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Analysis of Bacterial Adhesion Using a Gradient Force Analysis Method and Colloid Probe Atomic Force Microscopy

Xu Li and Bruce E. Logan*

Department of Civil and Environmental Engineering, Pennsylvania State University, University Park, Pennsylvania 16802

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The atomic force microscope (AFM) has been used to examine the stickiness of bacteria on the basis of the analysis of approach and retraction force curves between the AFM tip and the bacterial surface. One difficulty in analyzing approach curve data is that the distance between the AFM tip and the surface of the bacterium is difficult to define. The exact distances are difficult to determine because the surface of the bacterium deforms during force imaging, producing a highly nonlinear region in the approach curve. In this study, AFM approach and retraction curves were obtained using a colloid probe AFM for three strains of *Escherichia coli* (D21, D21f2, and JM109). These strains differed in their relative adhesion to glass surfaces, on the basis of measurements of sticking coefficients in packed bed flow through column tests. A gradient force curve analysis method was developed to model the interactions between the colloid probe and a surface. Gradient analysis of the approach curve revealed four different regions of colloid−surface interactions during the approach and contact of the probe with the bacterial surface: a noninteraction region, a noncontact phase, a contact phase, and a constant compliance region. The noncontact phase, which ranged from 28 to 59 nm for the three bacterial strains, was hypothesized to arise primarily from steric repulsion of the colloid by extracellular polymers on the bacterial surface. The contact phase, spanning 59−113 nm, was believed to arise from the initial pressure of the colloid on the outer membrane of the cell. The constant compliance region likely reflected the response of the colloid probe to the stiff peptidoglycan layer that confers strength and rigidity to gram negative bacteria. It was shown that the sticking coefficients reported for the three *E. coli* strains were correlated with the length of the noncontact phase but not the properties of the other phases. Sticking coefficients were also not correlated with any parameters determined from retraction force curves such as pull-off distances or separation energies. These results show that gradient analysis is useful for studying the contribution of the length of the exopolymers on the cell surface to bacterial adhesion to glass surfaces.

Introduction

Bacterial adhesion to a surface is a complex process that can be separated into two stages. First, a negatively charged bacterium approaching a negatively charged surface experiences repulsive or attractive forces that affect whether the bacterium will reach the surface. These forces consist of Lifshitz−van der Waals (LW) attractive forces, electrostatic (EL) repulsive forces, and acid−base (AB) forces arising from hydrogen bonding between two surfaces immersed in a polar solvent (e.g., water).[1,2] The second stage of adhesion occurs when the bacterium has reached the surface. At this point, the cell will either rapidly desorb from the surface or it will remain attached as the probability of desorption decreases exponentially with time spent at that surface.[3]

The atomic force microscope (AFM) has been used to study these forces in order to better understand the factors that affect adhesion.[4−6] Two AFM techniques have generally been used: either the bacteria are attached to the AFM tip[7] or the tip is used to probe the bacterium.[8] Ong et al.[6] demonstrated by using the first approach that the length of the lipopolysaccharide (LPS) molecules on two strains of *Escherichia coli* affected the repulsive force between the bacteria and a glass surface on the approach of the bacterium to the surface. *E. coli* strain D21f2, which has a truncated lipopolysaccharide layer, was repelled by the surface, while *E. coli* strain D21, which had a longer LPS molecule, was attracted to the surface. However, macroscopic measurements of the "stickiness" of the bacteria were made in packed bed (column) tests. Cell stickiness was quantified in terms of a sticking coefficient, calculated as the probability of attachment on the basis of the number of times a bacterium strikes a surface (predicted by a semiempirical model). Sticking coefficients for these two strains to glass beads measured in column tests[9] did not agree with AFM predictions that strain D21 was "stickier" than strain D21f2 under similar solution conditions of ionic strength and pH. Measurements made by others using the latter approach (using the tip to probe individual bacteria) failed to detect any difference in the repulsive interactions between the tip and these same strains.[8,9] Interpretation of the results reported by Ong et al. is complicated by the fact that they added glutaraldehyde to their bacteria which cross links proteins and can change the relative stickiness of the bacteria.

