Macroscopic and Nanoscale Measurements of the Adhesion of Bacteria with Varying Outer Layer Surface Composition

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The influence of the lipopolysaccharide (LPS) chain length on bacterial adhesion was investigated by measuring the collision efficiencies of three *Escherichia coli* K12 strains, each having a different length LPS, to silica glass beads in column tests (macroscale tests). Nanoscale interactions between the bacteria and a silicon nitride tip were probed utilizing atomic force microscopy (AFM). Adhesion results based on column tests indicated that collision efficiencies of the three bacteria were not consistently correlated to LPS length. Under conditions of low ionic strength (1 mM NaCl), collision efficiencies increased with LPS length for the three strains of *E. coli*. However, if cells were fixed with glutaraldehyde (2.5%), the strain with the shortest LPS chain had the greatest adhesion, while the bacterium with the mid-length LPS had the least adhesion to glass beads. Collision efficiencies increased when the solution ionic strength was increased from 1 to 100 mM as expected, and in most cases glutaraldehyde treatment also increased adhesion. AFM force curves failed to distinguish the adhesion characteristics of these bacteria measured in column tests, as all AFM force curves on the bacteria were identical. Changes in adhesion were also not predictable by more conventional measurements of bacterial properties based on ζ potential or contact angle. These results suggest that the LPS molecule length is not the sole determinant of adhesion of the three *E. coli* strains in porous media and that AFM force curve analysis, zeta potential, or contact angle data cannot yet be used to fully predict adhesion of these three strains to glass beads.

Introduction

Various models have been proposed to explain the forces affecting the initial adhesion of bacteria to a surface. The first models considered only electrostatic and van der Waals forces (DLVO theory)[1] and were originally developed to describe the stability of inorganic colloidal particles. It was found, however, that this approach did not account for the mid- to long-range polymer interactions observed between a bacterium and a solid particle.[1−4] For Gram-negative bacteria, more recent work has suggested that the lipopolysaccharide (LPS) layer on the outer bacterial membrane, and more loosely associated extracellular polymeric substances (EPS), can affect bioadhesion through steric interactions over long distances.[1−3,5−11] This has resulted in polymer-based models of adhesion.[1,12]

The outer membrane of Gram-negative bacteria is composed of phospholipids, proteins, and polysaccharides that serve to protect the cell as well as to give it shape and rigidity (Figure 1).[13] The LPS molecule is anchored in the outer membrane by a lipid end bonded to the rest of the LPS molecule consisting of ketodeoxyoctonate (KDO), a core polysaccharide, and a relatively larger O-antigen. The core and O-antigen region of the LPS consist mainly of six- and seven-carbon sugars. The hydrophilic portion of the LPS, which includes the ketodeoxyoctonate (KDO) through the o-antigen, extends into the surrounding aquatic environment. Molecular modeling has been used to examine the conformation and size of the LPS chain.[14] The length of the LPS chain up through the outer core is roughly 2−3 nm, whereas the length of the O-antigen portion may be as large as 40 nm, depending upon the number of repeating units, bending of the O-antigen chain, and solution conditions such as pH and ionic strength.

The emergence of new instruments for analyzing colloids, such as the atomic force microscope (AFM), has made it possible to elucidate molecular-level mechanisms of bacterial adhesion to solid surfaces and to probe steric interactions. The effect of LPS length on bioadhesion was

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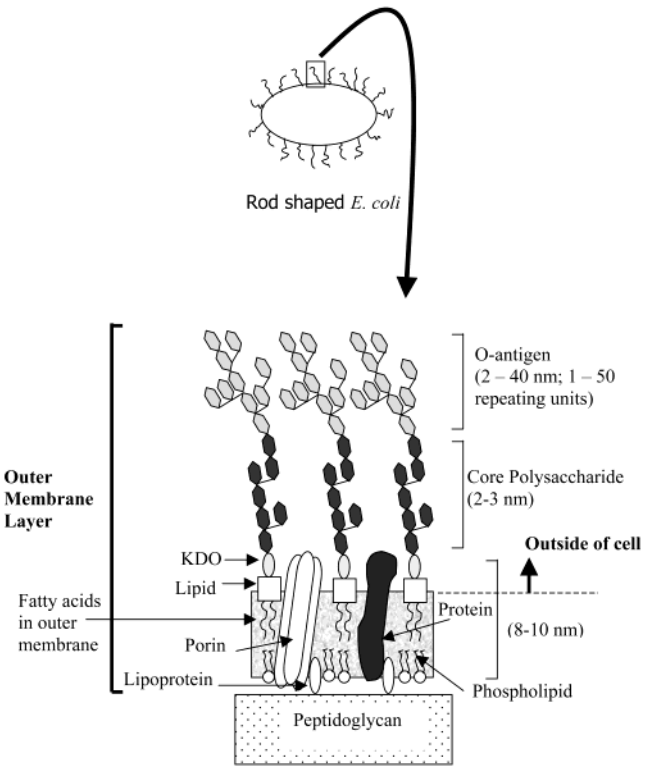

