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Adhesion characteristics of two *Burkholderia cepacia* strains examined using colloid probe microscopy and gradient force analysis

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Abstract

Colloid probe atomic force microscopy (CP-AFM) was used to investigate two strains of *Burkholderia cepacia* in order to determine what molecular scale characteristics of strain Env435 make it less adhesive to surfaces than the parent strain, G4. CP-AFM approach curves analyzed using a gradient force method showed that in a high ionic strength solution (IS = 100 mM, Debye length = 1 nm), the colloid probe was attracted to the surface of strain G4 at a distance of ∼30 nm, but it was repelled over a distance of 25 nm when approaching strain Env435. Adhesion forces measured under the same solution conditions during colloid retraction showed that 1.38 nN of force was required to remove the colloid placed in contact with the surface of strain G4, whereas only 0.58 nN was required using strain Env435. At IS = 1 mM (Debye length = 10 nm), the attractive force observed with G4 was no longer present, and the repulsive force seen with Env435 was extended to ∼250 nm. The adhesion of the bacteria to the probe was much less at low IS solution (1 mM) than at high IS (100 mM). The greater adhesion characteristics of strain G4 compared to Env435 were confirmed in column tests. Strain G4 had a collision efficiency of $\alpha = 0.68$, while strain Env435 had a much lower collision efficiency of $\alpha = 0.01$ (IS = 100 mM). These results suggest that the reduced adhesion of strain Env435 measured in column tests is due to the presence of high molecular weight extracellular polymeric substances that extend out from the cell surface, creating long-range steric repulsion between the cell and a surface. Adhesion is reduced as these polymers do not appear to be "sticky" when placed in contact with a surface in AFM tests.

Keywords: Atomic force microscopy (AFM); Colloid probe; Bacterial adhesion; EPS

1. Introduction

Bacterial adhesion is a phenomenon that has applications in many fields, including increasing the efficacy of wastewater treatment [1], preventing biofilm formation on medical implants [2], and improving bioremediation techniques [3]. An understanding of the molecular mechanisms responsible for adhesion would benefit these fields, but bacterial adhesion is a complex process consisting of several stages. First, the bacterium approaches a surface, either through diffusion, settling, hydrodynamics, or through its own motility [4]. Once near the surface, it interacts with the substratum via several different forces, both attractive (van der Waals, depletion, acid–base), and repulsive (electrostatics, solvation, steric). If the attractive forces dominate, they will cause the bacterium to contact the substratum (initial adhesion event). Once initial adhesion has occurred, a bacterium can either remain on the surface, or desorb due to Brownian motion or electrostatic repulsion. In general, lowering the solution ionic strength (IS) reduces the adhesion of bacteria due to increased electrostatic repulsion [5–7].

Atomic force microscopy (AFM) has been used to examine attractive and repulsive forces between surfaces at molecular scales [8,9]. Typically an AFM tip, or a colloid probe mounted on the cantilever, is pressed onto a bacterium and then retracted from the surface. The force required to pull the tip (or probe) from the surface is used as a relative measurement of a bacterium's adhesiveness. However, this analysis technique does not consider the impact that the initial adhesion event has on the overall tendency of bacteria to adhere to a certain substrate. It has also been shown that retraction pull-off forces do not always correlate with adhesion data obtained in packed column or flow chamber adhesion experiments [10].