* Corresponding author. Phone: 814-863-7908. Fax: 814-863-7304. E-mail: blogan@psu.edu.

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Our analysis of the literature indicates that most approach curves between AFM tips and bacteria exhibit repulsive, and not attractive, forces.[4,8−11] Instances where attractive forces between a surface and bacteria cause a jump-in of the tip to a surface are relatively rare.[12] When approach curve forces due to steric repulsion are considered, it has been possible to relate AFM derived forces to adhesion data in some cases. For example, using eight strains of *Streptococcus mitis*, it was shown that approach curve interaction forces measured with an AFM could be positively correlated with macroscopic adhesion data obtained using a parallel-plate flow through the chamber.[13]

Retraction AFM force curves have also been used to understand factors affecting bacterial adhesion,[10] but retraction curve data alone do not always correlate with macroscopic adhesion measurements. To obtain a retraction force curve, a tip is first brought into contact with the surface; the force holding the tip to the surface is then measured when the tip is retracted. Using this approach, Lower et al.[14] found that anaerobically grown cells of *Shewanella oneidensis* that respired using iron had greater adhesion to a surface than aerobically grown cultures. They interpreted this result as evidence of adhesion between respiratory enzymes that reduced the iron and the mineral surface. Retraction curves have also been used to explain differences in the adhesion of *E. coli* to surfaces treated with ethylenediamanetetraacetic acid (EDTA) that removed 80% of the LPS layer.[10] Vadillo-Rodriguez et al. found that macroscopic adhesion data for eight strains of *S. mitis* could only be correlated with approach curve data, not retraction curve data.[13]

The above studies provide evidence that both approach and retraction curve data must be considered in explaining macroscopic observations of bacterial adhesion to a surface. Here, we use a colloid probe AFM to demonstrate that, by using a gradient analysis method, the approach curve can be separated into four distinct sections: a noninteraction region, a constant compliance region; one section where the probe and surface undergo noncontact interactions; and one section where the colloid probe and bacteria are in contact. We compare our results from the analysis of approach and retraction curves to sticking coefficients reported for macroscopic adhesion tests in order to determine which of these AFM-based approaches are useful as predictors of microbial adhesion.

Methods

Bacterial Cultures. Three *E. coli* strains (D21, D21f2, and JM109) were used that have different LPS lengths.[6,9] A full length LPS molecule is composed of three components: keto-deoxy-octulonate (KDO); a core polysaccharide; and an O-antigen. Strain D21 contains both the KDO and the core polysaccharide but lacks the O-antigen, while strain D21f2 contains only the KDO molecule. *E. coli* K-12 strain JM109 has a complete LPS layer. Strains D21 and D21f2 were obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University, while strain JM109 was obtained from Shahriair Mobashery at Wayne State University.

Cyro-preserved cultures (100 μL; −80 °C) were revived in 20 mL glass tubes containing 5 mL of Luria broth (LB; 25 g/L) by incubating them for 15−18 h on a tube rotator at 26 °C (midexponential growth phase). A cell suspension (1 mL) was then transferred to a fresh medium (LB; 100 mL) in a 250 mL flask that was incubated for 4 h with shaking (150 rpm; 26 °C). Cells (10 mL) were washed three times in a phosphate buffer solution (PBS; 1.548 g/L KH_2PO_4 and 5.720 g/L K_2HPO_4; 100 mM ionic strength) at a pH of 7.2, centrifuged at 4200*g* at 20 °C for 10 min, and resuspended in fresh PBS.

