion. In the latter case, a description of the detachment forces caused by slight rinsing and dipping that is as detailed as possible should be given. Second, it is important to realize that bacterial detachment by a passing air-liquid interface is smallest when the interface moves at a higher velocity, as this allows no time for an in situ enumeration methods should be preferred for the study of bacterial adhesion to surfaces. First, since an unknown number of adhering bacteria is detached by passages of an air-liquid interface, as during slight rinsing and dipping, in situ enumeration methods should be preferred for the study of bacterial adhesion. In case in situ enumeration methods are not available, the terminology should be adjusted to emphasize that bacterial retention was measured rather than adhesion. In the latter case, a description of the detachment forces caused by slight rinsing and dipping that is as detailed as possible should be given. Second, it is important to realize that bacterial detachment by a passing air-liquid interface is smallest when the interface moves at a higher velocity, as this allows no time for a three-phase contact to form.

REFERENCES


