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Role of eDNA on the Adhesion Forces between *Streptococcus mutans* and Substratum Surfaces: Influence of Ionic Strength and Substratum Hydrophobicity

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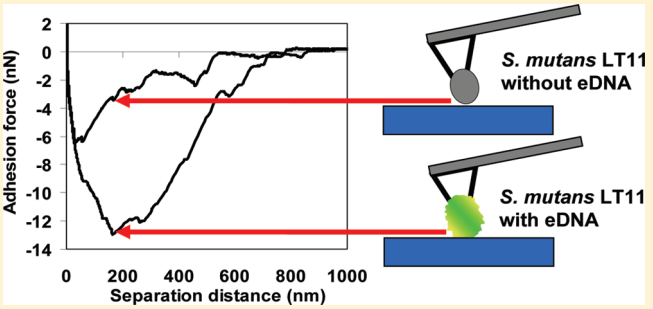
ABSTRACT: The aim of this study was to investigate the role of extracellular DNA (eDNA) on the adhesion strength of *Streptococcus mutans* LT11 on substrata with different hydrophobicities at high and low ionic strengths. AFM adhesion forces to a hydrophilic and hydrophobic substratum increased with increasing surface-delay times and ionic strength and were stronger on a hydrophobic than on a hydrophilic substratum. The presence of eDNA on the streptococcal cell surface enhanced its adhesion force to a hydrophobic substratum significantly more than to a hydrophilic substratum, especially after bond maturation. Bond maturation on a hydrophilic substratum was accompanied by an increasing number of minor adhesion peaks, indicating the involvement of acid−base interactions, whereas on the hydrophobic substratum surface the number of minor adhesion peaks remained low. More minor adhesion peaks developed on the hydrophilic substratum at low ionic strength than at high ionic strength. The final rupture distance in retraction force−distance curves was independent of ionic strength on a hydrophilic substratum and increased with increasing surface delay time. On the hydrophobic surface, the final rupture distance did not increase with surface delay time but was significantly smaller at low than at high ionic strength. Final rupture distances were different in presence and absence of eDNA, and the lower values of this difference coincided with the decrease in hydrodynamic radius of the streptococci upon increasing ionic strength, measured using dynamic light scattering. AFM also yielded higher values for the ionic strength induced difference in final rupture distance because in AFM rupture is forced, while in dynamic light scattering differences in radius are only induced by ionic strength differences.



INTRODUCTION

Bacteria adhere to almost any surface in nature, leading to the formation of biofilms.[1] Bacterial adhesion and biofilm formation are mediated by the bacterial production of extracellular polymeric substances (EPS). EPS is composed of multiple components, including proteins, polysaccharides, and extracellular DNA (eDNA).[2] EPS on a bacterial cell surface significantly influences its physicochemical properties, including cell surface charge and hydrophobicity, i.e., the cell surface free energy and its components.[3,4] Surface charge and free energy of bacteria and substrata are generally considered important factors governing bacterial adhesion and biofilm formation to substratum surfaces,[3,5−7] and both depend on the surface chemical composition of the interacting surfaces.[3,8]

Once adhering, bacteria often experience detachment challenges which decrease the chance of successful biofilm formation. Under almost all conditions allowing bacterial adhesion and biofilm formation, detachment forces can arise from fluid flow, like in aqueous systems, pipelines, food-processing equipment, and in the oral cavity due to salivary flow, mastication, and tongue movement. Therefore, not only adhesion but moreover the forces with which bacteria adhere to a substratum surface are essential for biofilm formation in many natural environments. Atomic force microscopy (AFM) is a rapidly emerging technique to measure the adhesion force to be overcome to cause detachment of adhering bacteria.[9] In AFM, a bacterium is immobilized on the AFM cantilever and brought into contact with a substratum surface upon which the bacterial probe is retracted from the surface and the force required to detach the bacterium from the surface is recorded. Usually, the retraction part of the force−distance curve displays a single main adhesion peak close to the substratum surface and several minor peaks at increasing distance.[10] These minor peaks relate to the further stretching of biopolymers upon retraction, like proteins and polysaccharides in EPS until final rupture occurs.[9]

Recently, it has been reported that eDNA is found in both Gram-negative (*Pseudomonas aeruginosa*)[11] and Gram-positive (*Bacillus cereus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Listeria monocytogenes*, *Enterococcus faecalis*)[12−16] bacterial strains as an essential part of EPS. eDNA plays an important role in enhanced bacterial adhesion,[12,13] aggregation,[14] architecture, and enhanced mechanical stability of biofilms.[11,16,17] The presence of naturally occurring eDNA molecules on both

