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13. ABSTRACT (Maximum 200 words)

ABSTRACT

Bacterial Flocculation: Focus on Bioengineering Applications

Subsurface microbial activity is critical in many bioengineering processes such as enhanced oil recovery, *in-situ* or enhanced bioremediation, and biomass plugging operations. To investigate biofilm formation, strain PH002 of the *Azoarcus* genus, which produces flocs in stationary growth phase was studied. Flocculation occurs as this gram-negative, denitrifying strain is grown anaerobically on a phenol carbon source. As stationary phase is approached, the phenol concentration diminishes and the hydrophilic cells become hydrophobic. When grown in a comparable manner, aerobically with succinate as the carbon source, flocculation does not occur and the cells remain hydrophilic. The main thrust of the research was to understand the molecular basis of flocculation in this strain by comparing the molecules most likely to contribute hydrophobicity to the bacterial surface. In this research, the outer membrane proteins from anaerobically and aerobically grown cells were extracted, separated by gel electrophoresis and compared. Reproducible results were not obtained from the outer membrane experiments. Cellular lipopolysaccharides were also extracted and separated by gel electrophoresis. Differences in lipopolysaccharides bands were observed between hydrophobic and hydrophilic cells but a complete study was not done. Further work remains to adequately study the outer membrane proteins and lipopolysaccharide components of strain PH002 as causative factors in flocculation.

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Bacterial Flocculation: Focus on Bioengineering Applications

A. Introduction

Subsurface microbial activity is critical in many bioengineering processes such as enhanced oil recovery, enhanced and *in-situ* bioremediation and biomass plugging operations. To advance these engineering applications, it is important to understand the properties of bacteria that operate in this subsurface zone and their growth habits, particularly biofilm or biofloc formation. These factors mitigate against bacterial migration or transport and are important in biomass plugging strategies. Surface molecules produced by the bacteria influence adherence to soil particles and cell-to-cell cohesiveness. A clear understanding of the cell surface components that contribute to biofilm formation is necessary for the development of methods to influence bacterial growth for a desired application. In this research, strain PH002, an organism selected because of its ability to use phenol as a sole carbon source functioned as a model organism to study processes associated with flocculation and biofilm formation and properties. The main thrust of this research was to understand the molecular basis of floc formation in this nitrate reducing strain. This study was to determine the chemical nature of the cell surface molecules that initiate flocking to help to understand the physiochemical basis for flocculation in the bacteria.

The following specific research efforts were pursued. Included is a brief description of the strategies followed.

Identify the surface components of strain PH002 that are responsible for flocculation.

a. Determine the types of biomolecules that are associated with flo cculation: The flocking process in PH002 follows an increase in cell surface hydrophobicity and is a surface phenomenon. Molecules likely to impart hydrophobicity to the cell surface in the flocking process were extracted, separated with an emphasis on outer membrane proteins and lipopolysaccharides.

b. Purify and characterize molecular species associated with flocculation: Lipopolysaccharides and the outer membrane were separated from whole cells and separated by gel electrophoresis.

B. Background

1. Biofilms and Exopolymers

Microbial cells growing in an aquatic environment can form biofilms or bioflocs. Biofilms are defined as a matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations with the pore spaces of porous media. Flocculation is studied particularly in industrial processes including brewing, biomass production, wastewater treatment, and bioconversions. Environments hospitable to

biofilms are ubiquitous and include flowing water systems for drinking water and waste water facilities, industrial applications, especially those involving surfaces and networks of pipes, food science installations for transfer and processing of food stuffs, natural and induced metabolism of ground water contaminants and in the human body, particularly in association with infections resulting from indwelling implants and in the formation of dental plaque. In each situation, interaction is because of cell surface properties and extracellular polymers produced by the cells provide stabilizing forces.

2. Strain PH002

The facultative anaerobic strain PH002 was originally isolated from the East River in New York in a search for organisms able to metabolize phenolic compounds under denitrifying conditions. It is a Gram-negative, rod shaped organism, mobile by means of one polar flagellum, which is present only during exponential phase (van Schie and Young, 1998). Strain PH002 cells swell during stationary phase because of the accumulation of poly-b-hydroxybutyrate in inclusion bodies. The strain has recently been found to belong to the *Azoarcus* genus and this identification is primarily based on 16S ribosomal DNA sequence analysis. The organism metabolizes benzoate, other benzyl- or phenyl- compounds, and succinate during aerobic growth. Under denitrifying growth conditions, flocs are formed by the organism in stationary phase. These fluffy white flocs settle to the bottom or entrapped nitrogen bubbles may cause them to float to the surface. No flocs have been observed in aerobic growth. Cell surface hydrophobicity and general cohesive forces between strain PH002 cells increases during growth. Flocculation of stationary phase cells follows the increase in cell surface hydrophobicity and /or cell-to-cell cohesion and occurs only in stationary phase when the sole carbon source is exhausted.