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The forces experienced by the colloid probe when approaching the bacterial surface can also be used to study bacterial adhesion. Repulsive forces between the tip and the bacterium surface are often observed, and changes in the type of interaction force can be explored using a new gradient force analysis [11,12]. This analysis consists of taking the derivative of the deflection versus distance AFM approach curve at each data point. The derivative is then plotted against the original distance. This technique highlights sudden changes in the slope of the deflection–distance curve that otherwise could be overlooked. Gradient force analysis has revealed significant differences between three different *Escherichia coli* strains with varying lipopolysaccharide (LPS) layer structures [11]. The analysis revealed four distinct regions in the approach curve: a noninteraction region, a non-contact phase, a contact phase, and a constant compliance region. The noninteraction region occurs over any separation distance where the AFM probe is not affected by the bacterium. The non-contact phase begins at the first point where the tip is affected by the bacterial surface, due to direct interactions with extracellular polymeric substances (EPS) on the cell surface or electrostatic repulsion [13]. The contact phase is evidenced by a sharp change in the slope of the gradient force curve, most likely due to the probe interaction with a different type of material. The contact phase therefore indicates when the probe has contacted the outer membrane of the bacterium and is now pushing into a different type of surface, eliciting a new response in the gradient. The constant compliance region is where the AFM cantilever is being deflected at the same rate that the piezo is traveling, indicating it is in contact with a relatively hard surface, which is most likely the peptidoglycan layer for Gram negative bacteria [4].

There have been several studies that have examined the approach curve between a surface and a bacterium (or multiple bacteria) [8,14]. In general, the approach curves show only repulsive forces and attractive forces are rare. In one study, the repulsion due to steric repulsion was able to be related to macroscopic adhesion in a parallel plate flow chamber [15]. Previous work has shown that both the approach and retraction curves should be analyzed when attempting to understand adhesion characteristics [12]. Therefore, in this study gradient force analysis method was used to interpret the approach curves while retraction forces were examined in terms of total pull-off force. Two strains of *Burkholderia cepacia* were used in tests: the wild-type strain G4, and a mutant (strain Env435) that was obtained by others using a screening test for non-adhesive bacteria. *B. cepacia* G4 is a strain of environmental interest as it is capable of degrading chlorinated hydrocarbons such as trichloroethylene. Although the characteristics of Env435 that make it non-adhesive are not well studied, Env435 lacks an *o*-antigen in its outer lipopolysaccharide (LPS) layer [16].

2. Methods

2.1. Bacterial cultures

B. cepacia strains G4 and Env435 were obtained from Mary DeFlaun at Envirogen Corp. Cells were preserved in a −80 °C freezer in a Luria Broth/glycerol solution (50%, v/v), and revived overnight on a tube rotator in 20 mL glass culture tubes containing 5 mL of Luria Broth (Miller's). Cells were transferred to fresh media (100 mL LB), grown at 30 °C until their mid-exponential growth phase (about 4 h), and then washed three times by centrifugation (2800 × *g*, 10 min, 20 °C) in a phosphate buffer solution (PBS, IS = 100 mM). PBS consisted of KH_2PO_4 (Fisher Scientific, 1.55 g/L for 100 mM) and K_2HPO_4 (Fisher Scientific, 5.72 g/L for 100 mM) in deionized (DI) water (MilliQ system, Millipore, Marlborough, MA) [17].

2.2. Packed column and filtration experiments

To confirm the different adhesive properties of the two strains of bacteria, filtration experiments were performed in two different IS solutions of PBS (IS = 1 and 100 mM). The experimental setup, which has been previously described in detail [18], consisted of mini-columns with either a glass fiber filter (Whatman, GF/D) placed at the bottom of the column, or a glass fiber filter supporting a packed column of 40 μm silica beads (Polysciences, Inc.). The bacterial suspensions were pulled by vacuum through each column, and the total effluent was collected and analyzed for cell concentrations. The difference between the number of bacteria in the original suspension and the number in the effluent was the number of bacteria that were retained in the column (either the filter alone or the filter and the packing combined). The fraction retained by the packing, corrected for the fraction of bacteria retained by the filter, can be calculated as:

$$F_P = 1 - \frac{1 - F_R}{1 - F_f} \tag{1}$$

where F_P is the fraction of bacteria retained in the packing, F_R the fraction retained over the entire column (packing plus filter), and F_f is the fraction of bacteria retained in just the glass fiber filter. Using F_P, the Rajagopalan and Tien filtration model [19] can be used to obtain collision efficiency (α) for the bacteria in the packed column as

$$\alpha = \frac{-4a_c \ln(1 - F_P)}{3(1 - \theta)\eta L} \tag{2}$$

In this equation, a_c is the collector radius (40 μm for this study), θ the porosity, η the collector efficiency, and L is the length of the column.