Figure 1. Schematic representation of a typical outer membrane of a Gram-negative bacterium. (Adapted from ref 13.)

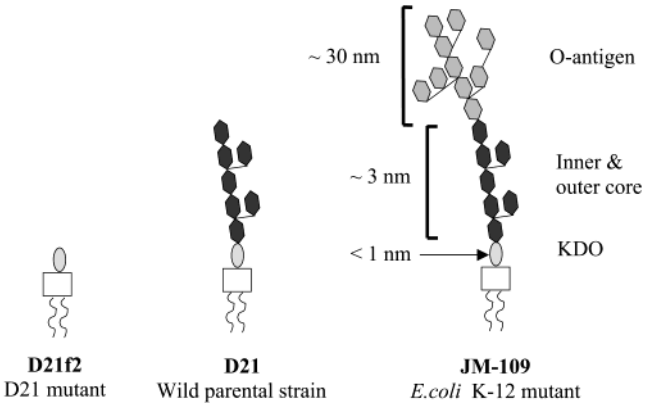

Figure 2. Lipopolysaccharide structures of *E. coli* strains used in study.

recently investigated by Razatos et al.[5,15] using two strains of *Escherichia coli* with progressively truncated LPS chain lengths. *E. coli* strain D21(wild type) expresses an LPS that extends from the lipid to the outer core region, while the mutant strain D21f2 has an LPS chain that is truncated just after the KDO carboxyl group (Figure 2). Using glutaraldehyde-fixed bacteria bound to an AFM tip, Razatos et al.[5,15] reported an attractive interaction between the wild-type strain (with the longer LPS chain) and a glass surface. In contrast, the mutant with the truncated LPS chain was repelled by the glass surface. This nanoscale interaction led to their prediction that bacterial adhesion increased with the length of the LPS chain. It was hypothesized that the repulsive force observed for the mutant strain was due to electrostatic repulsion between the negatively charged KDO molecules and negatively charged glass surface. The authors postulated that the longer LPS molecule of the wild strain shielded the negatively charged KDO moiety from the negatively charged glass surface. Additional support for the importance of steric interactions for bacterial adhesion was provided by demonstrating that coating a substrate with a polymer (poly(ethylene glycol), or PEG) created a physical barrier to bacterial adhesion by reducing both long-range attractive forces and providing a short-range steric hindrance to adhesion.[15]

There is great interest in the factors that affect bacterial adhesion to surfaces, particularly under conditions where bacteria are carried by advective flow through porous media. We were interested in whether the findings of Razatos et al.[5,15] would help us to better understand bacterial adhesion to mineral surfaces in groundwater aquifers. Bacterial adhesion in porous media can be important for reducing pathogen migration from water reinjected into the ground to recharge depleted aquifers. Although the studies of Razatos et al.[5,15] provided evidence of different molecular-scale interactions of the bacteria with surfaces, their findings of adhesion between a clump of bacteria on an AFM tip, or an AFM tip and a lawn of bacteria, were never directly tested by a non-AFM-based test. Thus, there was no confirmation that their findings could be used to understand adhesion of these two *E. coli* strains under different circumstances.

