**RELATIONSHIP BETWEEN THE ADHESIVE PROPERTIES OF BACTERIA
AND THEIR TRANSPORT AND COLONIZATION IN THE SUBSURFACE
ENVIRONMENT**

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Relationship Between the Adhesive Properties of Bacteria and Their Transport and Colonization in the Subsurface Environment

This research has focused on the attachment of bacteria to solid surfaces and the significance of their adhesion properties in their transport through porous media. Our work has focused on strains of *Pseudomonas* and a related species *Burkholderia cepacia*. Most of our experimental strains were isolated from subsurface environments at US DOE experimental field sites. The first portion of this project was conducted at the University of Maryland during 1994-1996, during which two graduates and one graduate student were supported by the award. The project was then continued under contract number DE-FG02-96ER62302 at the University of South Carolina, where one postdoctoral associate has been supported by the award.

This project has had 5 major components:

- (1) Transposon mutagenesis was used to generate strains with altered adhesion properties, and attempts were made to identify the cell surface changes responsible for the alterations in the mutants' attachment characteristics;
- (2) The relationship between adhesion characteristics and bacterial transport through porous media was assessed by determining relative transport characteristics of our experimental strains in bench-top sand columns;
- (3) The presence and adhesion characteristics of bacteria present in deep subsurface samples from the DOE field site at Cerro Negro, NM, were evaluated, so these could be related to the bacterial transport characteristics;
- (4) Cell surface properties of strains used in the DOE field transport experiments at Oyster, VA, were evaluated; and
- (5) The transport properties of our experimental strains in intermediate scale experiments were determined and evaluated in relation to models describing fluid flow.

These components are described in turn in the following sections, and publications covering portions of the work are indicated in parenthesis and listed at the end of the this report.

1. The Adhesion and Cell Surface Characteristics of Wild Type and Mutant *Pseudomonas* Strains.

The objectives of this work were (i) to use transposon mutagenesis to produce mutants of *Pseudomonas* (or closely related) strains that were altered in adhesion ability and transport through porous media and (ii) to identify the alterations in surface characteristics that were responsible for the changes in attachment. Our initial experiments were with an estuarine isolate of *P. fluorescens*. After working out the mutagenesis methodology, we obtained *Pseudomonas* strains isolated from the DOE Savannah River Site (Aiken, South Carolina) and the GEMHEX program site at the Hanford Reservation, Washington, so that our studies would include a range of subsurface isolates from different sampling sites relevant to the DOE.

Mutants of the test strains were generated with *TnphoA*, which enabled identification of those mutants that were altered in surface proteins. Transposon mutants were screened

for alterations in adhesion ability by attachment assays on hydrophobic polystyrene and water-wettable polystyrene. In our initial experiments with *P. fluorescens*, four *TnphoA* mutants with increased adhesion to the hydrophobic surface and decreased adhesion to the water-wettable surface were obtained. Transport of the strains through porous media was evaluated by passing suspensions of each mutant and the parent through columns containing quartz sand and determining the number of cells retained in the columns. The mutants all demonstrated increased adhesion and retention in the columns. Southern analysis demonstrated two types of mutants with separate transposon insertion sites. Polyacrylamide gel electrophoresis of the strains demonstrated that the O-antigen on the LPS was either attenuated or absent. Lack of this polysaccharide, and the consequent increased exposure of the lipid moiety of the LPS, is probably responsible for the increase in adhesion to the hydrophobic substrata and retention in the sand column. This work was the first report of LPS being involved in attachment to nonbiological surfaces. Combined with previous published studies of attachment of *P. fluorescens*, these results demonstrated that more than one type of polymer can mediate the adhesion of this organism to solid surfaces (Williams and Fletcher, 1996). The portion of this work relating to surface analysis or the organisms was supported by this award, whereas the production of these strains was supported by a contract from the Environmental Science Research Center at Pacific Northwest National Laboratories.

Eight strains of *Pseudomonas* that had been isolated from the DOE Savannah River Site and eleven strains of pseudomonads isolated from Hanford were obtained from the Subsurface Microbiology Culture Collection (SMCC), after consultation with Dr. David Balkwill. Adhesion assays were performed, using our standard laboratory assay, and there was a great deal of variation in adhesiveness among the strains. Some preferentially attached to hydrophobic surfaces, whereas others attached preferentially to the more hydrophilic surface. This variability is interesting, as it is not consistent with the concept that subsurface organisms have developed a particular adhesive phenotype conferring an advantage in an environment composed largely of solid surfaces. Instead, subsurface strains appear to have diverse adhesion properties (Fletcher, 1996).