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Gram-negative and Gram-positive bacterial cell surfaces has been shown to increase bacterial cell surface hydrophobicity,[13,14] i.e., the electron-donating and electron-accepting surface free energy parameters of the cell surface,[8] and therewith influences adhesion to substratum surfaces[12,13] and other bacteria (aggregation).[14,18] The increase in bacterial cell surface hydrophobicity in the presence of eDNA directly contributes to increased acid−base interactions with other surfaces, and indeed AFM retraction force−distance curves indicated stronger bacterial aggregation forces in the presence of naturally occurring eDNA than in its absence. Subsequent Poisson analysis of retraction force−distance curves confirmed that acid−base interactions dictate DNA-mediated bacterial aggregation.[14] Hitherto, physicochemical analyses of the role of eDNA have confined themselves to one ionic strength of the suspending fluid, while it is known that ionic strength has a significant impact not only on the magnitude of electrostatic interactions but also on the extension or collapse of possible cell surface biopolymers, including possibly eDNA.[7]

Therefore, the aim of this paper is to determine the influence of ionic strength on the role of eDNA in determining the adhesion force between a bacterium and substrata with different hydrophobicity. To this end, we used *Streptococcus mutans* LT11, an inhabitant of the oral cavity, as a model organism known to possess relatively large amounts of eDNA.[19]

MATERIALS AND METHODS

Bacterial Culture Conditions and Harvesting. *S. mutans* LT11, stored in 7% DMSO at −80 °C, was plated onto brain heart infusion (BHI, OXOID, Basingstoke, UK) containing 1.5% agar and incubated overnight at 37 °C in 5% CO_2. A single colony was used to inoculate 10 mL BHI preculture and incubated for 24 h. This preculture was used to inoculate a 200 mL main culture in BHI, which was grown for 16 h. Bacteria were harvested by centrifugation at 5000*g* for 5 min at 10 °C and washed twice with phosphate buffered saline (PBS: 150 mM NaCl, 10 mM potassium phosphate, pH 6.8; high ionic strength) or with 10 times diluted PBS (15 mM NaCl, 1 mM potassium phosphate, pH 6.8; low ionic strength). To remove the naturally present eDNA from the *S. mutans* LT11 cell surface, the bacterial suspension was pretreated with 50 units of DNase I (Fermentas life sciences, Roosendaal, The Netherlands) per 200 mL culture in the presence of 10 mM $MgCl_2$ for 45 min at 37 °C. Subsequently, the treated suspension was again washed twice in either high or low ionic strength PBS and finally resuspended in high or low ionic strength PBS. In order to break bacterial chains, suspensions were sonicated on ice for 3 × 10 s at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT) which did not affect bacterial viability. Disintegration of chains into single bacteria after sonication was confirmed microscopically.

Substratum Preparation. Microscopic glass slides (Menzel GmbH + Co KG, Braunschweig, Germany) were cleaned using 2% RBS solution (Omniclean RBS 35, Breda, The Netherlands) under sonication for 5 min. The glass slides were rinsed thoroughly with tap water, then with methanol, again with tap water, and finally with ultrapure water. The cleaned glass surface obtained had a low water contact angle of 4°. To obtain a hydrophobic surface, the cleaned glass slides were dip-coated in dimethyldichlorosilane (DDS, Merck, Germany) in trichloroethylene (0.05 w/v %) for 15 min and washed with trichloroethylene, methanol, and ultrapure water to yield a hydrophobic surface with a water contact angle of 104°. Prior to start of the experiments, both clean and DDS-coated glass slides were rinsed with the appropriate phosphate buffer.

AFM Interaction Force Measurements between Streptococci and Substratum Surfaces. Bacterially functionalized probes were prepared by immobilizing streptococci to tipless "V"-shaped AFM cantilevers (VEECO, DNP-0, Camarillo, CA), after immersion of the V-end of the cantilever for 1 min in a drop of 0.01% (w/v) poly-L-lysine (Sigma, Poole, UK) followed by air-drying for 2 min. Subsequently, the coated part of the cantilever was immersed in a suspension of *S. mutans* LT11 (3×10^8 bacteria mL^{-1}) prior to and after removal of eDNA in high or low ionic strength PBS for 1 min to attach the bacteria to the cantilever. For each AFM experiment, a new bacterial probe was prepared and used immediately after preparation. During transportation to the AFM the bacterial probe was stored in 100% humidity.