Collision efficiencies for the glass fiber filters were calculated by using a modified equation of the RT model (and F_f as the fraction retained), as follows [20]:

$$\alpha = \frac{-\pi a_c \ln(1 - F_f)}{2(1 - \theta)\eta L} \tag{3}$$

In this equation, the geometric factor for spherical collectors (4/3) has been replaced by the factor appropriate for glass fiber cylinders ($\pi/2$) [21]. For GF/D filters, $a_c = 1.35$ μm, $\theta = 0.55$, and $L = 675$ μm [20].

2.3. AFM preparation

Washed cell suspensions were allowed to adhere to a glass cover slip (24 mm × 60 mm, Corning No. 1) for analysis in AFM experiments [10]. The cover slips were cleaned by rinsing in an acid bath (3:1 35% HCl:65% HNO_3) for 3 h in a glass boat (Cole-Parmer). The solution was decanted off and the cover slips were rinsed with copious amounts of DI water. The slides were put back into the glass boats and soaked for 24 h in 200 mL piranha solution (4:1 98% H_2SO_4:30% H_2O_2). The slides were removed, rinsed with copious amounts of DI water, and stored in DI water at 4 °C [22].

Bacteria were held on the slide (both surfaces are negatively charged) using poly-D-lysine (PDL), a positively charge poly-electrolyte. A 15 μL drop of PDL (5 mg/mL, Sigma–Aldrich, 70–150 kDa) was allowed to air dry on the cover slips. The cover slips were then rinsed with DI water and blown dry using pure nitrogen gas. A small drop (0.5 mL) of the bacterial suspension was then added directly on top of the treated portion of the cover slides and allowed to adhere for 30 min. The cover slides were then washed with DI water, and stored in 100 mM PBS at 4 °C prior to use. Colloid probes were prepared using tipless cantilevers (Veeco NanoProbe NP-020) and glass beads (4 μm diameter, Polysciences, Inc., Warrington, PA) as previously described [11].

2.4. AFM procedure

AFM force curves were obtained using a BioScope AFM (Digital Instruments, Santa Barbara, CA) with a Nanoscope IIIa controller, software version 5.30r1, and attached to an optical inverted microscope (Olympus IX70). Force curves were obtained on individual bacteria that were located by first performing large area scans (25 μm × 25 μm; Fig. 1) in tapping mode (drive frequency ~ 8.4 kHz). Once a suitable bacterium was located, 10 force curves were taken on the center of the cell, with at least five bacteria examined for each experimental condition. The loading force (~5.4 nN) was held constant by setting a trigger at 90 nm of cantilever deflection.

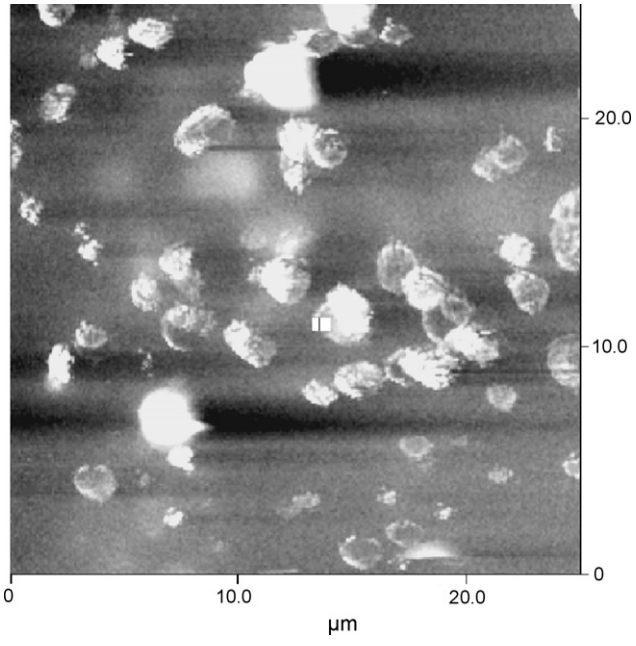

Fig. 1. AFM image (25 μm × 25 μm scan) of *B. cepacia* G4 cells on a slide.