Several strains that were highly adhesive or that produced a mucoid colony (indicating extracellular polymer production) were selected for mutagenesis. Considerable effort was put into generating adhesion mutants, quantifying the adhesion changes, and assessing stability of the new phenotypes. Eventually, work focused on *P. fluorescens* R8, a spontaneous rifampin-resistant mutant of *P. fluorescens* B0267 (US DOE Subsurface Microbial Culture Collection, Florida State University), and mutant strain M1, which had reduced adhesion properties and retained those properties for several years in the laboratory. This pair of strains was used for subsequent transport experiments (Sections 2 and 5, below). We also attempted to determine the biochemical basis for the reduced adhesion in strain M1, but could detect no changes in outer membrane proteins or LPS. Presence of the transposon in the mutant was confirmed by Southern and Western blots.

In a collaborative study with Dr. Mary DeFlaun at Envirogen Inc., we investigated the surface characteristics of *Burkholderia cepacia* G4 and several adhesion variants isolated

by selection of nonadhesive strains (DeFlaun et al., 1999). *B. cepacia* G4 is a soil isolate, which is of particular interest because of its bioremediation capacities. Our focus was on a stable adhesion-deficient mutant, strain ENV435, which had increased transport compared to strain G4. Strain ENV435 was compared to the wild-type strain for its adhesion to silica sand and transport through two different aquifer sediments varying in sand, silt, and clay content. Longitudinal transport of the wild-type and adhesion mutant was compared to a conservative chloride tracer in 25 cm-long, glass columns. The wild-type strain was severely retarded compared to the conservative tracer in a variety of aquifer sediments, while the adhesion mutant traveled at the same rate as the conservative tracer. An intact sediment column study showed similar results; ENV435 was transported at a faster rate and in much greater numbers than G4. Results of hydrophobic interaction chromatography revealed that G4 was significantly more hydrophobic than ENV435, and polyacrylamide gel electrophoresis revealed significant differences in the LPS O-antigen of the adhesion mutant compared to the wild-type.

Thus, like *P. fluorescens* (above), alterations in the LPS of *B. cepacea* appeared to influence the adhesion properties of the organism. However, the similarity ends there. With *P. fluorescens*, attenuation of the LPS resulted in **increased** adhesion to the hydrophobic test surface and to sand surfaces in transport columns (Williams and Fletcher, 1996). In contrast, the *B. cepacea* strain with attenuated LPS demonstrated **decreased** adhesion to sand and thus increased transport in sand columns (DeFlaun et al., 1999). These disparate results -- combined with previous reports that outer membrane proteins and flagella are involved in attachment, as well as our inability to detect any differences in LPS or outer membrane proteins in a large number of adhesion mutants -- all indicate that a number of cell surface polymers can influence the attachment characteristics of bacteria and that at times relevant polymer alterations may be subtle and difficult to detect without detailed analysis.

2. Relationship Between Cell Adhesiveness and Transport Through Porous Media.

The relationship between attachment properties of bacteria and their transport through porous media has been assessed by measuring transport of characterized *Pseudomonas* strains and adhesion mutants derived from them through both silica sand and sand from the DOE field site at Oyster, VA (Robb and Fletcher, in preparation). The transport characteristics of strains R8 and M1 were determined using mini- (13 ml of sand) and medium-scale (2 l of sand) columns. These were packed with (a) Accusand (AS), a commercially available, relatively pure, homogenous sand, or (b) "Oyster" sand (OS), a non-homogenous sand with a relatively high iron content collected from a site at Oyster, Virginia, which has been used for DOE field experiments on bacterial transport. The bacteria were loaded onto the packed columns and "chased" through the column with artificial groundwater. Enumeration of the bacteria recovered from the mini-columns was achieved by a fluorometric method we developed to detect low densities of cells. A cell-permeant fluorescent dye, Syto 11 (Molecular Probes, cat. # S-7573), which only fluoresces when intercalated into DNA, was added to the effluent. The fluorescent intensity of the sample was measured, and the amount of bacteria in the sample was calculated vs. a known standard. Enumeration of bacteria retained by the sand particles

after transport experiments was determined separately by radiolabeling R8 and M1 with $^{14}\text{-C}$ and $^3\text{-H}$, respectively, before loading them onto the columns, and determining the amount of label associated with sand samples taken at the end of the experiment. Both strains R8 and M1 were retained in greater numbers near the top of the OS columns. Strain R8 was also retained near the top of AS columns, whereas M1 was distributed throughout the column. In the AS columns, total retention of M1 was considerably lower than that of R8. These distributions were similar when the strains were added as pure cultures or when they were added as a mixed suspension, i.e., the presence of either strain did not appear to enhance or interfere with the attachment of the other.