In this study, we therefore tested whether *E. coli* bacteria having different lengths of LPS would affect bacterial adhesion to porous media in the manner predicted by Razatos et al.[5,15] We tested the same two *E. coli* strains used by Razatos et al.[5,15] plus an *E. coli* K-12 mutant (JM-109) that expressed an entire LPS (a full O-antigen) to see if the longer LPS layer would further reduce adhesion (Figure 2). It was assumed that the LPS were uniform in length for each strain. Due to our interest in understanding bacterial transport in porous media such as groundwater aquifers, column tests were done under conditions of controlled ionic strength using an indifferent electrolyte (NaCl). The use of low ionic strength solutions has been shown to increase the transport of bacteria in porous media.[16,17] We also used an AFM to measure forces between the silicon nitride tip and the surface of individual bacteria. Because Razatos et al.[5,15] only examined bacteria that had been treated with glutaraldehyde, we examined AFM force curves obtained in both the presence and absence of glutaraldehyde treatment of these bacteria. Razatos et al.[5,15] argued that glutaraldehyde did not affect bacterial adhesion because both contact angles and ζ potentials before and after glutaraldehyde treatment remained unchanged. Glutaraldehyde is known to affect the elasticity of the bacterial surface.[18,19] The fact that glutaraldehyde treatment was necessary to attach bacteria to the AFM tip suggested that it might change cell adhesion properties in some manner.

Methods

Bacterial Cultures. Genetically modified *E. coli* K-12 strains D21 (parental wild-type) and D21f2 (mutant) were obtained from the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, CT). JM109, a mutant K-12 strain, was donated by Shahriar Mobashery (Wayne State University, Detroit, MI). All strains were nonmotile. Cells were grown in Luria broth (20 g/L) unless stated otherwise. Cells were stored at −80 °C in a tryptic soy broth/glycerol solution (50% v/v), revived

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by overnight growth in 5 mL of Luria broth (Miller's) at 26 °C, transferred to fresh medium (100 mL), and harvested in the mid-exponential growth phase (~10^8 cells/mL). Bacteria were washed by centrifugation (2800*g*, 20 °C, 10 min) 3 times in the test solution before being used in experiments. For the mini-column tests, washed cells were radiolabeled with tritiated leucine (5.81 nM, with a specific activity of 6.36 TBq/mmol) for 3 h in 100 mM NaCl solutions. Uptake of the label was measured by using a liquid scintillation counter (Wallac LKB, Turku, Finland). Cells suspended in 1 mM TRIS (tris(hydroxymethyl)aminomethane, Sigma) did not take up label, and cells washed repeatedly (10 times) in 1 mM TRIS buffer ruptured. For column experiments with 1 mM TRIS, cells were therefore labeled in PBS (100 mM) buffer. For low ionic strength experiments with 1 mM NaCl, cells were grown at 37 °C and labeled in PBS. Cells suspended in high ionic strength solutions (100 mM NaCl) were grown at 26 °C in the same NaCl solution. Cell suspensions were diluted to 10^6 mL^{-1} in 40 mL of the test solution prior to bioadhesion tests. Cells were counted by using acridine orange in order to determine the amount of radiolabel per cell and to verify the absence of cells in flocs.

In some experiments, radiolabeled cells (1 mL) were fixed in glutaraldehyde (2.5% v/v final concentration) for 2 h and then washed prior to being used. Glutaraldehyde was kept at −80 °C and thawed immediately before use to prevent polymerization.[20]

Phosphate-buffered saline (PBS; pH 7.2; ionic strength 171 mM) used in one experiment consisted of the following: Na_2HPO_4 (10 mM), KH_2PO_4 (1.37 mM), KCl (2.58 mM), and NaCl (136 mM).[5]

Bioadhesion Tests. The stickiness of a bacterium was quantified in terms of a bacterial collision efficiency using the microbe and radiolabel kinesis (MARK) minicolumn test. The bacterial collision efficiency (α) is the probability of attachment calculated by using a filtration model (see below). The MARK method allows for rapid collection of bioadhesion data and is particularly useful for bacteria with low collision efficiencies (α < 0.01); it is described in detail elsewhere.[16,21] Briefly, 3-mL plastic syringes (0.8 cm ID) mated to a vacuum box were used as columns. Glass beads (soda lime; Polysciences Inc., Warrington, PA) used to pack the column were cleaned by agitation in a 10% sulfuric acid solution for 3 h and rinsed with DI water prior to use. Beads were packed as a wet slurry for a final bed volume of ~1.5 mL. The porous medium was stirred with a spatula (to minimize entrapment of air bubbles) and rinsed with 10 pore volumes of deionized water followed by 10 pore volumes of the test solution (1 or 100 mM NaCl or TRIS) at flow rates between 4 and 6.4 mL/min (110−170 m/day). It has been shown using other bacteria that a fluid velocity in this range is not an important factor in cell adhesion.[21] The cell suspension (2 mL) was pulled through the packed bed followed by a cell-free rinse with the test solution (6 mL) to remove loosely attached cells. The packing was dried in the column by continuing to pull a vacuum for 15 s. The top of the column was extruded, cut, and placed into a scintillation vial (20 mL) for liquid scintillation counting. The number of bacteria retained in the column was calculated from the mass of retained radiolabel.