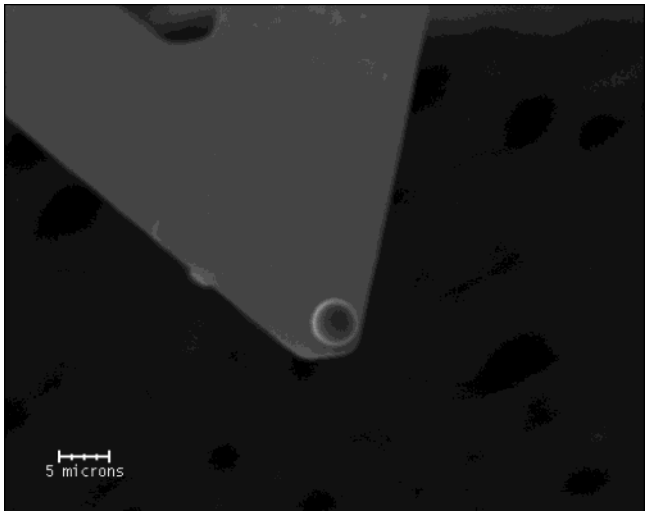

Figure 1. Scanning electron micrograph image showing the glass colloid probe mounted on a tipless AFM cantilever. (The micrograph was obtained at Penn State using a gold/platinum vapor deposited layer).

Bacteria were bound to glass cover slips for AFM imaging as previously described.[8] Slides were prepared by adding 1 mL of polyethyleneimide (PEI) to cleaned cover slides (24 × 60 mm^2), rinsed with Milli-Q water, and blown dry using N_2 gas. A bacterial suspension (1 mL) was placed on top of the treated slide for 20 min, the slides were rinsed with Milli-Q water, and then the slides were used immediately for AFM analysis.

AFM Experiments. AFM force curves were obtained using a BioScope AFM (Digital Instrument, Santa Barbara, CA) equipped with an AFM head, a Nanoscope IIIa control system (version 4.32r3), and an optical inverted microscope (Olympus IX70). Colloid probes were prepared using tipless cantilevers (Veeco NanoProbe NP-020) and glass beads (diameter, 3−10 μm; Polysciences, PA). Glass beads were rinsed three times with Milli-Q water, and a drop of the beads was placed directly on a microscope slide (75 × 25 mm^2; Corning 2948, NY). The beads were then dried by blowing them with N_2 gas. A drop of UV light cured glue (Norland Optical Adhesive, NJ) was added to the other end of the same glass slide and spread out into a thin layer using N_2 gas. Glue was placed onto the cantilever by lowering it into the glue and then retracting it, leaving a patch of glue on the cantilever end. Using the BioScope optical microscope, the cantilever was positioned over a glass bead (∼4 μm diameter) and the cantilever lowered to contact the bead. The cantilever containing the bead was then retracted and placed in the BioForce UV/ozone cleaner for 40 min to cure the glue. An example of a colloid probe AFM tip is shown in Figure 1. Force curves were obtained on individual bacteria. Each bacterium was first located using tapping mode imaging, and then, force curves were obtained by switching to contact mode.[8] The loading force applied to each bacterium was fixed at ∼4.5 nN by controlling the maximum deflection of the cantilever on the surface to be ∼150 nm. Unless stated otherwise, each force curve used in the analysis is the average of six force curves, with each force curve an average of 10 measurements taken on the top of the bacterium. The force curve data were imported into a spreadsheet and analyzed as described below.

Deflection distances were converted to forces (nN) using Hooke's law, or $F_s = -k_c d_d$, where k_c is the spring constant (nN/nm) equal to 0.03 as specified by the manufacturer and assumed to be a constant for all cantilevers (obtained from the same wafer) and d_d is the deflection of the cantilever (nm). Forces are positive when the cantilever is deflected upward from the sample and negative with the cantilever is drawn down to the sample. The

