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FINAL TECHNICAL REPORT

DE-FG02-94ER61814.A000

Title: Relationship Between the Adhesive Properties of Bacteria and Their Transport and Colonization in the Subsurface Environment**Principal Investigator: Madilyn Fletcher, Center of Marine Biotechnology, University of Maryland Biotechnology Institute****PROPOSAL SUMMARY**

The adhesion of bacteria to sediment particle or rock surfaces considerably affects their transport in subsurface environments. This research focuses on the macromolecular properties of bacteria that determine their adhesiveness and on the significance of adhesion in transport of subsurface bacteria. Specific objectives include (1) to obtain adhesion mutants of subsurface *Pseudomonas* species altered in surface adhesives; (2) to determine alterations in adhesives in selected mutants; (3) to evaluate the effect of adhesiveness on transport and long-term distribution and colonization of bacteria in porous media. Primary methods will be transposon mutagenesis to generate adhesion mutants, biochemical analyses of cell surface polymers, and the use of laboratory columns containing subsurface materials to study the distribution and transport of bacteria along flow paths over time.

PROGRESS OVER TWO YEARS

Over two years, we generated and investigated adhesion mutants of *Pseudomonas fluorescens* to determine the surface polymer(s) that was responsible for changes in attachment properties of adhesion mutants, and we determined that lipopolysaccharide influenced both adhesion and transport through porous media (1 below). We have obtained at least one adhesion mutant from *Pseudomonas* species isolated from the DOE Savannah River Site (Aiken, South Carolina), and we applied mutagenesis to strains of *Pseudomonas* obtained from the GEMHEX program at the Hanford Reservation, Washington, so that our studies would include a range of subsurface isolates from different sampling sites (2 below). We have been evaluating the relationship between adhesion and transport properties of adhesion mutants in both silica sand and sand from the Oyster field site in the Bacterial Transport program (3 below). We also extended our adhesion and surface analyses to a collection of pseudomonad adhesion variants housed at Envirogen, Inc., in collaboration with Dr. Mary DeFlaun (4 below). Finally, we attempted to evaluate the attachment properties of bacteria in samples from Cerro Negro by treating crushed rock samples with different chemical solutions and determining numbers of extracted cells by direct counts (5 below). Research progress is summarized below:

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1. Adhesion and Transport Through Porous Media of *Pseudomonas fluorescens* is Affected by Lipopolysaccharide Composition.

The objectives of this work were (i) to use transposon mutagenesis to produce mutants of *Pseudomonas fluorescens* 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. Mutants of *P. fluorescens* 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. 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 combined with previous studies of attachment of *P. fluorescens* demonstrates that more than one type of polymer can mediate the adhesion of this organism to nonbiological surfaces. The portion of this work relating to surface analysis of the bacteria was supported by this award, and followed the mutagenesis, adhesion, and transport studies, which had been previously carried out under a contract from the Environmental Science Research Center at Pacific Northwest Laboratories. This total piece of work has been written up as a manuscript and was published in *Applied and Environmental Microbiology*, Vol. 62, January 1996.

2. Evaluation of Adhesion Properties and Mutagenesis of Subsurface Isolates from Savannah River and GEMHEX.

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. For the Savannah River Site strains, adhesion assays were performed, using our standard laboratory assay, and *Pseudomonas* strain B333 was selected for subsequent mutagenesis because of its tendency to adhere to surfaces and its mucoid colony morphology, which might facilitate identification of alterations in mutants that affect adhesion. Mutagenesis was conducted with mini-*TnphoA*. Seven mutants were eventually identified: (i) three that attached in higher numbers to test substrata, and (ii) three that attached in lower numbers to hydrophobic substrata but were not altered with respect to hydrophilic polystyrene. Adhesion

properties of these mutants after starvation for 48 hours in artificial groundwater (AGW) was evaluated, and two were selected for further transport experiments. However, these strains proved to be unsuitable for measurement of transport through columns containing porous media using our standard protocol, which evaluated transport after starvation. Starvation induced flocculation of the cells, which would in itself affect transport and thus would prevent any direct evaluation of the effects of adhesive properties on transport. Because of the time that had been invested in developing these mutants and the need to apply our mutants to transport experiments, we devised a different approach aimed at determining the effect of starvation-induced flocculation on longer-term transport. Bacteria were introduced into the columns and then starved for 48 hours, followed by elution as done in the standard protocol. However, there was poor reproducibility in these experiments, and we decided to abandon this approach, and attempted again to obtain suitable adhesion mutants.