Raw data from AFM experiments (mV) were imported into a spreadsheet for analysis. The sensitivity was measured for each force curve, defined as the change in deflection–distance (nm) per change in signal (mV) during constant compliance. This ratio was then used to convert the raw data into deflection data using the force constant as determined by the thermal drift method (0.061 ± 0.0003 nN/nm) [23]. For the gradient force analysis, the derivative of the deflection data was taken at each point, and then plotted against distance as previously described [19]. Pull-off distances were calculated as the distance between the jump-off point (the most negative point of the retraction curve) and the point at which the tip returned to the baseline, consistent with previous studies [24–27].

3. Results

3.1. Approach curves

In the high IS = 100 mM solution, the approach curves for the highly adhesive parent strain G4 showed an attractive (a negative deflection) interaction force, whereas the approach curves for non-adhesive mutant strain Env435 showed only repulsive interactions (a positive deflection) (Fig. 2). With strain G4, there was an attractive force observed approximately 39 nm before contact. In the low IS solution (1 mM), however, the approach curves were decidedly different (Fig. 3) as the repulsive (strain Env435) and attractive (strain G4) forces were absent.

There were several distinct regions of tip–surface interaction observed using the gradient analysis curve for strain G4. The contact distance (defined here as the origin) was first set using a geometric approach, based on extrapolating the line in the constant compliance region (region D) to the *x*-axis, as done in previous studies [22,28]. As the tip approached the surface, there was at first no interaction between the tip and the cell beyond 35 nm (region A, using the top graph of Fig. 2 as the example). There was a non-linear interaction observed when the colloid probe first started interacting indirectly with the surface, likely through electrostatic and steric interactions with EPS at distances from 35 to 0 nm (region C_e). In this region the probe should be primarily be interacting electrostatically with the bacterial polymers and not the surface due to the high ionic strength, and therefore the region is referred to as the non-contact phase since this interaction occurs before the two surfaces are actually in contact. At distances of 0 to −35 nm (C_s) there was more linear response of the tip indicating contact with the surface (but not a constant compliance), followed by a region of constant compliance (D, where the gradient is constant due to deflection and distance varying linearly). The point of contact (origin) defined by the geo-

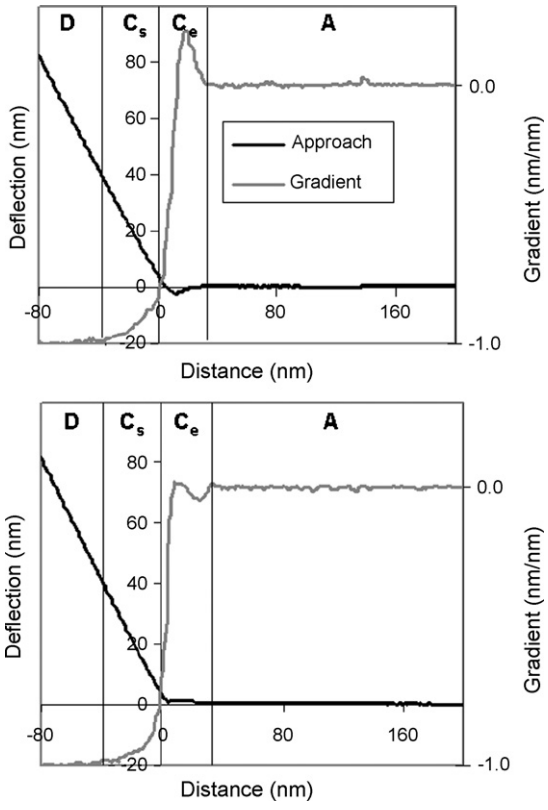

Fig. 2. Approach curves of silica colloid probes probing strain G4 (top) and strain Env435 (bottom) in IS = 100 mM solution. Region A is where there is no interaction between the colloid probe and the bacterium. Region C_e is the non-contact phase, where there is interaction, but the surfaces are not in direct contact. Region C_s is where the surfaces are in contact, and the bacterial surface is deforming due to the force of the probe. Region D is the constant compliance region, where deflection is proportional to distance.