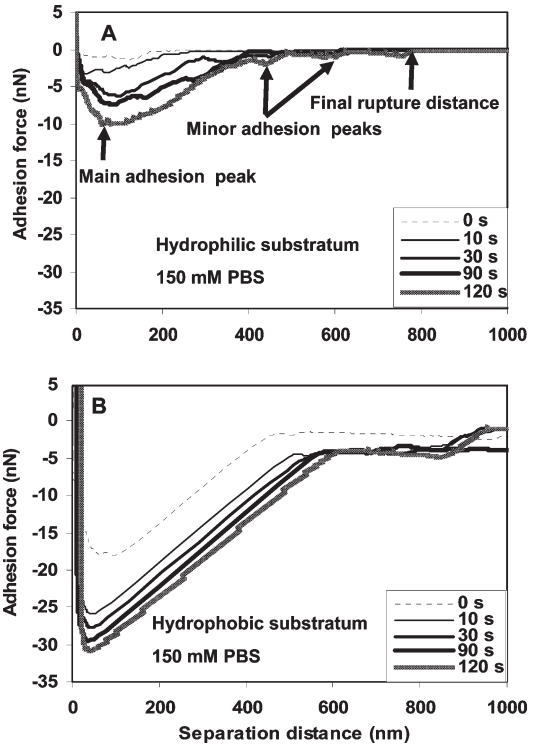

Figure 1. (A) Retraction force−distance curves between *S. mutans* LT11 with eDNA and the hydrophilic substratum in 150 mM PBS at different surface delay times showing the main adhesion force peak, minor adhesion peaks, and the final rupture distance. (B) Retraction force−distance curves between *S. mutans* LT11 with eDNA and the hydrophobic substratum in 150 mM PBS at different surface delay times.

All AFM adhesion force measurements were performed at room temperature in high or low ionic strength PBS using a Dimension 3100 system (Nanoscope V, Bruker, Santa Barbara, CA). For each bacterial probe, force−distance curves were measured with a surface delay time, i.e., the contact time allowed between bacterium and substratum surface, ranging from 0 up to 120 s at a scan rate of 1 Hz and at a constant load of 5 nN. The adhesion force (F) was calculated from the AFM cantilever deflection data using

$$F = K_{sp}D \quad (1)$$

where K_{sp} is the spring constant of the bare cantilever and D is the deflection of the cantilever. The spring constant of each cantilever was determined in air using the thermal method.[20]

For each bacterial probe, force curves were measured for different surface delay times on the same, randomly chosen spot on a hydrophobic or hydrophilic substratum surface. The integrity of the bacterial probe was monitored during the experiments by measuring adhesion forces with 0 s surface delay at the onset of each measurement cycle and again after completion of a cycle (0−120 s surface delay times). If the maximum adhesion forces at 0 s at the start and after 120 s did not coincide within 1 nN, all data from that measurement cycle were discarded,