The collision efficiency was calculated by using the filtration model of Rajagopolan and Tien:[22,23]

$$\alpha = \frac{-2d_c \ln(1 - R)}{3(1 - \theta)\eta L} \quad (1)$$

where $d_c = 40\,\mu m$ is the diameter of the glass beads, R the fraction of bacteria retained in the column, $\theta = 0.46$ the porosity, η the collector efficiency, and L the length of the slice of glass beads extruded from the column (0.25−1.0 cm) and analyzed by scintillation counting. The collector efficiency is calculated as

$$\eta = 4A_s^{1/3}N_{Pe}^{-2/3} + A_s N_{Lo}^{1/8} N_R^{15/8} + 0.00338 A_s N_G^{1.2} N_R^{-0.4} \quad (2)$$

with

$$A_s = \frac{2(1 - \gamma^5)}{2 - 3\gamma + 3\gamma^5 - 2\gamma^6} \quad (3)$$

where $N_{Pe} = Ud_c/D_p$ is the Pectlet number, $N_{Lo} = 4H/(9\pi\mu d_p^2 U)$ is the London−van der Waals number, $N_R = d_p/d_c$ is the interception number, $N_G = U_p/U$ is the gravitation number, $\gamma = (1 - \theta)^{1/3}$, U is the fluid velocity, D_p is the diffusion constant of the bacterium calculated by using the Stokes−Einstein equation, $H = 10^{-20}$ J the Hamaker constant (a value consistent with previous studies[10]), μ is the viscosity of water, and d_p is the equivalent diameter of the bacterium (0.65 μm, D21; 0.59 μm, D21f2; 0.56 μm, JM109). The first term in eq 2 indicates the contribution of diffusion to adhesion while the other two terms include the effects of interception, gravitational settling, and London−van der Waals forces. For the conditions examined here, diffusion accounted for approximately 50% of the collisions.

AFM Experiments. Force curves were obtained on liquid samples in 1 mM TRIS buffer (pH 8.3) with an AFM (Bioscope; Digital Instruments) using a fluid cantilever holder and DNP−S silicon nitride cantilevers (Digital Instruments) with a force constant of 0.045 N/m as measured by the Cleveland method.[24] Cantilevers and tips were cleaned by using a BioScope UV/ozone TipCleaner before being used. Unless stated otherwise, a 1 mM concentration of TRIS (pK = 8.3) was used in AFM measurements to be consistent with that used by Razatos et al.[5,15] However, Razatos et al.[5,15] reported a lower pH of 7.5, presumably obtained by adjustment of pH using acid. The isoelectric point of the AFM tip has been reported to range from 4.2 to 7.6, depending on the specific composition of silanol and silylamine surface groups in the silicon nitride tip,[25] while glass beads are negatively charged at a pH above 2.[26] By using a pH above the isoelectric point of both the AFM tip and glass in force curves, we ensured that both the tip and the bacterium were negatively charged, a condition that reflected the negative charges between the bacteria and glass beads in column tests.

Bacteria were attached to polyethyeleneimine (PEI)-coated glass slides.[19,27] PEI (1 mL, 0.2%) was added to each slide for 3−5 h. After copious rinsing (10−50 mL), 1 mL of the bacterial suspension (10^8 mL^{-1}) was added to the slide. The bacteria-coated slide was rinsed to remove any weakly bound bacteria and used the same day.

Before obtaining a force curve the bacterium was imaged in tapping mode to locate the center of a cell.[19] The tip was withdrawn 10 μm from the surface of the bacterium, the scanning mode was then set in contact mode, and the tip was brought down toward the surface in 1-μm increments until deflection of the cantilever was observed. The force curves were obtained with a scanning frequency and scan size of 1 Hz and 1 μm, respectively. Multiple force curves were obtained to ensure reproducibility of the force curve and to confirm that this force curve represented the interaction between the tip and the top (and not the sides) of the bacterium.[19] The slope of the constant compliance region of the force curves was used to convert the deflection in millivolts to deflection in nanometers. There was no difference in force curves obtained for glass or PEI-coated glass slide.

