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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Systems have been developed for detecting adhesion of bacteria on metal surfaces in a sterilizable system at different shear force environments (Fowler cell adhesion module). With this system mutants created of different colony morphologies or adhesion tendencies have been examined. Development of ultrasensitive detection of the phospholipid ester-linked fatty acids (PLFA) utilizing GC/CIPIMS has enabled detection of a very few bacteria. Monocultures show distinct differences in PLFA and ratio of glycocalyx to protein (by FT/IR DRIFT) between unattached, attached in high shear environment, and attached in lower shear environment. Advances in attenuated total reflectance ATR-FT/IR technology have allowed monitoring of attachment and bacterial growth on surfaces of ATR crystals. The attachment "foot" of different Caulobacter has been shown to be different. These techniques can be applied to monitor the chemistry of biofilm formation and dynamics.

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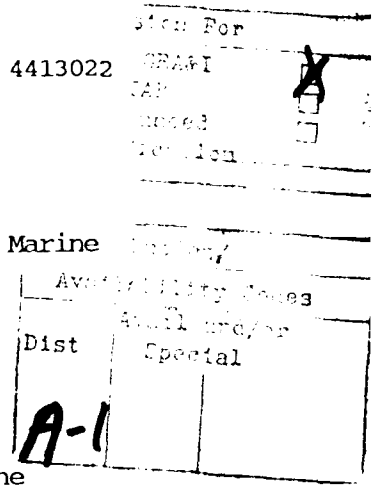


Research Objectives:

(1) To develop the most sophisticated means of examining the composition and activity of biofilms and the specific mechanisms by which microbes change their activities on attachment to surfaces. The methods will involve destructive analysis of great resolution and sensitivity and on-line non-destructive analysis by which processes involved in biofouling, biodeterioration, surface effects on flow and other properties can be examined.

(2) The techniques developed will be utilized to define the molecular mechanisms involved in the adhesion of specific bacteria to substrata to gain insight into the mechanisms by which adhesion is modulated in bacteria that are involved in biofouling and microbially facilitated corrosion.

(3) To genetically manipulate and analyze specific mutants of marine bacteria that have different properties relative to attraction and adhesion to substrata.

(4) To train graduate and post-doctoral researchers in the application of techniques and instrumentation to important problems of marine research.

Progress (Year 2): This year has been a period of developing the methods by which the chemistry and physiology of adhesion can be examined and has resulted in several significant advances that will result in publications and insight into biofilm adhesion.

The Fowler cell adhesion module is a sterilizable device in which the adhesive strength of bacteria can be determined by growth in a laminar shear force gradient. The inoculum, the composition of the media, temperature, nature and topology of the substratum in the test disk can be controlled. The major breakthrough this year was developing a system by which the level of aeration could be controlled in the shear force gradient could be maintained for the aerobic bacterial biofilms. C. Low was able to utilize mutants of Pseudomonas atlantica created at Agouron with different colonial morphology and exopolymer composition to demonstrate different adhesive strengths. One mutant which produces excessive exopolymer shows remarkable increases in the ratio of exopolymer to protein when it attaches in higher shear force

gradients than at lower gradients (1). Preliminary evidence using diffuse reflectance Fourier transform-infrared spectroscopy (DRIFT) showed different attachment chemistry for P. atlantica variant T6c-m (test surface 316 stainless steel with autogenous weld and heat affected zone) in higher shear gradients compared to lower shear gradients. Examination of the phospholipid ester-linked fatty acid (PLFA) patterns of the attached and unattached components of the P. atlantica monocultures show distinct differences.

Building on the preliminary studies of C. Low, a continuous culture system was designed by M. Mittelman (a new Ph.D. student) for maintaining wild-type and mutant strains of P. atlantica in a metabolically active adherent state. Temperature, aeration, culture agitation, and medium addition were controlled in this system. Preferential selection of adherent organisms was made by utilizing flocs of bacteria growing at the air/medium interface as continuous culture inocula. It was determined that standardization of test conditions would be essential for comparisons of environmental effects on biomass components. A recirculation rate through the Fowler Cell Adhesion Measurement Module (CAMM) of 60 ml/min was utilized for subcritical shear stress biofilm studies. This flow rate provided an adequate biofilm on 316 stainless steel coupons (600 grit finish) for qualitative and quantitative analyses.

In addition to the improvements in the continuous culture system, an in situ extraction procedure was devised for analysis of biomass indicators on coupon surfaces. An extraction template containing several 0.28 $cm^2$ glass o-ring joints was designed to provide a means for chemically extracting biomass constituents from surfaces, thus obviating the need for mechanical scraping procedures. In situ extraction of total protein, carbohydrate, and radiolabeled lipids using a Bligh-Dyer extraction cocktail is possible with this system.