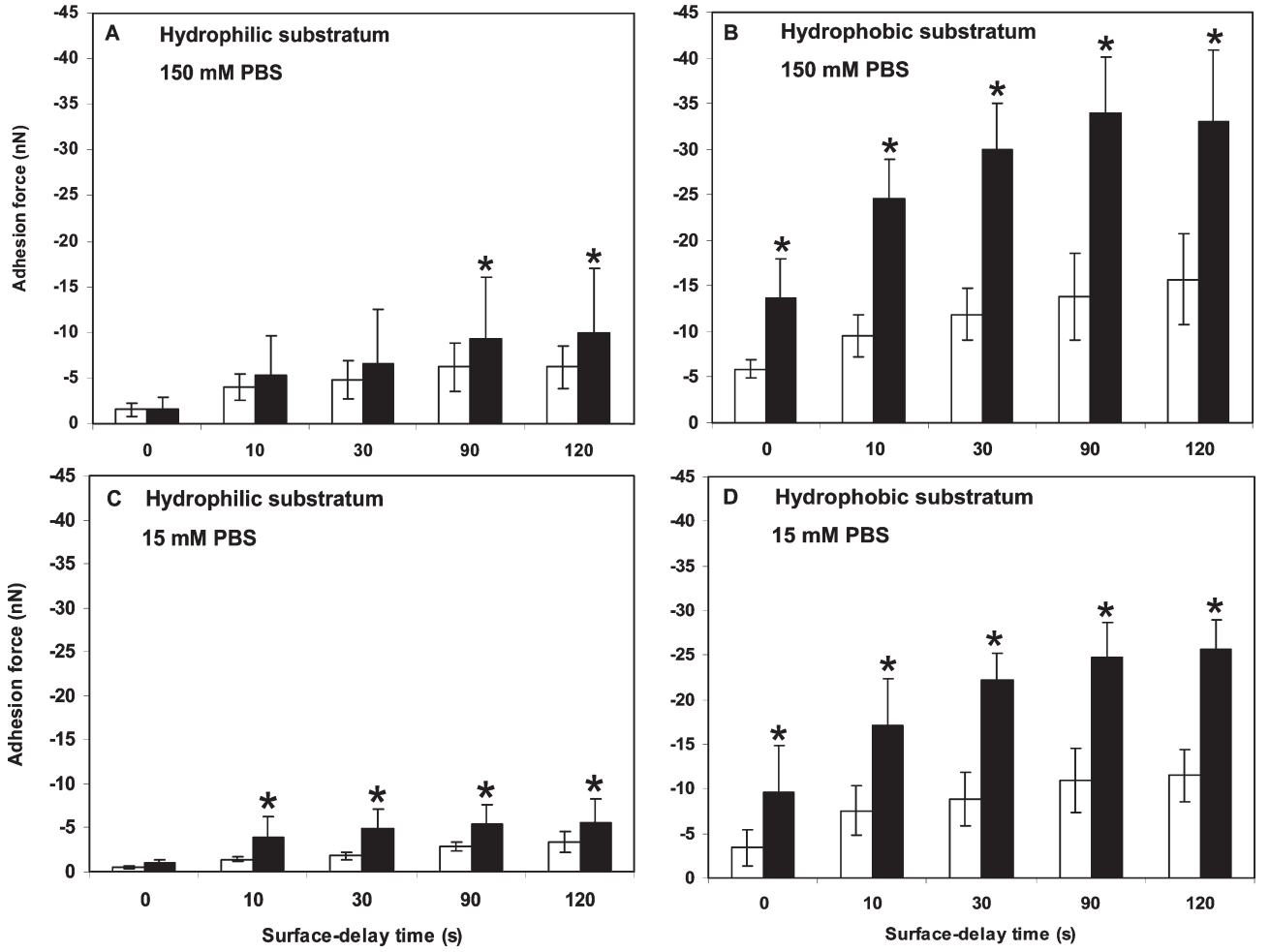

Figure 2. Maximum adhesion force (main adhesion peak) in the retraction force−distance curves as a function of the surface delay time for *S. mutans* LT11 with (black bars) and without (white bars) eDNA on its surface at different ionic strengths. Error bars represent the standard deviation over 35 force curves from three independent bacterial cultures. Asterisk indicates a statistically significant difference ($p < 0.05$) in adhesion force between *S. mutans* LT11 adhesion forces prior to and after removal of eDNA.

and a new bacterial probe was prepared. In total, 35 force−distance curves were measured for each surface delay time, using nine bacterial probes, prepared out of three separate bacterial cultures.

Retraction force−distance curves were used to determine the adhesion force, number of adhesion peaks, i.e., the main and minor adhesion peaks, and the final rupture distance, i.e., the separation distance at which the most remote, minor peak was observed (see Figure 1A).

Hydrodynamic Radius of *S. mutans* LT11. The hydrodynamic radius of *S. mutans* in high and low ionic strength PBS was measured using a Zetasizer nano series (model ZEN3600, Malvern Instruments Ltd., UK) at 25 °C for streptococci prior to and after removal of eDNA from three independent cultures (3×10^8 bacteria mL^{-1}). Backscattered light from a monochromatic laser (633 nm wavelength) after passing through the bacterial cell suspension was detected at an angle of 173°. Subsequently, the autocorrelation of the time-dependent intensity fluctuations of the scattered light was used to derive the Brownian motion velocity of the bacteria, from which the hydrodynamic radius can then be calculated using the Stokes−Einstein equation.[21]

Statistical Analysis. The influence of naturally present eDNA on the adhesion force, number of adhesion peaks, and final rupture distance between *S. mutans* LT11 and the two substratum surfaces with different hydrophobicities and at high and low ionic strength was analyzed using a two-tailed Student's *t* test. A similar analysis was carried out on the hydrodynamic radii of *S. mutans* LT11 with and without eDNA in different ionic strength suspensions. Differences were considered significant if $p < 0.05$.