ζ Potentials and Contact Angles. Cell suspensions prepared as described above were resuspended in 1 or 100 mM NaCl solutions or 1 mM TRIS. Zeta potentials were measured (ZetaPALS analyzer, Brookhaven Instruments Corporation, Holtzville, NY) 5 times using 15 cycles per analysis (triplicate samples). Contact angles were measured on bacterial lawns

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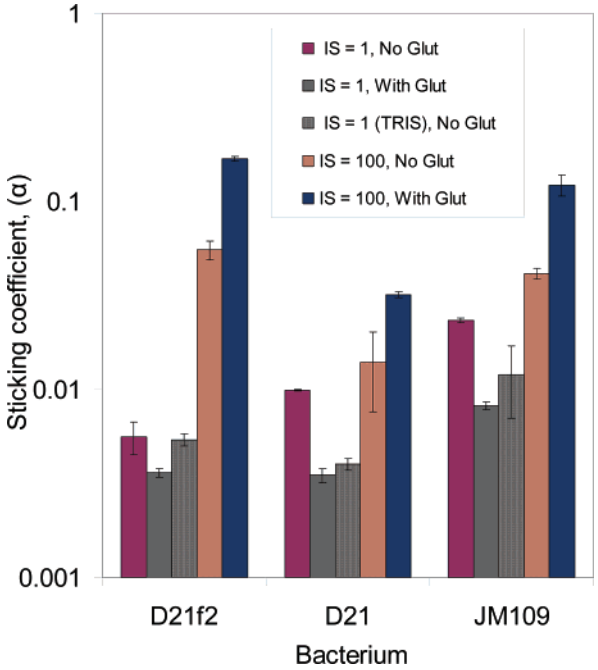

Figure 3. Effect of glutaraldehyde and ionic strength on collision efficiency. All experiments performed with washed cells resuspended in NaCl at the given ionic strength (IS) except as noted (Glut indicates cells fixed in 2.5% glutaraldehyde).

prepared by filtering 3-mL of a washed cell suspension (1 mM NaCl) onto aluminum oxide filters (Anodisc; 0.2-μm pore diameter, Whatman Corp.). Each filter, containing approximately 20 layers of bacteria, was rinsed with 5 mL of deionized water and dried in a Petri dish for 24 h. Contact angles were measured by applying a 3-μL drop of deionized water on top of the bacterial lawn. An image of each drop was magnified, photographed, and printed in order to directly measure the contact angle.

Results

Collision Efficiencies. The collision efficiencies of the three strains of *E. coli* to glass bead surfaces were not consistently correlated with the length of the LPS molecule (Figure 3). When cells were suspended in 1 mM NaCl (in the absence of glutaraldehyde fixation), collision efficiencies increased with LPS length (i.e. α's increased in the order D21f2, D21, and JM109). When glutaraldehyde was used to fix the cells or cells were suspended in TRIS buffer (1 mM; no glutaraldehyde) there was no significant difference in the collision efficiencies of D21f2 and D21. Strain JM109 had the highest collision efficiency of the three strains in the presence or absence of glutaraldehyde in a 1 mM NaCl solution.

The solution ionic strength affected the adhesion of all three strains of bacteria. In all cases, collision efficiencies were significantly increased when the solution ionic strength was increased from 1 to 100 mM regardless of glutaraldehyde treatment (Figure 3). The largest increase in the collision efficiency (almost 2 orders of magnitude) due to ionic strength was found for strain D21f2 treated with glutaraldehyde.

The effect of glutaraldehyde fixation on the collision efficiency was inconsistent for tests run at the two different ionic strengths. When a 100 mM NaCl solution was used, glutaraldehyde increased the adhesion of bacteria to glass beads (Figure 3). However, at a lower ionic strength of 1 mM, the addition of glutaraldehyde produced exactly the opposite effect and reduced the bacterial collision efficiencies of the three strains.

AFM Measurements. To try to understand the different effects of ionic strength and glutaraldehyde on the adhesion of these different strains, we used the AFM to measure interaction forces between the AFM tip and bacteria bonded to glass. We were able to obtain force curves on bacteria attached to glass, even though they were not fixed with glutaraldehyde, under conditions of low ionic strength (1 mM TRIS buffer; pH 8−9). The force curves obtained for the three different strains were not any different (Figure 5). A force curve obtained for D21 at a lower pH (7.5) in 1 mM TRIS buffer also failed to demonstrate any adhesion between the tip and the bacterium (data not shown). Because all force curves measured with the AFM were identical, it was not possible to relate differences in collision efficiencies measured in column tests to AFM force curves.