Examination of 4-5 day biofilms on stainless steel coupons by direct-count epifluorescence microscopy (DCEM) demonstrated that microcolony formation was positively correlated with shear stress over a range of 0.2 to 9.5 dynes/$cm^2$. At relatively low shear stresses, bacterial cells were more homogeneously distributed across the coupon surfaces. The significance of higher densities of microcolonies at the higher shear stresses may be in the concentration of nutrients, metabolic by-products, and charged ions at discrete areas on stainless steel.

The DCEM observations were paralleled in diffuse reflectance Fourier transform-infrared spectroscopy (DRIFT) measurements at the coupon surface. Peak heights at the amide I and II, phosphate ester, and C-O stretch (carbohydrate) regions were positively correlated with shear stress. A calculation of peak height ratios in the amide and C-O stretch regions demonstrated that a significant positive correlation existed between carbohydrate:protein ratios and shear stress. Biofilm analysis by DRIFT provides a nondestructive, semi-quantitative measure of significant biomass markers on test surfaces. Results obtained by DRIFT analyses can be correlated with those obtained from more quantitative assays of such biomass indicators as total protein, carbohydrate, and fatty acids.

A determination of microbial activity at varying applied shear stresses was made employing $^{14}C$-acetate as the labeled carbon source. Incorporation of

the radiolabel into cellular lipids was significantly greater at the higher shear stresses. Uptake ranged from $5 \times 10^{-5}$ umoles/cm$^2$ at 0.2 dynes/cm$^2$ to $18 \times 10^{-5}$ umoles/cm$^2$ at 2 dynes/cm$^2$. Preliminary experiments employing total protein measurements indicate that the observed increases in label uptake resulted from greater cell densities at the higher shear stresses rather than from increased activity on a per cell basis. Increased attachment at higher shear stresses could be a function either of nutrient polarization or of an adaptive mechanism for dealing with applied shear forces.

The attachment process of bacteria can be monitored on-line and non-destructively using the technique of attenuated total reflectance Fourier transforming infrared spectroscopy (ATR-FT/IR). D. Nivens has pushed ATR-FT/IR technology to detect approximately $10^5$ bacterial cells attached on the surface of a germanium crystal. The attachment process was monitored by ATR-FT/IR to provide chemical information in the form of infrared absorption bands. Proteins are defined from the Amide I and Amide II bands (~1650 and 1550 cm$^{-1}$), and the carbon oxygen stretch (~1080cm$^{-1}$) for polysaccharides. The attachment of C. crescentus and C. subvibrioides isolates provided by J. Smit, was monitored by resuspending the bacteria in water and pumping the cell suspension into an ATR flow cell. Results showed that the detected attachment chemistry of the two species were different (2). With this system it may be possible to monitor in real time the rates at which biocides penetrate into a living biofilm. ATR-FT/IR provides a means to examine the interaction between bacteria and metal surfaces by utilizing ATR crystals sputter coated with metal. The metal can be used as an electrode for experiments in spectroelectrochemistry. This will provide means for a direct examination of the effects of cathodic protection on the attachment and subsequent development of microbial biofilms. The ATR-FT/IR has been developed into a powerful analytical technique for examination of the molecular basis of adhesion. The chemistry observed will be correlated to the genetic analysis of the genes involved in attachment in our collaborative studies with Agouron.

Our laboratory has developed the "signature" biomarker technique for determining the biomass, community structure, nutritional status and metabolic activities of microbes based on the analysis of PLFA (as reviewed 3-6, papers supported by this grant). In the past year and a half A. Tunlid has developed miniaturized extraction and derivatization techniques which with new detection involving chemical ionization mass spectrometry (GC/CIMS) with detection of positive ions for the PLFA patterns with sensitivities of $10^3$ bacterial cells. This is critical as at $10^{-15}$ molar sensitivity it is impossible to find uncontaminated solvents. This was solved by requiring transesterification to form esters rather than direct esterification of free fatty acids. Even higher sensitivities were achieved for the detection of polar head groups of polar lipids or the hydroxy fatty acids of the lipopolysaccharide lipid A after derivitization of the alcohol or amino groups of esters with electron withdrawing reagents and detection by negative ions. With this technique it is becoming possible to achieve detections at the $10^{-17}$ molar level which is the level of a single bacteria. This required the combination of elegant microchemistry and a dedicated 300K mass spectrometer operating optimally. It should now be possible to extract and detect bacteria from specific areas of corrosion or attachment coupons with a micromanipulator. Our laboratory's past work has shown the utility of

knowing the community structure, biomass and nutritional status from the lipids and the $^{13}C$ labelling the metabolic activities can be determined.