RESULTS

The basis of the results presented in this article is formed by retraction force−distance curves between *S. mutans* LT11 and hydrophilic and hydrophobic substrata at high and low ionic strength, deriving adhesion forces, numbers of minor peaks, and final rupture distances from such curves (see also Figure 1A). As examples, retraction force−distance curves of *S. mutans* LT11 with eDNA present from the hydrophilic (Figure 1A) and the hydrophobic (Figure 1B) substratum measured in 150 mM PBS are shown. Note that in both examples adhesion forces increase with increasing surface−delay times, while extending over separation distances of several hundreds of nanometers until final rupture occurs.

Adhesion forces of *S. mutans* LT11 with eDNA were always stronger than after removal of eDNA for all surface-delay times and regardless of substratum hydrophobicity and ionic strength (Figure 2). However, the presence of eDNA enhanced the

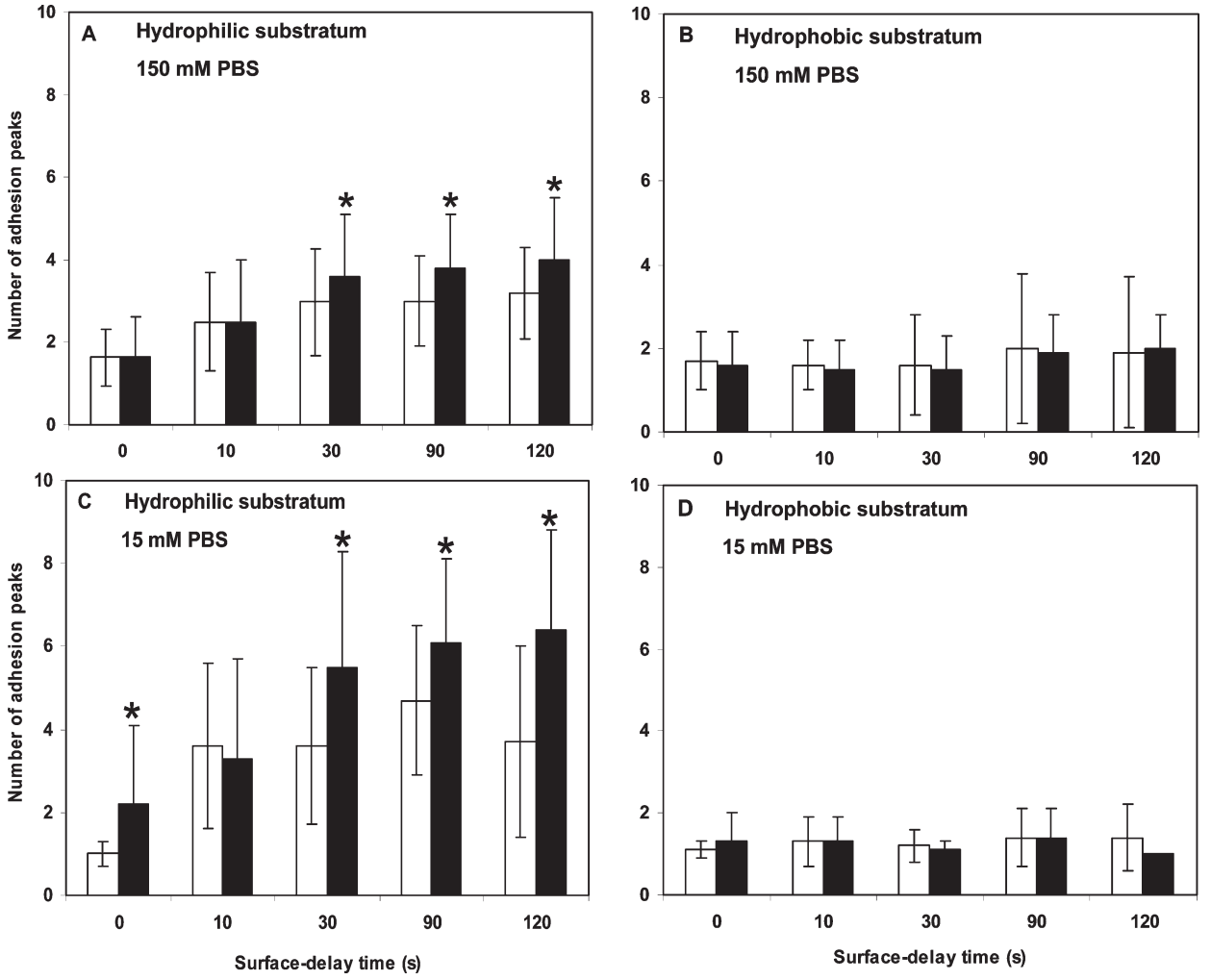

Figure 3. Number of adhesion peaks in the retraction force−distance curves as a function of the surface delay time for *S. mutans* LT11 with (black bars) and without (white bars) eDNA on its surface at different ionic strengths. Error bars represent the standard deviation over 35 force curves from three different bacterial cultures. Asterisk indicates a statistically significant difference ($p < 0.05$) in the number of adhesion peaks in *S. mutans* LT11 adhesion forces prior to and after removal of eDNA.

streptococcal adhesion forces significantly ($p < 0.05$) more on the hydrophobic substratum than on the hydrophilic substratum, especially after bond maturation. Adhesion forces increased significantly ($p < 0.05$) with ionic strength for both hydrophilic and hydrophobic substrata both prior to and after removal of eDNA from the streptococcal cell surface.