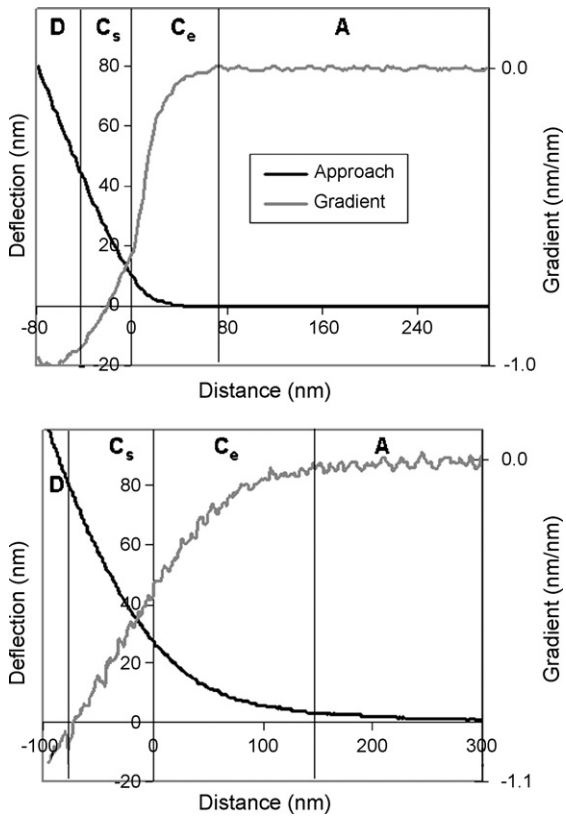

Fig. 3. Approach curves for silica colloid probes probing G4 (top) and Env435 (bottom) in 1 mM.

metric extension method is in agreement with that point of contact obtained with the gradient analysis which is defined as the interface of regions C_e and C_s [28], a finding in agreement with previous results using this gradient analysis method [11].

At low IS there was a much longer (105% longer) non-contact phase for Env435 than for G4 (Fig. 3). There was also a long non-linear region that continued for 79 nm, where the slope of the gradient changed. After that region, constant compliance was reached. A summary of the average distances for the non-contact (C_e) and contact (C_s) regions is given in Table 1.

3.2. Retraction curves

The force required to retract the tip from the surface of strain G4 (1.38 nN) was much greater than that needed for strain Env435 (0.58 nN) in 100 mM PBS (Fig. 4). The pull-off distances showed the opposite trend, with those for strain G4 (71 nm) being much shorter than those for strain Env435 (147 nm). There were significant changes to the retraction curves when the ionic strength was reduced to 1 mM (Fig. 5). The pull-off force for G4 was reduced to 0.28 nN, whereas for Env435 the attractive force was eliminated completely, indicating that there was no attraction between the silica colloid probe and the bacterial surface. Pull-off forces and distances obtained in retraction curves are summarized in Table 2. The general trends that can be observed in the detachment data are that G4 is more adhesive than Env435 at both high and low ionic strengths, and

Table 1
Summary of the region lengths in the AFM approach curves between a silica colloid probe and a bacterium (strain G4 or strain Env435)

	Contact phase, C_s (nm)	Non-contact phase, C_e (nm)	Total non-linear distance (nm)
1 mM			
G4	52 ± 6	63 ± 7	115 ± 13
Env435	79 ± 3	129 ± 17	208 ± 20
100 mM			
G4	42 ± 4	39 ± 6*	81 ± 10
Env435	31 ± 6	34 ± 1	65 ± 7

The uncertainties shown are 95% confidence intervals ($n \geq 50$). The (*) indicates there was an attractive force prior to a repulsion.