New technology besides the systems described above are in place or being built at IAM to focus on the molecular basis of adhesion with genetically modified bacteria. The surface enhanced Raman spectrometer (SERS) is being constructed at IAM with funds from the DOEURIP. This should allow a whole new technology to be focused on localization of specific molecular structures. Our colleague T. Ferrell of ORNL has developed microbases that intensify the Raman spectra at least $10^6$. We will try attaching bacteria to the microbases. Raman is complementary to the FT/IR (Raman lines are related to molecular polarizability whereas IR is more a function of molecular dipholes). Our VG trio-3 tandem mass spectrometer has a fast atom bombardment (FAB) which is an elegant way to sensitively examine the differences in structures of polymers such as the extracellular polysaccharides formed by attaching bacteria. We participated in a special course in FAB/MS/MS at the American Society for Mass Spectrometrists this summer and will use this on the mutants as they attach in different shear environments.

Work Plan (Year 3): The instrumental tools have been developed in the Fowler cell adhesion module, the ATR-FT/IR, DRIFT-microscope, the GC/CIPIMS and GC/CINIMS for detecting signatures, the experimental SERS, the supercritical fluid extraction system which could increase sensitivity and the FAB/MS/MS technology can be applied to an expanded series of mutants from our colleagues.

Research activities proposed for the next twelve months will focus on evaluations of the relative attachment strength of mutants provided by Dr. Doug Bartlett at The Agouron Institute. We have cultures of both adherent ($EPS^+$) and non-adherent ($EPS^-$) mutants of P. atlantica and anticipate receiving several more characterized Vibrio parahaemolyticus mutants. Environmental factors affecting attachment strength at critical shear forces will be evaluated using the CAMM unit. The influence of bulk media content (C:N ratio, ionic strength, pH, etc.) and primary surface conditioning films on attachment activities will also be examined. The role of substratum inhomogeneities such as welded and heat affected zones will be measured using test coupons provided by the Welding Sciences Group at the University of Tennessee.

M. Fletcher has supplied us with three mutants of P. fluorescens with different adhesive properties. John Smit has supplied us with at least eight strains of Caulobacter and a mutant with much increased holdfast material that will provide a standard for the IR signatures we have detected.

This year we will concentrate with the cell adhesion module on the relationship between metal stress in a smooth coupon and adhesion in controlled shear environments. Our newly delivered DRIFT microscope, which will enable non-destructive FT/IR evaluations of areas of coupons 10 um in diameter, will greatly increase our resolution as will the increased sensitivity of the PLFA, polar head group, and LPS-lipid A hydroxy fatty acid assay. The ATR-FT/IR will be used with new strains to examine the holdfast by shortening the evanescent wave penetration into the bacteria. We will begin using the system to study penetration of biocides and nutrients into

the biofilm. We will begin using the FAB/MS/MS to examine the structural differences in adhesions in the different mutants and the monocultures that attach in different shear environments.

Inventions: The development of new ultrasensitive methods of detecting bacteria based on PLFA and LPS-lipid A using supercritical fluid extraction is being discussed with venture capitalists this summer. A company, TBAC, Inc., has been formed and is currently developing its relationship to the University to exploit these techniques and the SERS technology. A micro-extraction system for quantitative recovery of surface biomass constituents designed by D. Nivens is being thoroughly tested and will be described in a forthcoming methods paper.

Publications and Reports:


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6. White, D. C. 1988. Microbial community structure and function as indicators of environmental health. Eighth Life Sciences Symposium, Int. Conf. on Bioindicators, Knoxville, TN, March 21-23, pp. 1-20.

7. Nivens, D. E., A. Tunlid, M. J. Franklin, J. Q. Chambers, and D. C. White. 1988. Development of attenuated total reflectance Fourier transform infrared spectroscopy as an on-line monitoring device to study the attachment of living bacteria. Abs. Am. Chemical Society-Southeastern Region (Atlanta), Abstract #279.

Training Activities:

D. E. Nivens is a graduate student in Analytical Chemistry at UTK doing spectroelectrochemistry of corroding biofilms for his research. He has passed his preliminary and cumulative examinations. He has pushed the ATR-FT/IR to levels of sensitivity and biological specificity greater than published literature. He will optimize the DRIFT microscope.

M. J. Franklin, M.S., is a Ph.D. graduate student in Microbiology at UTK.

Marc Mittelman, M.S., joined us in August for a Ph.D. program. Marc has been in industry for 10 years and is author of the book "Biological fouling of industrial water systems," with G. Geesey.