More recently an adhesion mutant of strain BQ267 (*P. fluorescens* group, Congaree formation) has been identified. This mutant adheres very poorly to both adhesion assay substrata. We conducted extensive surface analyses of these organisms and were unable to determine any polymer changes that could be responsible for alterations in adhesion.

Adhesion assays have been conducted on all eleven isolates from the GEMHEX samples. There was a great deal of variation in adhesiveness among the strains: some attached in higher numbers to the hydrophobic test substratum than the hydrophilic one, while others demonstrated the reverse pattern. Two demonstrated very poor attachment to both types of surface. There was clearly no general trend in attachment ability for these subsurface strains. Two were selected for mutagenesis, and several potential adhesion mutants were identified. We are currently extending studies with these organisms.

3. Bacterial Transport Through Columns Containing Silica Sand and Sand from the Virginia Oyster Field Site.

We have been working with the Bacterial Transport Working Group on the Oyster field experiment and participated in developing protocols for identifying suitable test organisms. We also evaluated surface properties of two strains that were selected as prime candidates for the field test. Since being involved in the Oyster field experiment, we have been including Oyster sediment in our standard column transport assays.

4. Alterations in Adhesion, Transport, and Membrane Polymers in an Adhesion-Deficient *Pseudomonad*.

Envirogen, Inc. has an extensive collection of adhesion variants that have a range of transport properties. Thus, in a collaboration with Dr. Mary DeFlaun at Envirogen, we conducted a study of the surface properties of *Burkholderia cepacia* G4 and an adhesion-deficient strain that had been selected by serial passage through a sand column. This variant was compared to the wild-type

strain for its adhesion to silica sand and transport through a number of different aquifer sediments varying in sand, silt, and clay content. Longitudinal transport of the wild-type and adhesion variant 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 variant traveled at the same rate as the conservative tracer. Polyacrylamide gel electrophoresis revealed significant differences in the lipopolysaccharide O-antigen of the adhesion variant compared to the wild-type. Differences in this cell surface polymer may explain the decreased adhesion in this strain.

This work was presented at the Annual Meeting of the American Society for Microbiology in New Orleans in 1996, and a manuscript for publication is in preparation.

5. 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 recent drilling at Cerro Negro provided us with a rare opportunity to investigate adhesion properties of *in situ* bacteria.

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. Samples were crushed and blended with three solutions to influence different mechanisms of bacterial adhesion: (i) AGW, (ii) AGW with increased sodium concentration, to influence electrostatic interactions, and (iii) AGW with detergent (Tween 80 and 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 the region influenced by the 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 their 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 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 thesis in July 1995.

DELIVERABLES

Williams, V. and Fletcher, M. 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Appl. Environ. Microbiol.* 62:100-104.

American Society for Microbiology Annual Meeting, 1995, poster: Transposon Mutants of *Pseudomonas fluorescens* with Altered Adhesion Ability and Transport Through Porous Media. M. Fletcher, V. Williams, and S.R. Oppenheimer.

American Society for Microbiology Annual Meeting, 1996, poster: Alterations in Adhesion, Transport, and Membrane Polymers in an Adhesion-Deficient *Pseudomonad*. S.R. Oppenheimer, C.C. Condee, M.F. DeFlaun, and M. Fletcher.

American Society for Microbiology Annual Meeting, 1996, poster: Selection and Characterization of Indigenous Strains for a Bacterial Transport Study in a Coastal Plain Aquifer. M. Fletcher, W. Holben, R.J. Steffan, and M.F. DeFlaun.

Master's Thesis: Nicholas J. Landau, Analysis of Bacterial Numbers and Mechanisms of Bacterial Adhesion in the Terrestrial Deep Subsurface, University of Maryland.

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