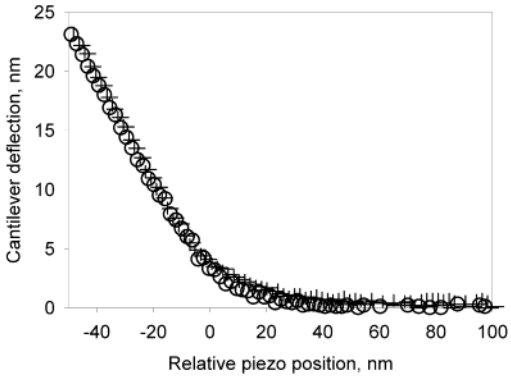

Figure 4. Deflection of AFM cantilever vs peizo movement when silicon nitride tip interacts with D21 (○), D21f2 (−), and JM109 (l). Cells prepared in 1 mM Tris.

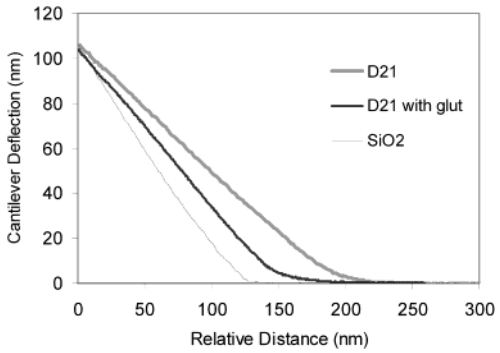

Figure 5. Effect of glutaraldehyde on forces between D21 and a silicon nitride tip.

Force curves for all three strains exhibited a region of constant compliance, with a linear slope of approximately −0.5 nm/nm (no glutaraldehyde) and a nonlinear region that extended about 40 nm (Figure 4). The linear portion of the force curve represents a region of linear elastic deformation of the surface of the bacterium. From this linear region, it was calculated that the spring constant of the bacterium was approximately 0.4 N/m.[19]

Although there were no differences in force curves measured for the three bacteria prepared and analyzed under identical conditions, the addition of glutaraldehyde consistently increased the rigidity of the cell surface. As shown in Figure 5 for strain D21, glutaraldehyde treatment increased the slope of the linear elastic region from −0.5 to −0.75, indicating the stiffness of the cells increased by 50% compared to cells that were not treated with glutaraldehyde. The same changes following glutaraldehyde treatment were observed for the other two strains (D21f2 and JM109; data not shown). The finding that glutaraldehyde makes cells stiffer was expected based on the effects of glutaraldehyde observed in other AFM force

Table 1. Contact Angles of the Three *E. coli* Strains

bacterium	contact angle in water	
	Ong et al.[7]	this study
D21f2	31 ± 4	33 ± 5
D21	19 ± 3	25 ± 2
JM109	*a*	17 ± 1

[a] Not reported.

Table 2. Measured ζ Potentials for *E. coli* Strains with (2.5% v/v) and without Glutaraldehyde Treatment

solution	glutaraldehyde, %	ζ potentials of *E. coli* strains, mV		
		D21f2	D21	JM109
1 mM NaCl	0	−50 ± 2	−43 ± 3	−58 ± 2
1 mM NaCl	2.5	−58 ± 1	−51 ± 2	−45 ± 1
1 mM TRIS	0	−43 ± 3	−40 ± 3	−50 ± 1
1 mM TRIS	2.5	−43 ± 5	−41 ± 3	−50 ± 2
100 mM NaCl	0	−27 ± 1	−21 ± 1	−26 ± 1
100 mM NaCl	2.5	−36 ± 1	−18 ± 1	−24 ± 2

measurements on liver endothelial cells,[28] kidney cells,[18] and *E. coli* in one study[19] but not in another study.[29]

Contact Angle and ζ Potential. D21f2 was more hydrophobic (contact angle = 33 ± 5°) than D21 (contact angle = 25 ± 2°), in general agreement with previous results by others[7] (Table 1). The lowest contact angle (17 ± 3°) was measured for strain JM109.