In that hydrophobic state, strain PH002 has been shown to include soil particles within cell clusters when soil fines are incubated with the organism. When applied to glass beads or packed soil columns in the hydrophobic state, the organism is not transported through the column in an eluate of simulated ground water because of the aggregation of the culture and increased soil adhesion potential of the bacteria.

The true native state of PH002 is unknown: it has only been studies in pure culture. It is attractive to speculate that the potential to transform the cell surface from having hydrophilic to hydrophobic properties and to flocculate is a survival strategy adopted by the organism that occurs as carbon sources are depleted. The development of hydrophobic surface features and the presence of the floc matrix may provide the organism with an ecological advantage. This research is significant because it focuses on understanding the chemical and physical interactions, which contribute to biofilm stability, and may function as a model system for floc formation and the survival advantages of flocculation.

C. Preliminary Studies

Preliminary studies were originally undertaken (Bechtold, unpublished) to study the effect of nitrate concentration during anaerobic growth on the hydrophobicity and flocculation of the organism, strain PH002. It was shown that when insufficient nitrate ion concentration was present to permit the stoichiometric depletion of the phenol substrate, the cell surface hydrophobicity was decreased and flocculation was prevented. These experiments showed that nutrient type and concentration influenced floc formation in the organism.

D. Research Design

In this project, the types of outer membrane molecules that may support the increase in cell surface hydrophobicity were studied. In gram-negative bacteria the outer membrane contains lipopolysaccharides and protein molecules among other molecules that could contribute to surface hydrophobicity. The rational of the following experiments in that cells grown under anaerobic conditions exhibit surface molecules that are different than those found after aerobic growth, those molecules that are different might contribute to the surface hydrophobicity of the cells. The molecules could then be further characterized. The proteins and lipopolysaccharides obtained from cells that had been grown under anaerobic conditions were studied.

F. Methods

Bacterial medium: Strain PH002, belonging to the genus *Azoarcus* was grown in a minimal salts liquid medium (Taylor et al., 1970) as a pure culture. Phenol (1mM) or succinate (1mM) were used as the sole carbon source. Sodium nitrate (5mM) was added to the media. All anaerobic solutions were purged with argon and argon filled the headspace. Cultivation occurred in serum bottle with butyl rubber stoppers or in Balch tubes. Purity of culture was checked by visual inspection after plating on tryptic soy agar plates, and microscopic observation.

Growth curves: Bacterial growth was followed by measuring the absorbance, which is an indicator of turbidity, of a culture at 600 nm in the growth tubes or in 1 cm cuvets with a spectrophotometer (Bausch and Lomb, Spec. 20).

Proteins and lipopolysaccharides: Outer cell membranes will be prepared by cell sonication and centrifugation, and outer membrane proteins prepared from these membranes by detergent treatment (Tagawa et al., 1993). Gel permeation chromatography was done with polyacrylamide gels (Bio-Rad Laboratories, Richmond, CA). Protein separation for visualization of bands was done by SDS-PAGE (Laemmli, 1970) and stained with Coomassie brilliant blue for visualization of protein bands. Lipopolysaccharides were prepared from whole cells using proteinase K digestion, separated by SDS-PAGE get electrophoresis, and visualized by silver staining (Hitchcock and Brown, 1983; Aucken and Pitt, 1993).

F. Results

Strain PH002 was grown both aerobically and anaerobically and the outer membrane proteins were isolated and separated on polyacrylamide gels. However, it was not possible to obtain consistent patterns among like experiments. The cells grown under aerobic and anaerobic conditions were treated in an identical fashion in like experiments. The cell culture was grown on tryptic agar plates, examined under a microscope and considered to be pure strain PH002. Because of this discrepancy among like samples, additional experiments to determine if the outer membrane proteins were involved in conferring hydrophobicity to the outer surface of strain PH002 were postponed and the planned work with lipopolysaccharides was initiated.

Strain PH002 was grown aerobically and anaerobically, the cells were isolated and the lipopolysaccharides separated on polyacrylamide gel electrophoresis. The cell cultures were examined under a microscope and grown of tryptic agar plates to confirm purity of the culture. The results indicated a difference between the lipopolysaccharides grown in aerobic and anaerobic circumstances. However, before it was possible to perform the continuing and confirming experiments, the project ended.