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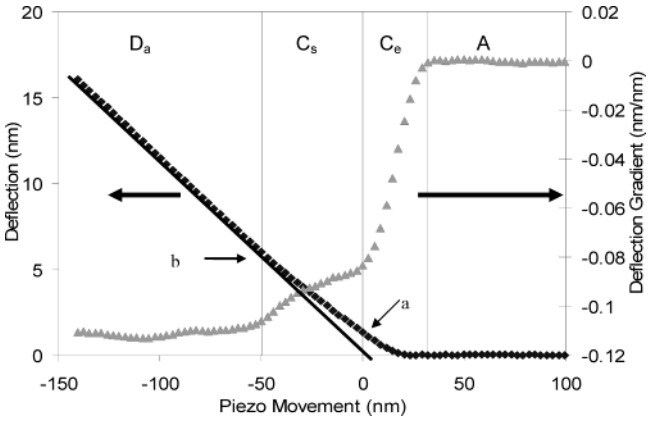

Figure 2. A sample AFM approach curve (diamonds) and the corresponding gradient analysis curve (triangles) for *E. coli* strain JM109. The four phases are defined on the basis of the linear regions observed in the gradient analysis data: *A*, noninteraction region; C_e, noncontact phase; C_s, contact phase; D_a, constant compliance. (The force curve shown is an average of 10 measurements.)

cantilever deflection, d_d (nm), was calculated as $d_d = (nS)/2^{16}$, where n is the value of the digitized signal, S is the total deflection distance in the z-direction (nm), and 2^{16} is the number of signal intervals (bytes for analog to digital conversion).

Force Curve Analysis. Approach force curves were analyzed using a gradient analysis method. The gradient at each point in the approach curve was calculated as the slope of a line based on the x and y values of the point and the locations of the two points preceding and succeeding the point.

Retraction curves were analyzed in terms of three different factors: the separation force, F_s, the pull-off distance, P_r, and the separation energy, E_s. The maximum separation force, $F_{s,max}$, was defined as the maximum force on the basis of the maximum deflection distance, $d_{d,max}$, experienced by the cantilever during retraction. The pull-off distance, P_r, is the distance the cantilever moves from the point of the maximum separation force to the point where the tip is no longer deflected. The separation energy, E_s, was obtained as the product of the force holding the probe to the surface and the distance moved by the cantilever during probe retraction. Each force at each point, $F_{s,i}$, was calculated for a scan split into $a = 512$ intervals where the distance moved by the cantilever in each interval, l, was calculated as $l = L/512$, where L is the total scan distance. The total separation energy is thus calculated as the total area in the attraction-force region (area below the baseline) in a retraction curve as

$$E_s = \sum_{i=1}^{a} lF_{s,i} = -\sum_{i=1}^{a} lk_c d_{d,i} \quad (1)$$

Results

Gradient Analysis of Approach Curves. An example of a gradient analysis of an approach curve using a colloid probe is shown in Figure 2 for a single *E. coli* JM109 bacterium in PBS. The x-axis indicates the distance that the piezo moved, and the primary y-axis indicates the deflection data measured on the basis of the location of the colloid probe. The gradient analysis of the cantilever, shown on the secondary y-axis, indicates four separate and approximately linear phases. In region *A*, the glass bead is too far away from the bacterium to be deflected. Thus, both the approach and gradient curves have a slope of zero. In phase D_a, the colloid probe is fully in contact with the bacterium, and as it pushes down on the bacterium, there is a linear response of the tip (i.e., a constant compliance region). The slope of the approach curve in phase D_a can be used to determine the spring constant of the bacterium on the basis of the spring constant of the cantilever as previously described.[8]

Table 1. Sticking Coefficients and Parameters Measured Using Approach and Retraction Curve Data (Average ± SD) for *E. Coli* Strains D21, JM109. and D21f2

	bacterium		
parameter	D21	JM109	D21f2
sticking coefficient[a]	0.019 ± 0.009	0.047 ± 0.003	0.066 ± 0.008
length of the LPS layer (nm)[b]	4	34	1
Approach Curve Data			
total contact distance, $C = C_e + C_s$ (nm)	172	95	112
noncontact length, C_e (nm)	59	36	28
contact length, C_s (nm)	113	59	84
Retraction Curve Data			
maximum detachment force, $F_{s,max}$ (nN)	0.16 ± 0.03	0.16 ± 0.02	0.34 ± 0.03
pull-off distance, P_r (nm)	706 ± 69	100 ± 8	275 ± 104
separation energy, E_s ($1000kT$)	14.7 ± 2.5	2.1 ± 0.3	7.7 ± 0.4

[a] Data from ref 9. [b] Lengths estimated on the basis of the LPS components known to be present for each strain and typical values reported in ref 21.