The ζ potential of D21f2 was always equal to, or more negative than, that of D21, regardless of the ionic strength, type of solution (NaCl or TRIS), or glutaraldehyde treatment (Table 2). The ζ potentials measured here for D21f2 and D21 (−58 ± 1 mV and −51 ± 2 mV, respectively) in a 1 mM NaCl solution were larger than those measured by Ong et al.[7] (36 ± 4 mV and 28 ± 2 mV) but they used a different solution (1 mM phosphate buffer). ζ potentials were smaller and not different for D21f2 and D21, when bacteria were suspended in a 1 mM TRIS buffer.

The ζ potentials of the three bacteria were lowered when the solution ionic strength was increased from 1 to 100 mM as expected (Table 2). There was no consistent effect of glutaraldehyde on overall cell charge. In most cases (except for strain JM109 in 1 mM NaCl) the ζ potential increased or stayed the same following glutaraldehyde treatment. There was also no consistent relationship between ζ potential (Table 2) and collision efficiencies (Figure 4).

Discussion

Bioadhesion tests using glass bead packed minicolumns demonstrated no consistent correlation between LPS length and adhesion of the three *E. coli* K-12 strains under the various conditions of ionic strength and glutaraldehyde fixation. The only case where a correlation between LPS length and collision was observed for the three strains of bacteria was when (*i*) cells were examined under low ionic strength (1 mM) conditions in NaCl and (*ii*) cells were not fixed with glutaraldehyde. However, strain D21f2 (the bacterium with the shortest LPS chain) had a collision efficiency equal to or greater than that for D21 in four of the five paired comparisons of collision efficiencies (Figure 4). Our findings for glutaraldehyde-treated cells was different than that of Razatos et al.,[5] as their AFM-based adhesion method indicated that glutaraldehyde-fixed D21 was stickier than D21f2. They found an attractive force between D21 and a glass surface (or an AFM tip), while they measured a repulsive force between D21f2 and a glass surface. Our tests (Figure 3) indicated that glutaraldehyde changed the adhesion of the different strains to the glass surface under conditions present in porous media transport tests.

We also observed that increasing the ionic strength of the suspension increased the collision efficiency of all three strains with the glass surfaces (Figure 3). This effect of ionic strength on collision efficiency is consistent with that found for other bacteria in column-based adhesion tests.[16,17,21,30] Similarly, Ong et al.[7] (their Figure 5) found that increasing the ionic strength through addition of 100 mM NaCl to the test solution reduced the repulsion between D21f2 and a mica surface and therefore concluded this interaction was due to electrostatic interactions.

It was observed in column tests that the adhesion of D21 increased with ionic strength when it was changed from 1 to 100 mM (NaCl). This finding, however, differs from the AFM-based results of Ong et al.[7] They found that for D21 there was no change in the attractive force measured between cells on the AFM tip and a mica surface in 1 mM TRIS or after addition of 100 mM NaCl. There are several possible explanations for these different results. The physical interactions produced between the bacterium and glass beads in our column-based bioadhesion experiments may be sufficiently different from those conditions produced between the lawn of cells and the flat surfaces used by Ong et al.[7] for their AFM based tests. Because there was a lack of change in adhesion with solution ionic strength for D21, Ong et al.[7] hypothesized that the interaction of D21 with a surface must be due to van der Waals interactions, bridging, or hydrophobic interactions. However, in our glass bead experiments, surface roughness may also be important. It has been shown, for example, that bacterial adhesion increases with molecular-scale roughness of glass.[31] In addition, about half of the collisions between the bacteria and surfaces in column tests are produced by Brownian motion, while interception, gravitational settling, and London−van der Waals forces account for other collisions. Once a bacterium collides with a surface in a column test, it can be removed from the surface by shear forces. These different conditions may produce results in column adhesion tests that are sufficiently dissimilar to AFM adhesion tests.

AFM force curves measured here for D21 and D21f2 were not different, while Razatos et al.[5] reported that D21 was attracted to the AFM tip (or glass surface) and D21f2 was repelled by the tip. The different AFM findings could be due to differences in solution chemistry, such as pH and ionic strength, cell preparation techniques, or the different atomic force microscopes used. While we both used a 1 mM organic buffer (TRIS) for AFM measurements, the pH used in our study was in the range of 8−9 versus 7.5 reported by Razatos et al.[5] However, we did not measure an attractive force between a cleaned AFM tip and D21 under conditions of a low (1 mM) or high (100 mM) ionic strength (using NaCl) or when we adjusted the pH of TRIS to 7.5. It is more likely that our different results are due to some unidentified cell preparation technique. We washed the bacteria by centrifugation three times at 2800*g*, while in other studies cells were "rinsed repeatedly"[5] or "rinsed copiously"[7] at 8000 rpm (*g*-forces were not given). We chose our centrifuge conditions and washing