These experiments have confirmed that the adhesion differences of strain R8 and the adhesion-deficient mutant, M1, appear to influence their comparative transport through sand columns. The transport rate of M1 significantly exceeds that of R8. The results also indicate that each strain transport independently of the other, with no interference or facilitation between the two.

The effect of different electrolyte solutions on transport of R8 and M1 through Accusand and Oyster sand mini-columns was also determined. Cells were washed and resuspended in either (a) artificial ground water (AGW) or in (b) AGW supplemented with NaCl , CaCl_2 , Na_2SO_4 , and Na_2HPO_4 . For the former (a), cells were loaded onto mini-columns pre-equilibrated with AGW and were eluted with AGW supplemented with the appropriate electrolytes. For the latter (b), cells were loaded onto mini-columns equilibrated and eluted with AGW supplemented with the appropriate electrolytes additional salts. Transport of strain R8 through OS and AS columns was increased when loaded onto and eluted from columns with AGW supplemented with $0.1\text{ mM Na}_2\text{HPO}_4$. Transport of strain M1 was increased by Na_2HPO_4 only in OS columns. The mechanism by which phosphate affected transport is not clear, particularly since no other ions had an effect. We had anticipated that the cations would influence attachment because of their ability to reduce electrostatic repulsion by compressing the electric double layer. However, their lack of effect of Ca^{2+} or Na^+ indicated that electrostatic charge interactions may not be significant in bacterial-sand interactions in this system. The phosphate may have been entering into more specific interactions, on either the sand or bacterial surfaces, but that is yet to be determined.

3. Evaluation of Bacteria from Cerro Negro Samples.

There is almost no information on the adhesion characteristics of subsurface bacteria, even though adhesion to porous media surfaces must have a considerable influence on the transport and distribution of bacteria in the subsurface. The US DOE drilling at Cerro Negro, NM, in 1992 provided us with a rare opportunity to investigate adhesion properties of in situ bacteria. The Cretaceous sediments at Cerro Negro were penetrated by an igneous intrusion ~3 million years ago. The stratigraphy is composed of alternating layers of sandstone and shale from marine and coastal depositions.

We attempted to assess adhesion directly by treating Cerro Negro samples with chemical solutions that would be expected to disrupt adhesion that is based on electrostatic or hydrophobic interactions (Landau, 1995). Samples were crushed and blended with three solutions to influence different mechanisms of bacterial adhesion: (a)

AGW, (b) AGW with increased sodium concentration, to influence electrostatic interactions, and (c) AGW with detergent (Tween 80 or Tween 40) to disrupt hydrophobic interactions. The particles and any attached bacteria that had not been removed by treatment in the test solutions were separated by filtration from bacteria that had been extracted. Microbes present in the filtrate were then counted by epifluorescent microscopy and viable counts. The effects of the sampling location, geological formations, extraction solution, and formation composition were determined by least squares ANOVA. Viable counts were very low, indicating few aerobic heterotrophic organisms in the unattached community. Direct counts of the most shallow formation, the Mancos Shale, were consistently higher than average. Numbers were also higher than average in a specific region that had been influenced by an igneous intrusion. This suggested that these organisms originated from cells recently transported to their present location, as this area should have been sterilized by the intrusion. Similar numbers of cells were observed in all three chemical extractions, so it was impossible to reach any conclusions about the physicochemical basis of the bacterial adhesion mechanisms. The direct counts of extracted organisms were very low and so near the level of detection it was difficult to reach any firm conclusions about the numbers of bacteria and relationships between bacterial distribution and geological formation. We concluded that microscopic direct counts were an unsuitable method for evaluating subsurface bacteria in rock samples where numbers were so near the level of experimental detection.

This work formed the basis of thesis research for Nicholas Landau, who successfully defended his Masters thesis in July 1995, and went on to work for a Ph.D. degree at Rutgers University. Because of the very "negative" nature of the results due to the very low number of bacteria, no publications arose from the work.