Gradient analysis of the nonlinear portion of the approach curve, C, reveals two separate linear phases, C_e and C_s. We interpret phase C_e to be the noncontact phase. Over a distance of $C_e = 32$ nm, the probe is deflected away from the surface of the bacterium by a maximum of 1.35 nm (point a in Figure 2), by a combination of electrostatic repulsion between the negatively charged cell and/or polymers and the glass bead, and steric forces arising from extracellular molecules on the surface of the cell.

Phase C_s is defined by another linear region in the gradient data. The maximum force exerted on the cell during the contact phase is 0.20 nN (point b). In this contact phase, it can be assumed that the colloid probe has reached and contacted the outer membrane of the cell. However, exactly what happens in phase C_s is not completely clear. The length of the contact region is 56 nm, and thus, the colloid probe moves some distance before achieving a constant compliance region (phase D_a). Due to the length of phase C_s, it is likely that the probe is pushing into various polymers and proteins on the surface of the cell until it finally reaches material which provides a single constant repulsive force, such as the peptidoglycan layer. In gram negative bacteria, such as *E. coli*, the peptidoglycan layer is embedded in the outer membrane and provides cell rigidity. The maximum distance from the edge of the LPS layer to the peptidoglycan layer is 60−63 nm, based on 8 nm for the outer membrane thickness, 12−15 nm for the periplasm,[15] and 40 nm for a full LPS layer.[16] Thus, either this contact region of 56 nm could include the LPS layer or the peptidoglycan layer moves some distance before constant compliance is achieved. The contact length does vary for the bacterial strains (Table 1). However, we would expect strain JM109 to have the longest contact

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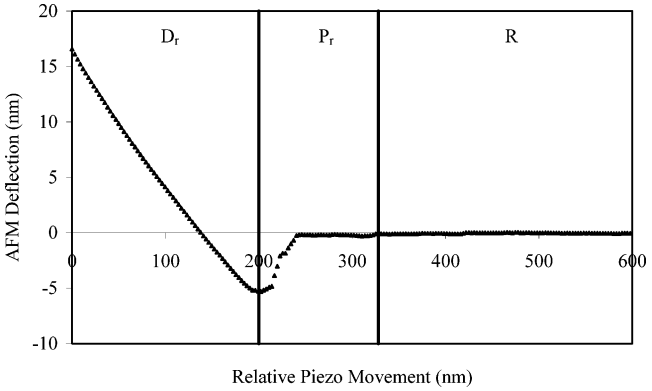

Figure 3. A sample retraction curve for *E. coli* strain JM109. Phase D_r is the constant compliance region observed during retraction, P_r is the maximum pull-off distance, and R indicates a region where there is no longer any interactions between the colloid probe and the surface. The distance of the largest trough is defined as $d_{d,max}$. (The force curve shown is an average of 10 measurements.)

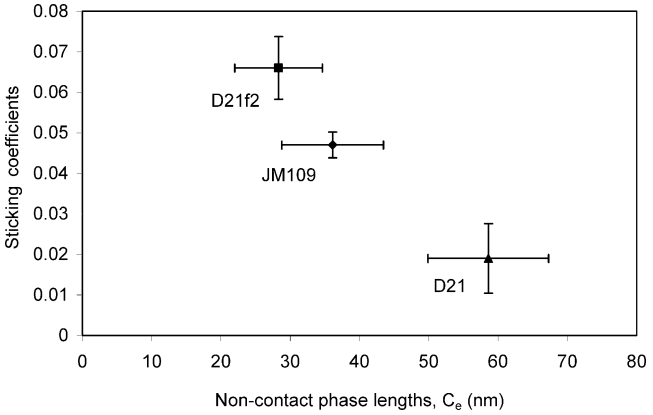

Figure 4. Correlation between the noncontact phase lengths of the three strains of bacteria and sticking coefficients. Error bars for noncontact lengths are based on ±SD for 50 AFM force curves, while sticking coefficients to glass beads are based on triplicate measurements in column tests as reported ref 9.