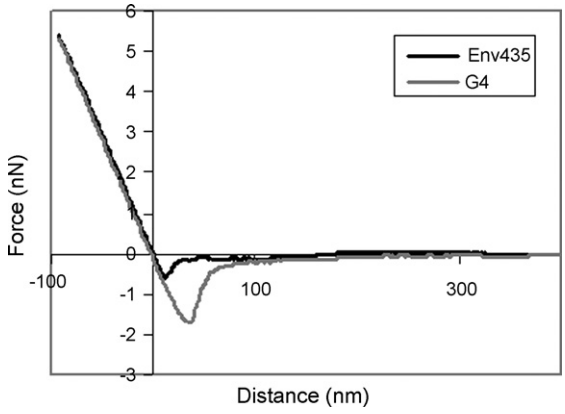

Fig. 4. Retraction curves of a silica colloid probe measured in IS = 100 mM solution for strains G4 and Env435.

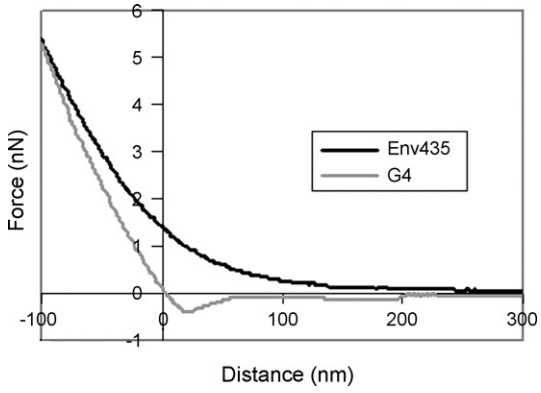

Fig. 5. Retraction curves of silica colloid probes in 1 mM PBS for G4 and Env435.

that reducing the ionic strength reduces the required pull-off force.

3.3. Filtration experiments

Column experiments using glass beads were used to verify that strain Env435 was less adhesive that strain G4 in both 1 and 100 mM IS solutions. The collision efficiencies in packed columns for strain Env435 were 0.048 ± 0.008 and 0.023 ± 0.003 in 100 and 1 mM IS solutions (95% CI, $n \geq 3$).

Table 2
Summary of pull-off forces and distances between colloid probes and bacteria (strain G4 or strain Env435) measured in retraction curves

	Pull-off force (nN)	Distance (nm)
1 mM		
G4	0.28 ± 0.07	135 ± 59
Env435	–	–
100 mM		
G4	1.38 ± 0.23	71 ± 40
Env435	0.58 ± 0.03	147 ± 32

There was no attractive force observed in the retraction curves for strain Env435 (IS = 1 mM). Uncertainties shown are 95% confidence intervals ($n \geq 50$ for each number). Each number presented is the average of at least 10 force curves per bacterium on at least 5 different bacteria.

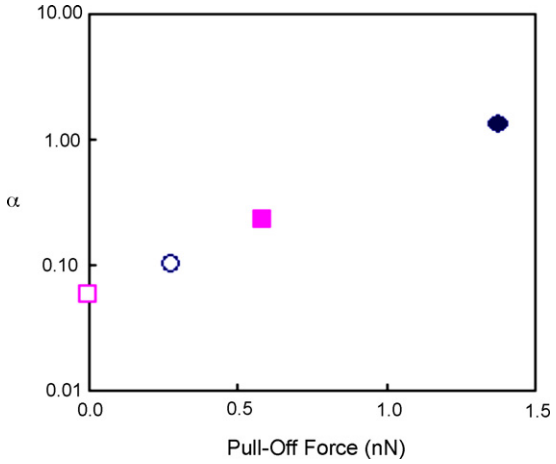

Fig. 6. Collision efficiencies (α) of bacteria retained by a glass fiber filter (F_f) vs. CP-AFM pull-off force for both G4 and Env435 strains of *B. cepacia*. The circles are data for G4, and the squares are for Env435. Open symbols are at low IS, and filled symbols are at high IS.