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steps to minimize the chance of cell damage. It is known that high-pressure filtration of bacteria, for example, can affect membrane integrity and can release amino acids from the cell and alter cell activity.[32]

There are some similar findings in our study and that of Razatos et al.[5] The distances over which we observed nonlinear forces for *E. coli* (30−50 nm) are consistent between the two studies. These distances are somewhat larger than those calculated for the electrostatic double layer for silica for our experimental conditions. At a pH of 6, the Debye length, at an ionic strength of 1 and 100 mM, is roughly 10 and 1 nm, respectively.[33] These distances are also larger than the LPS molecule length for D21 (3 nm) and D21f2 (<1 nm),[5] and therefore electrostatic forces might be expected to play a role in adhesion. However, we were unable to observe a change in AFM force curves as a function of ionic strength.[19]

It is clear that differences in collision efficiencies observed for the three different *E. coli* strains in our column studies were not reflected by any differences in our AFM force curves, as all force curves for the three bacteria were identical (Figure 5). It is this lack of a change in the AFM measurements that is of primary concern here, as our goal was to determine if we could use AFM force curves to understand adhesion of these different strains to glass and mineral surfaces during their transport in porous media. One reason for the similarity of force curves for the three *E. coli* strains may be that the forces between a tip and a bacterium are too small (less than several nN) to measure in these AFM tests. If the main factor governing bacterial adhesion were electrostatic forces, the interaction forces between the tip and the bacterium would be <1 nN and therefore not measurable by our system.[19] If bacterial adhesion was primarily a result of polymers on the bacterial surface, it may be that these polymers were brushed away by the pyramid-shaped tip during imaging and therefore were not detectable by our AFM scans.

More conventional approaches than AFM force curves for predicting different adhesion properties of colloids, such as ζ potential, also did not provide a clear prediction of adhesion of the three *E. coli* strains to glass beads. For example, D21f2 suspended in a solution with an ionic strength of 100 mM had a more negative ζ potential than D21 or JM109 (Table 2) and, therefore, should have the lowest collision efficiency of the three strains. In contrast, we measured the largest collision efficiency for this strain under these conditions of glutaraldehyde fixed cells (Figure 3).

Contact angles are known to be positively correlated with bacterial adhesion,[34] and water contact angles measured for each bacterium (Table 2) were significantly different. Our measurements suggest that D21f2 would have the largest collision efficiency because it had the highest contact angle with water of the three bacteria and JM109 the smallest collision efficiency because it had to lowest contact angle. However, JM109 was not consistently the least adhering of the three strains. In general, large changes in ζ potential and contact angle are needed to see significant differences in bacterial adhesion.[34] It may be that the range of values observed here for the three strains was too small to adequately predict adhesion differences between these different bacteria or that these methods are not sufficient to detect the factors responsible for adhesion differences.

It is well-known that glutaraldehyde stiffens a bacterium by cross-linking proteins and amino acids in the peptidoglycan layer[20] but the effects of fixation on cell properties related to adhesion have not been well-studied. Others have found that glutaraldehyde caused yeast cells to become more hydrophobic.[35] Razatos et al.[5] assumed that glutaraldehyde did not affect the bacteria because they did not measure any changes in the ζ potentials or contact angles of the bacteria following glutaraldehyde treatment. However, we did find differences in ζ potentials of the three strains following glutaraldehyde treatment (Table 3). Our column test results also show that the collision efficiencies of D21 and D21f2 strains were affected by glutaraldehyde, and therefore it is clear that glutaraldehyde changed the bioadhesion properties of these two strains.

Taken together, the results from our study indicate that the differences measured in adhesion in column-based bioadhesion tests between three *E. coli* strains that vary only in their LPS configuration cannot yet be fully explained either by existing nanoscale surface measurements such as AFM force curves or by more conventional techniques such as contact angles measured on bacterial lawns and ζ potentials of bacteria. While we believe it is likely that polymer configuration of both EPS and LPS play a role in bacterial adhesion, these steric factors cannot yet be fully understood with the current level of AFM analysis used here (i.e., simple force curves). Clearly, new approaches will be necessary to detect differences in bacterial surface properties to an extent that makes it possible to explain differences in adhesion between these three *E. coli* strains.

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