region if LPS was the main reason for the long contact distance, but strain D21, which lacks the long O-antigen, has the largest contact length of the three strains. The observation that D21 has the largest contact length suggests that molecules other than the LPS molecules are involved in determining the length of the contact phase. These could include proteins on the surface or other non-LPS associated polysaccharides.

Analysis of Retraction Curves. A retraction curve between the colloid probe and a bacterium exhibits a number of features that have previously been described in the literature.[17] During the colloid retraction, there are three general regions: D_r, P_r, and R (Figure 3). In phase D_r, the slope of the retraction curve is constant as the piezo is moved off the cell. Phase P_r (separation phase) is defined as the distance from the point where the tip jumps away from the bacterial surface to the point where the tip no longer deflects due to the presence of the surface. It includes the region where the probe exhibits a series of attractive forces that result in the held below a position expected for no interaction. The length of 128 nm of phase P_r reflects the sum of all adhesive forces that hold a bacterium to a surface. The maximum deflection distance, $d_{d,max}$, is used to calculate the maximum separation force using Hooke's law. The separation energy, E_s, is calculated using eq 1 from the deflection data obtained from phase P_r. The saw-tooth pattern exhibited in phase P_r is typical of the stepwise breaking of multiple bonds as the colloid probe is retracted.[18]

The length of phase P_r is larger than $C = C_s + C_e$ (88 nm) measured in the approach curve. This indicates that during the colloid's approach to the surface, molecules on the cell surface were oriented in a compact manner so that they were not fully extended to their maximum lengths. During retraction, however, the molecules on the cell surface stuck to the colloid probe were stretched to their maximum lengths as the colloid was pulled away from the cell surface. Thus, interaction distances were measured to be smaller during the colloid approach than during retraction. In phase R, there is no longer any contact or interaction of the cell surface polymers and the colloid probe.

Comparison of Approach Curve Distances with the Sizes of LPS Molecules. The average distances measured for the total contact, noncontact, and contact lengths from the approach force curves for the three strains of bacteria are given in Table 1. There was no apparent correlation between any of these approach curve distances and the estimated lengths of the partial and full lengths of the LPS molecules on the surfaces of the cells. Strain JM109 has a complete LPS layer, and therefore the longest LPS molecules, but the contact and noncontact distances measured for this strain were not the largest. This suggests that other molecules on the surfaces of these bacteria were responsible for the measured distances.

Correlation of Force Curve Results with Sticking Coefficients. The sticking coefficients of the three *E. coli* strains increase in the order of D21, JM109, and D21f2. Of the six parameters measured in the approach and retraction curves, only the noncontact length, C_e, was correlated with the sticking coefficient (Table 1). As shown in Figure 4, as the noncontact length increased, the sticking coefficient decreased. This suggests that the adhesion of these three strains to glass surfaces is controlled primarily by the initial electrostatic and steric (electrosteric) interactions experienced by the cell before they can fully contact a surface. It can also be seen in Figure 4 that there were large variations in noncontact phase lengths and sticking coefficients even among a monoclonal population of bacteria examined with the AFM colloid probe.

While the length of the contact phase did not predict variations in the sticking coefficients of the three strains, the contact phase was correlated with the pull-off distances and separation energies (Figure 5). As the length of the contact phase increased, more energy was required to fully detach the colloid from the bacterial surface. In addition, the pull-off distance also increased with the length of the contact phase. This suggests that, as the size of the contact phase increased, more polymers on the surface of the bacteria attached to the colloid probe, thus increasing the energy required to pull it from the surface.