4. Cell Surface Analyses on Strains Used for Oyster Field Transport Experiments.

Analysis of surface characteristics potentially significant in adhesion properties were analysis for bacterial strains that were candidates for the field transport experiment at Oyster, VA, in collaboration with Dr. Mary DeFlaun (Dong et al., in preparation). The following strains were used for analysis of outer membrane proteins and lipopolysaccharide (LPS): DA001 and two adhesion variants DA001R and B2-4, PL2W31 and its adhesion variant MACH1, and OYS50 and its adhesion variant OYS2-A. Outer membrane preparations were run on SDS-PAGE gels and visualized with Coomassie blue staining, and LPS preparations were run on PAGE gels and silver-stained. No significant differences were found between any of the parent-mutant pairs. The electrophoretic mobilities of strains DA001, DA001R, PL2W31, and MACH1 were measured with a Zeta-meter, an instrument that assesses bacterial surface charge. Electrophoretic mobility (EM) of DA001 and DA001R were below detectable limits (between 0 and 0.3 microns per second/volt per cm). The EM of PL2W31 was -1.51 and that of MACH1 was -0.527. Thus, it was not possible to ascribe the adhesion differences between the paired strains to any particular surface property. The only potentially relevant difference was surface charges of strains PL2W31 and MACH 1. These results further emphasize the diversity of adhesion mechanisms and surface components involved in the attachment mechanisms of bacterial strains. It is increasingly clear that it

is impossible to identify any single bacterial surface characteristic that determines adhesiveness.

5. Transport of Different Adhesion Phenotypes in Intermediate Scale Experiments.

Experiments in transport at a scale larger than that possible with bench-top columns were conducted in collaboration with Dr. Steve Silliman (Notre Dame) (Silliman et al., submitted). The transport of bacteria through heterogeneous porous media was investigated (a) in benchtop columns packed with sand and (b) in a tank specially designed to control local hydraulic conductivity, which varied as a two-dimensional, log-normally distributed, second-order stationary, exponentially correlated conductivity field. The bacteria were *Pseudomonas fluorescens* R8, a strain demonstrating appreciable attachment to surfaces, and strain M1, a transposon mutant of strain R8 with reduced attachment ability. In benchtop, sand-filled columns, transport was determined by measuring intensity of fluorescently stained cells in effluent, or by measuring cells, which had been labelled with radioisotope, that were retained in the sand columns. Results demonstrated that strain M1 was transported more efficiently than strain R8 through columns packed with either a homogeneous silica sand or a more heterogeneous sand with iron oxide coatings.

In experiments measuring transport through the larger laboratory tank, two experiments were conducted, in which bacteria were introduced and their transport to wells in the tank were determined in time by sampling from the wells and sample ports in the tank, and measuring bacterial numbers by colony counts on plate media. At the end of the first experiment, the distribution of the bacteria in the sediment was also determined by destructive sampling and plating. The two experiments produced bacterial breakthrough curves that were quite similar even though the similarity between the two porous media was limited to first and second-order statistical moments. This result appears consistent with the concept of large scale average behavior such as has been observed for the transport of conservative chemicals. The transported bacteria arrived simultaneously with the conservative chemical tracer (although at significantly lower concentration). However, the bacterial breakthrough curves showed significant late time tailing. This tailing is probably related to high concentrations of bacteria remaining on the grain surfaces in the regions of low hydraulic conductivity (and high degree of coating of the mineral surface with iron oxides). The concentrations of bacteria attached to the sediment surfaces showed considerably more spatial variation than do the concentrations of bacteria in the fluid phase. This contrast between behavior in the fluid phase and on the solids is consistent with initial modeling of these heterogeneous media.

6. Conclusions.

Bacterial attachment to surfaces clearly involves a diverse range of polymers and/or polymer chemistries. In two cases, we were able to demonstrate that LPS played a role in attachment processes, but adhesiveness apparently depends on the chemistry of the polymer, not on the simple presence or absence of subcomponents, such as the O-antigen chain. Moreover, in many more cases, LPS was not implicated in adhesion. Adhesiveness correlates with transport ability, and the tendency for bacteria to be transported through porous media depends on their tendency to remain unattached in the

water phase. Additional environmental factors, however, also impact on bacterial transport, and sediment mineralogy has a significant effect. Solution chemistry, such as electrolyte concentration, may be less important. The transport of non-adhesive bacteria also appears to track chemical tracers in the flow field, and in the absence of complicating factors, may be described by fluid flow models. Thus, the movement of nonadhesive bacteria through porous media may be relatively easy to predict, while bacteria with moderate or strong adhesive capabilities have complex behaviors that may change in time with physiological processes, or in space with sediment mineralogies.

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