Bond maturation on a hydrophilic substratum was accompanied by an increase in the number of minor adhesion peaks which was more pronounced at low ionic strength than at high ionic strength (Figure 3). The increase in the number of minor peaks was higher in the presence of eDNA on the streptococcal cell surface, especially at low ionic strength. In contrast, on a hydrophobic substratum often only one single, main adhesion peak was observed regardless of the presence of eDNA. When present, the number of minor adhesion peaks did not increase with increasing surface delay times and was dependent neither on the eDNA nor on ionic strength.

The final rupture distance on a hydrophilic substratum increased with increasing surface delay times and was always larger for *S. mutans* LT11 with eDNA than for *S. mutans* without eDNA, independent of ionic strength (Figure 4). On average over both ionic strengths, rupture distances were between 97 and 173 nm larger with eDNA than without eDNA. On the hydrophobic substratum, final rupture distances did not increase with surface delay times, while differing significantly with ionic strength. Final rupture distance was significantly larger prior to removal of eDNA from the streptococcal cell surface than after removal of eDNA, and this eDNA-associated difference in rupture distance was larger (166 ± 102 nm) at high ionic strength than at low ionic strength (77 ± 12 nm).

The hydrodynamic radius of *S. mutans* LT11 at high ionic strength (480 ± 30 nm) was not affected by the absence or presence of eDNA on the bacterial cell surface. However, removal of eDNA from the bacterial surface caused a significant ($p < 0.05$) decrease in the hydrodynamic radius at low ionic strength by 72 ± 40 nm.

DISCUSSION

In this paper, we describe the role of eDNA in determining the adhesion forces of *S. mutans* LT11 on a hydrophilic and hydrophobic substratum at high and low ionic strengths. The presence