G. References

Aucken, H. M. and Pitt, T.L. (1993) Lipopolysaccharide profile typing as a technique for comparative typing of Gram-negative bacteria. J. Clin. Microbiol., 31, 1286-1289.

Hitchcock, P. J. and Brown, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol., 154, 269-277.

Laemmli, U.K., (1970) Cleavage of structure proteins during the assembly of the head bacteriophage T4. Nature (London) 227, 680-695.

Tagawa, Y., Ishikawa, H. and Yuasa, N. (1993) Purification and partial characterization of the outer membrane protein of *Haemophilus somnus*. Infect. Immun., 61, 91-96.

Taylor, B.F., Cambell, W.L., and Chinoy, I. (1970) Anaerobic degradation of the benzene nucleus by a facultatively anaerobic microorganism. J. Bacteriol., 102, 430-437.

Van Schie, P.M. (1997) Physiology and taxonomy of phenol-degrading denitrifying bacteria. Ph.D. Thesis, Rutgers University.

van Schie, P.M. and Young, L.Y. (1998) Isolation and characterization of phenol degrading denitrifying bacteria. Appl. Environ. Microbiol., 64, 2432-2438.

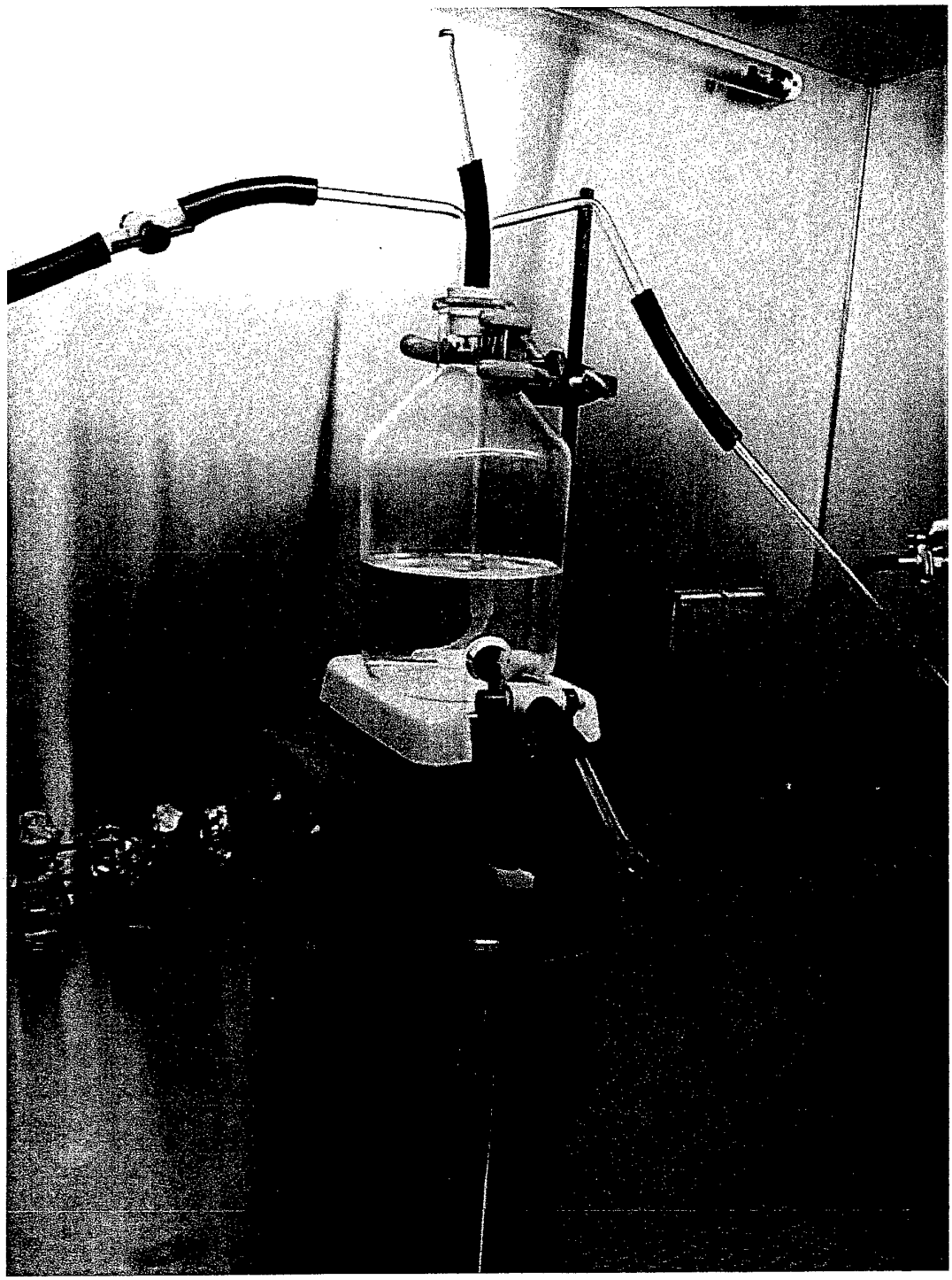
H. Appendix

Slide 1. Apparatus used to grow strain PH002 under anaerobic conditions. Sterile media was prepared in aspirator bottle. Accessory tubing and connectors and media in aspirator bottle were autoclaved, cooled and sparged with argon gas. After cooling media was inoculated with PH002 culture. The culture was dispensed into argon filled Balch tubes or serum bottles through outlet stopcock using light pressure from argon gas. Strain PH002 was grown anaerobically in Balch tubes or serum bottles.

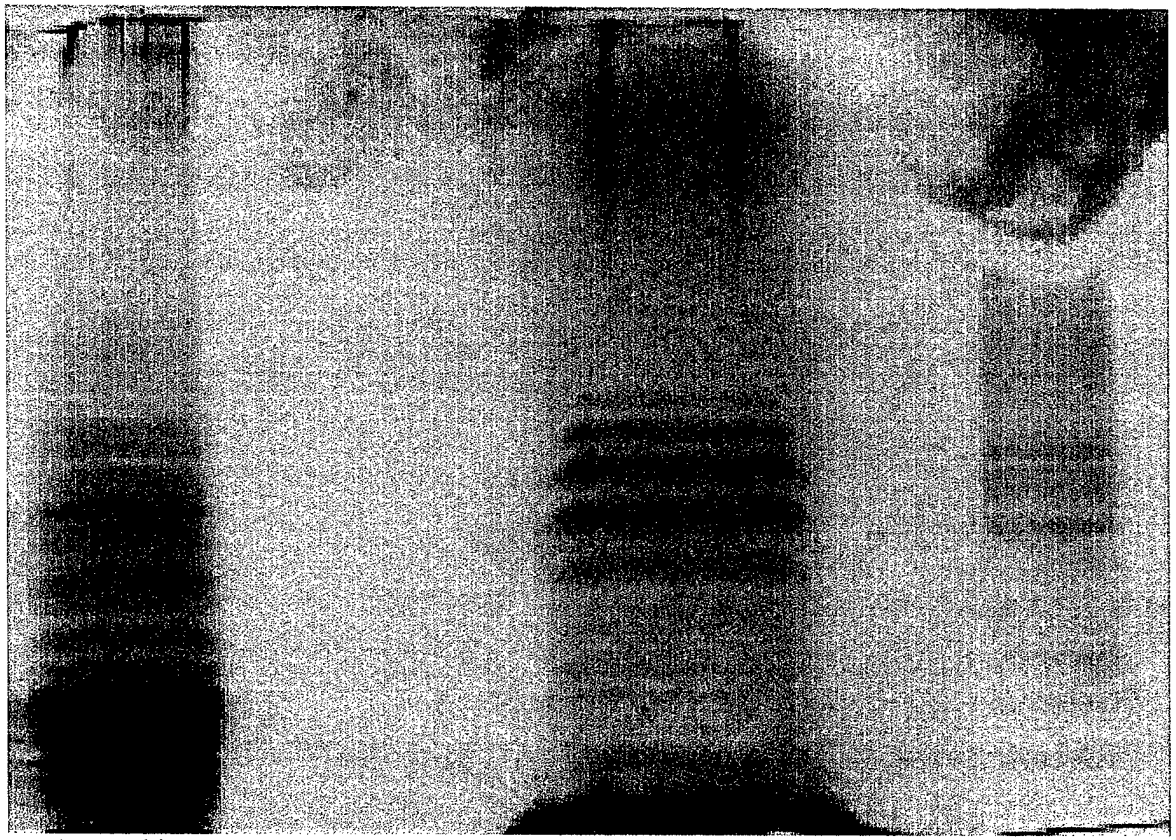
Slide 2. Representative gels patterns obtained from electrophoresis of outer membrane protein samples from strain PH002 grown under both anaerobic and aerobic conditions.

Slide 3. Representative gel patterns obtained from electrophoresis of lipopolysaccharides from strain PH002 grown under anaerobic and anaerobic conditions.

Slide 1



LPS of Anaerobic and Aerobic PHOO2



Anaerobic Aerobic MWM

Note: MWM-Molecular Weight Mark