Effect of Loading Force. An increase in the loading force, F_l, also increased the amount of energy needed to pull the colloid completely off the bacterial surface. As shown in Figure 6, as the loading force was increased from 0.66 to 7.4 nN, the separation energy increased from 14.7K kT to 346.5K kT. Thus, constant loading forces must be used when comparing different strains of bacteria.

Discussion

Using a gradient analysis method, it was shown that there were two separate components of the nonlinear portion of the approach curve: a noncontact phase and a contact phase. These two phases, which are not evident

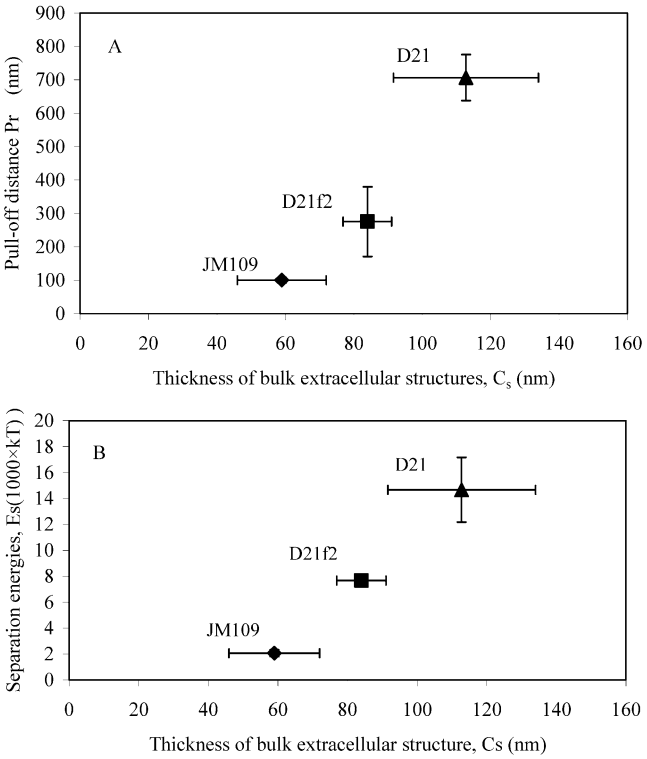

Figure 5. The thickness of the contact phase, C_s, measured in approach curves is correlated to (A) the pull-off distance, P_r, and (B) the separation energies measured between the probe and the bacterial surfaces in retraction curves.

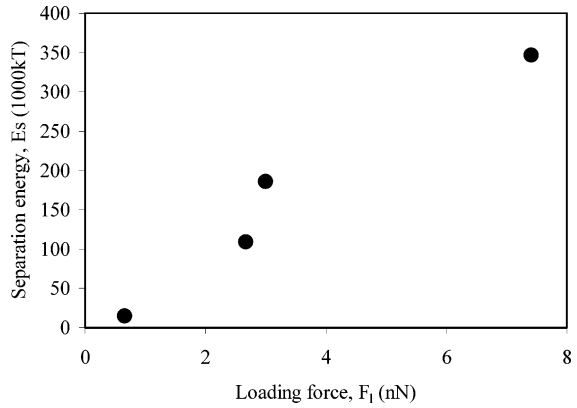

Figure 6. Loading force applied to the colloid probe can affect the measured separation energy.

in the approach curve itself, are identifiable as two distinct components using a gradient analysis method. The gradient analysis also provides information on the location of the bacterial surface during force imaging. The origin, or zero-distance location, in a force curve is often defined using a geometric approach on the basis of the intersection of two lines: one line drawn along the baseline and a second line drawn along the constant compliance.[4] As can be seen in Figure 2 (point a), the location that separates the contact and noncontact regions, indicated by a sudden change in the slope, coincides with the origin on the basis of the geometric approach. Therefore, we conclude that the point that separates the contact and noncontact phases is indeed the "zero" location of the cell surface.