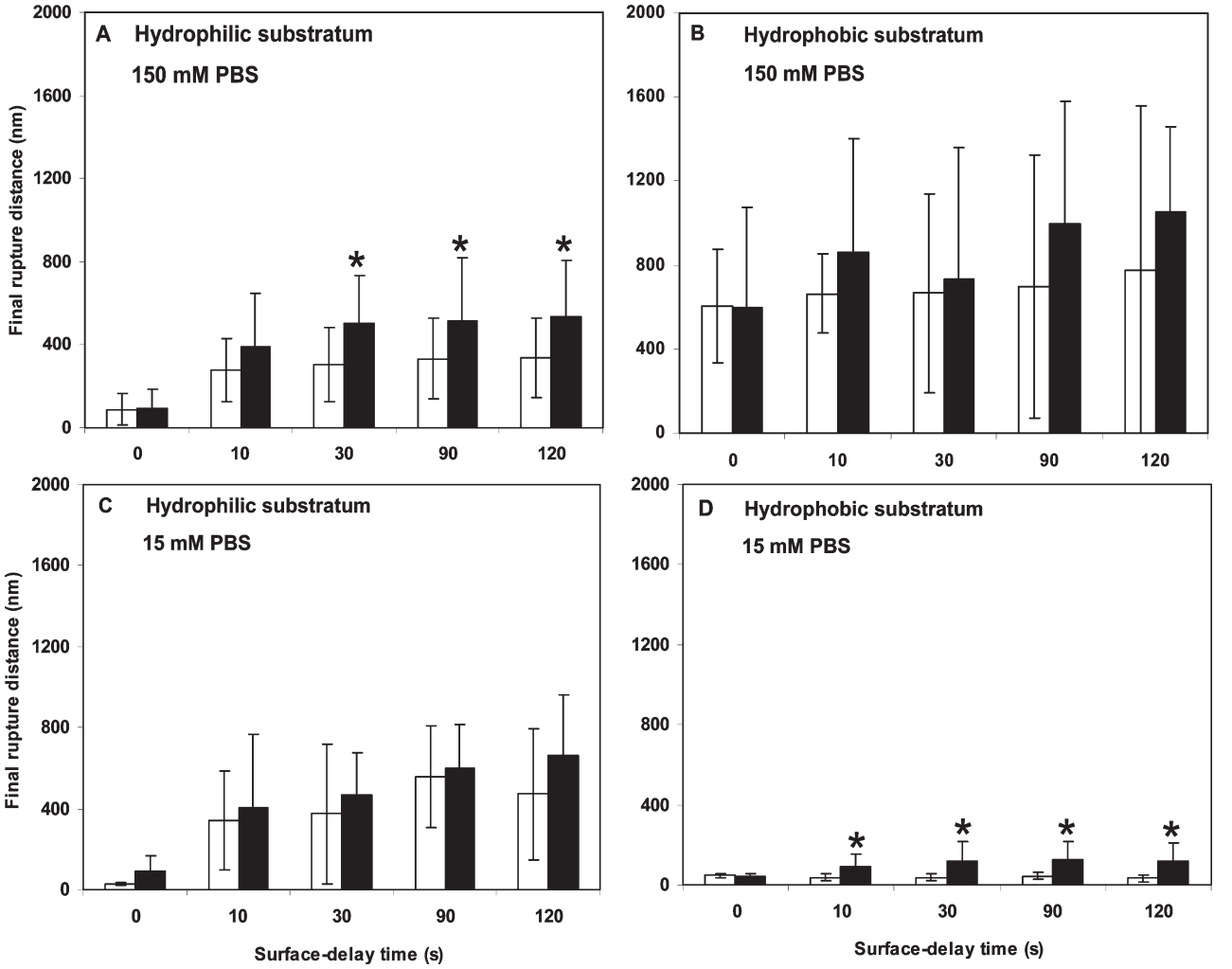

Figure 4. Final rupture distance of the bonds (the last peak) as a function of the surface-delay time for *S. mutans* LT11 with (black bars) and without (white bars) eDNA on its surface at different ionic strengths. Error bars represent the standard deviation over 35 force curves from three independent bacterial cultures. Asterisk indicates a statistically significant difference ($p < 0.05$) in final rupture distance of *S. mutans* LT11 forces prior to and after removal of eDNA.

of eDNA on *S. mutans* LT11 cell surfaces enhanced its adhesion force to a hydrophobic substratum significantly more than to a hydrophilic substratum, especially after bond maturation, while being stronger on the hydrophobic substratum and at high ionic strength. Bond maturation is a consequence of removal of interfacial water facilitating an increase in number of contact points between the bacterium and the substratum surface over time. Here it was demonstrated that bond maturation involves an increase in both the number of minor adhesion peaks and final rupture distance on the hydrophilic substratum, while on the hydrophobic substratum the number of minor adhesion peaks as well as the final rupture distance had reached their final value virtually immediately upon contact.