The collision efficiency for strain G4 was 0.68 ± 0.03 at IS = 1 mM. At an IS = 100 mM however, G4 was so adhesive to the filter alone ($F_f > 0.95$), that a statistically significant value of the collision efficiency could not be obtained in tests using the glass beads. Based on column tests using only the glass fiber filters, the collision efficiencies of bacteria that were retained by the filters showed a direct correlation with CP-AFM retraction pull-off forces (Fig. 6).

4. Discussion

Both the approach and the retraction AFM curves were analyzed to test the importance of an initial adhesion event to the overall adhesiveness of bacteria. Retraction curves were consistent with macroscopic column experiments as both showed that strain G4 was more adhesive than strain Env435 [29]. In the IS = 100 mM solution, the pull-off force was 2.4× larger for G4 than for Env435. In the IS = 1 mM solution, G4 showed a reduced pull-off force compared to the high IS solution, whereas with Env435 the pull-off force disappeared completely in the low IS solution. This was probably due to the presence of EPS, as further discussed below.

The approach curves also showed differences between the two strains. In high IS solution (Fig. 2) the colloid probe was attracted to the surface of strain G4, likely due to van der Waals attractive forces. Upon further approach, the repulsive electrostatic and steric forces then dominated and repelled the probe. In low IS solution (Fig. 3), the attraction is not seen, most likely because the stronger electrostatic repulsion forces prevented the attraction. Lowering the ionic strength from 100 to 1 mM increases the Debye length from 1 to 10 nm [30]. This means that the electrostatic repulsion would be significant at a much longer separation distances at low IS. Low IS can also cause the LPS molecules to become more extended, which creates more steric repulsion for the colloid probe [10], which would also explain the lower adhesion values upon retraction [31].

In high IS solution, the colloid probe is repelled by the surface of Env435 upon approach (Fig. 2). Since the range over which this occurs (∼35 nm) is much longer than electrostatics can act with this small of a Debye length (∼1 nm), repulsion must be due to another effect. It is likely that Env435 produces extracellular polymeric substances (EPS) which act as a steric buffer between the bacterial surface and the colloid probe. This layer would explain the repulsion upon approach (due to steric hindrance), as well as the small (in magnitude), yet long-range adhesion force upon retraction.

In low IS solution (Fig. 3), very long-range repulsion (170 nm) was observed upon approach of the colloid probe to Env435 bacteria. A possible explanation for this is the presence of a thick EPS layer. It is known that many bacteria produce more EPS in a stressed environment, such as when there are no nutrients or when the ionic strength is low [32,33]. Since the experiments were run with the bacteria experiencing both of these stresses, it is possible they were producing more EPS. This would also explain the absence of an attractive force upon retraction, since the colloid probe would never have been able to get close enough to the actual bacterium for van der Waals forces to dominate. Another possibility is that the EPS is simply more extended and loosely packed, accounting for the long-range forces seen during both approach and retraction of the colloid probe.

5. Conclusions

It has been shown that two *B. cepacia* strains behave differently when contacting a surface. By using a gradient force analysis of atomic force microscopy approach curves, we showed that at high IS strain G4 was attracted to a silica colloid probe upon approach of that surface, whereas the Env435 strain was repelled. Both strains adhered much more strongly at high IS, likely due to a decrease in electrostatic repulsion with the increased solution IS. The AFM results show G4 has much higher pull-off forces than Env435 upon retraction of the colloid probe and much less repulsion upon approach of the probe. These AFM results agreed with data from the macroscopic adhesion tests, which showed that strain G4 was retained much more readily in columns packed with glass beads than strain Env435.

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