The factors that contribute to the contact and noncontact regions of the approach curve cannot be determined solely on the analysis provided here. We hypothesize that the noncontact region arises primarily from steric interactions between the probe and polymers on the cell surface and that the contact region is based on the distance the probe moves into the outer membrane until it reaches the peptidoglycan layer. The noncontact region cannot be due solely to electrostatic effects, as the length of this layer (28−59 nm) is substantially larger than the electrostatic repulsive layer thickness (∼1 nm based on the Debye length for a 100 mM ionic strength solution[19]). In a study by Camesano and Logan,[4] it was shown that the thickness of a repulsive layer around bacteria was a function of pH and ionic strength. The thickness of this layer was much larger than that expected solely from electrostatic repulsion, consistent with the results presented here. Further experiments by Abu-Lail and Camesano,[20] showed that polymer lengths on bacteria changed in a manner consistent with how polymers change their conformation and length in response to ionic strength.

In both of these studies by Camesano and co-workers, the origin was defined using a geometric method where the origin is defined on the basis of extending two lines along the constant compliance line and the noninteraction approach line. Data in the force curve to the right of the origin were used for determining the repulsive layer thickness, while data to the left of the origin were discarded. As a result, the origin in their studies would have been the separation point between the contact and noncontact regions. Thus, we can infer (on the basis of the location of their origin) that the repulsive layer thickness measured by Camesano and Logan was essentially a noncontact phase distance, and did not include the contact phase distance.

Taken together, these results indicate that the length of the noncontact phase is an important factor that determines the relative stickiness of a bacterium to a glass surface. Steric repulsion was primarily due to extracellular polymers present on the outside of these three strains of bacteria, not the relative lengths of the LPS molecules of the bacteria. While this correlation of noncontact phase distance with stickiness does not rule out a role for separation energies in determining bacterial adhesion, it does indicate that the sizes of these polymers can be a dominant factor that can be used to distinguish the relative adhesion of bacterial strains to a surface.

Appendix 1. Notation

A = length of the noninteraction region in the approach curve, defined as the distance the tip moves until the point at which the tip deflects due to interactions with the surface (nm)

C = total contact distance in the approach curve defined as the distance between the first location where the tip deflects due to the surface and the starting point of the constant compliance region; $C = C_e + C_s$ (nm)

C_e = noncontact distance in the approach curve, assumed to be due primarily to electrostatic forces, defined on the basis of a gradient analysis of the approach curve (nm)

C_s = contact distance in the approach curve, assumed to be due primarily to steric interactions, defined on the basis of a gradient analysis of the approach curve (nm)

D_a = constant compliance region of the approach curve (nm)

D_r = constant compliance region of the retraction curve (nm)

d_d = distance the cantilever is deflected downward in retraction curves (nm)

$d_{d,max}$ = maximum value of d_d, e.g., maximum distance the cantilever is deflected during colloid probe retraction (nm)

d_u = distance the cantilever is deflected upward in retraction curves (nm)

E_s = separation energy calculated as the area under the retraction force curve in the negative (attractive) deflection region (kT)

F_l = loading force exerted by the colloid probe on a bacterium (nN)

F_s = separation force needed to completely separate the tip from the surface (nN)

$F_{s,max}$ = maximum separation force needed to separate the tip from the surface (nN)

k = the Boltzmann constant (J/K)

k_c = spring constant of the cantilever (nN/nm)

L = total scan distance moved by the piezo (nm)

l = distance between two consecutive data points in a force curve (nm)

n = value of the signal based on a possible range of 216 values

P_r = Pull-off distance in a retraction curve defined as the point from the jump of the tip from the surface to the point where the tip no longer deflects due to the presence of the surface (nm)

R = distance in the retraction curve defined as the point at which the tip no longer shows interactions with the surface to the tip starting point (nm)

S = total deflection distance in the z-direction (nm)

T = temperature (K)

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