Effect of Substratum Hydrophobicity on eDNA-Mediated Bacterial Adhesion. Bond maturation is expected to change according to a different mechanism on hydrophobic than on hydrophilic substrata, as bond maturation is only accompanied by an increase in the number of minor adhesion peaks (see Figure 3) and final rupture distance (see Figure 4) on the hydrophilic substratum. The hydrophobic substratum lacks polar groups on its surface and therefore cannot offer acid−base interactions to an approaching surface. In addition, hydrophobicity makes it easy to remove interfacial water.[8] Therewith the increase in adhesion force with surface delay time observed for *S. mutans* on a hydrophobic surface may be due to an increase in Lifshitz−van der Waals forces caused by removal of interfacial water from the bacterial cell surface, facilitating a closer approach. As this enables more intimate contact, also in the presence of eDNA, the bacterial cell surface will bind more strongly to the substratum and thus extend farther upon retraction of the AFM probe until final rupture, although not to the extent as on the hydrophilic substratum. The increase in final rupture distance may not only due to eDNA but could possibly involve other biopolymers like proteins[19] and polysaccharides[15] associated with eDNA. The hydrophilic substratum, on the other hand, has a lot to offer an approaching surface in terms of acid−base interactions, and bond maturation is clearly mediated by an increase in the number of acid−base bonds, as evidenced by the increase in the number of minor adhesion peaks, previously identified to contribute to acid−base interactions.[14] Stronger adhesion forces on the hydrophilic surface allow more extensive stretching of eDNA and other biopolymers until final rupture occurs, as forced by the AFM (see Figure 4).

Effect of Ionic Strength on eDNA-Mediated Bacterial Adhesion. Biopolymers on bacterial cell surfaces, like proteins, polysaccharides, or eDNA, stretch themselves from a collapsed configuration at high ionic strength due to the absence of electrostatic stabilization to a more elongated configuration at low ionic strength.[7] In line, adhesion forces of *S. mutans* LT11 increase with increasing ionic strength due to decreased electrostatic repulsion, regardless of substratum hydrophobicity and eDNA. The stretched state of eDNA molecules at low ionic strength probably expose more binding sites to the substrata, and as a consequence more bonds can be formed due to acid−base interactions which explains why on the hydrophilic substratum the number of minor adhesion peaks increased more than at high ionic strength.

Differences in forced biopolymer stretching in the retraction force−distance curves at low and high ionic strength are supported by the differences in hydrodynamic radius of the bacterial cell at low and high ionic strength as compared with the final rupture distances. Differences in final rupture distances in the absence and presence of eDNA on the streptococcal cell surface were independent of ionic strength and varied between wide ranges from 97 to 173 nm, if we neglect minor differences between hydrophobic and hydrophilic substrata. This indicates that under the forced stretching conditions of the AFM eDNA can be forced to stretch up to 173 nm, which is considerably smaller that due to ionic strength changes only. Dynamic light scattering indicated that under the sole influence of ionic strength the collapse of eDNA contributes only 72 nm to the change in hydrodynamic radius, which corresponds with the lower values of the difference in final rupture distance observed as forced by the AFM. A difference in final rupture distance due to the presence of eDNA of 173 nm is not unlikely because eDNA has a size of around 30 kb (3.4 Å per base). eDNA of 30 kb would correspond to a length of around 10 000 nm; that is adsorbed in loops and trains on the bacterial cell surface.[22] The length of these loops and trains will become available upon rupture.

Biofilm formation on substratum surfaces heavily depends on the ability of the initially adhering bacteria to withstand environmental detachment forces.[23,24] Since adhesion forces between *S. mutans* as our model organism with substratum surfaces are stronger in the presence of eDNA, the current data indicate that biofilm formation under environmental conditions may depend heavily on the presence of eDNA.

CONCLUSIONS

Adhesion forces of *S. mutans* LT11 to both hydrophilic and hydrophobic substrata increase with increasing surface delay times and ionic strength and were stronger on a hydrophobic than on a hydrophilic substratum. The presence of eDNA on *S. mutans* LT11 cell surfaces always enhanced its adhesion force to a hydrophobic substratum significantly more than to a hydrophilic substratum, especially after bond maturation. As a consequence of bond maturation, an increase in the number of minor adhesion peaks was observed on a hydrophilic substratum, previously associated with the involvement of acid−base interactions, whereas on the hydrophobic substratum the number of minor adhesion peaks remained low also during bond maturation. More minor adhesion peaks developed on the hydrophilic substratum at low ionic strength than at high ionic strength. The final rupture distance in retraction force−distance curves was independent of ionic strength on a hydrophilic substratum and increased with increasing surface delay time. On the hydrophobic surface, the final rupture distance did not increase with surface delay time but was significantly smaller at low than at high ionic strength. The lower values for the difference in final rupture distance at low and high ionic strength coincided with the decrease in hydrodynamic radius of the streptococci upon increasing ionic strength. AFM also yielded higher values for the ionic strength induced difference in final rupture distance because in AFM rupture is forced, while in dynamic light scattering differences are only induced by ionic strength differences.

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