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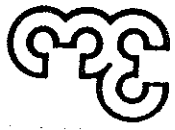

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New Methods

Non-Destrucitve Biofilm Analysis by Fourier Transform Spectroscopy (FT/IR)

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INTRODUCTION

Microbes existing in complex consortia of interacting physiological groups found in environments present a complex problem for assays. The problems involved in the classical means to determine the biomass of bacteria, the viable count, requires both that of providing a universal growth medium in the petri plate, and that the organisms must be quantitatively removed from the surfaces and from each other. Direct microscopic methods that require quantitative release of the bacteria from the biofilm can have the problem of inconsistent removal from some surfaces and the attachment and activity of microbes at surfaces is an extremely important feature of microbial ecology (18). Not only do microbes attach to sufraces, but there is abundant evidence that they exist in consortia of multiple metabolic types. Microcolonies of mixed bacterial types bound together with extracellular polymers are readily detectable in marine sediments by transmission electron microscopy (19).

Biomass and Community Structure. Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of cultural selection of the classical plate count is eliminated. Since the total community is examined in these procedures without the necessity of removing the microbes from surfaces, the microstructure of multi-species consortia is preserved. The method involves the measurement of biochemical properties of the cells and their extracellular products. Those components generally distributed in cells are utilized as measures of biomass. Components restricted to subsets of the microbial communities can be utilized to define the community structure. The concept of "signature" biomarkers for subsets of the community based on the limited distribution of specific components has been validates by using antibiotics and cultural conditions to manipulate the community structure. The resulting changes agreed both morphologically and biochemically with the expected results (39). Other validation experiments that involved isolation and analysis of specific organisms and finding them in appropriate mixtures, utilization of specific inhibitors and noting the response, and changes in the local environment such as the light intensity are summarized in reviews (35, 36).

The ester-linked fatty acids in the phospholipids (PLFA) are presently both the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed (2, 13, 42). The specification of fatty acids that are ester-linked in the phospholipid fraction of the total lipid extract greatly increases the selectivity of this assay as most of the anthropogenic contaminansts as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids (37). Phospolipids are not found in storage lipids and have a relatively rapid turnover in some sediments so the assay of these lipids gives a measure of the "viable" cellular biomass (38). The specificity and sensitivity of this assay has been greatly increased by the determination of the configuration and position of double bonds in monoenoic fatty acids (23) and by the formation of electron-capturing derivatives which after separation by capillary GLC can be detected after chemical ionization mass spectrometry as negative ions at femtomolar sensitivities as described in this symposium (28). This makes possible the detection of specific bacteria in the range of 10 to 100 organisms. Since many environments such as marine sediments often yield 70 ester-linked fatty acids derived from the phospholipids, a single assay provides a large amount of information. By

utilizing fatty acid patterns of bacterial monocultures, Myron Sasser of the University of Delaware in collaboration with Hewlett Packard has been able to distinguish between over 8000 strains of bacteria (30).

Nutritional status. The nutritional status of biofilms or microbial consortia can be estimated by monitoring the proportions of specific endogenous storage compounds relative to the cellular biomass. The nutritional status of microeukaryotes (algae, fungi, or protozoa) in biofilms can be monitored by measuring the ratio of triglyceride glycerol to the cellular biomass (7). Certain bacteria form the endogenous lipid poly betahydroxyalkanoate (PHB) under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (4, 5, 6, 16, 24). The sensitive assay of PHB has proved a useful means of defining the nutritional status of microbes in various environmental habitats. Unbalanced growth detected in the accumulation of PHB has been detected in the epiphytic microbiota on sea grass blades (16), as the result of the chelating activity of the tannin-rich brown runoff water from the pine plantations of north Florida in estuarine detrital microbiota (24), in contrasting rhizosphere bacteria attached to and in the areas away from the roots of the rape plant *Brassica napus* (L.) (33), and in uncontaminated subsurface aquifer sediments (31, 40). In all these examples the stimulation of bacillary growth with cell division decreases the PHB level.

The ratio of the rate of formation of PLFA to PHB from ^{14}C-acetate has been shown to be an exraordinarily sensitive measure of the nutrient environment in the bacterial habitat (5,6).

Extracellular polysaccharide glycocalyx measurement based on the specific content of uronic acids (3). Increases have been shown to indicate nutritional status of monocultures (32) and uncontaminated subsurface aquifer sediments (40). The microfouling community formed on metal surfaces exposed to rapidly flowing seawater shows a rapid accumulation of uronic acid containing extracellular glycocalyx as a response to mechanical or chemical countermeasures (25, 26, 41).

A marked increase in the proportions of monoenoic PLFA with the double bond in the *trans* configuration has been shown to be a part of the formation of mini-cells in marine monocultures undergoing prolonged starvation and in microbial assemblies by (14). Preliminary evidence indictes the same phenomenon may be demonstated in the highly oligotrophic environment of uncontaminated sub-surface aquifer sediments.

Metabolic activity. The rates of formation or turnover of "signature" components or nutritional status indicator molecules can be measured with the incorporation of labelled precursors. Rates of formation of bacterial DNA (20), 35-S-sulfate into sulfolipid (in the microeukaryotes (21, 39) an 32-P-phosphate into phospholipids (21) can be utilized as measures of the activity of the total microbiota. Analysis of signatures by GC/MS makes possible the utilization of mass labeled precursors that are non-radioactive, have specific activities approaching 100%, include isotopic marker for nitrogen, and can be efficiently detected using the selective ion mode in mass spectroscopy. The high specific activity makes possible the assay of critical reactions using substrate concentrations in the biofilms that are just above the natural levels. This is not possible with radioactive precursors. Improvements in analytical techniques have increased the sensitivity of this analysis. Utilizing a chiral derivative and fused silica capillary GLC with chemical ionization and negative ion detection of selected ions, it proved possible to detect 8pg (90 femtomoles) of D-alanine from the bacterial cell wall(the equivalent of 10^3 bacteria the size of *E. coli* with a reproducibility of 1% (30).

ROLE OF FT/IR IN BIOFILM ANALYSIS

The methods based on quantitative analysis of components of the microbiota and its extracellular polymers for biomass, community structure, nutritional status, and metabolic activity show responses to a variety of anthropogenic and natural perturbations (36). However to truly understand the interactions within microbial consortia the analysis should be nondestructive, sensitive and continuous as well as have the resolution on the scale of micrometers-the sizes of microbial consortia themselves. The technique currently with the greatest potential for non-destructive biofilm analysis utilizes FT/IR.

The infrared portion of the spectrum is extraordinarily rich in information regarding the vibrational and rotational motions of atoms in molecules. Not only can specific infrared absorption be assigned to particular types of covalent bonds but the modifications of these bonds by the local electronic environment can be detected in the details of the spectra (1, 11,12, 29). The infrared spectrum of a compound has long been accepted as one of the best nondestructive identification techniques. One of the problems restricting the application of infrared spectroscopy has been that the atomic interactions sensed in the infrared portion of the spectrum are at relatively low energies and the detection is rela-

tively inefficient. This has precluded the full usage of the power of the analysis using complex materials isolated from the environment.

The advent of fast computers has made possible a new type of infrared spectral analysis. This has provided the technology to utilize the far infrared portions of the spectrum, to follow rapid reaction rates with changes in spectral intensity, and to utilize different types of sample exposures such as photoacoustic spectroscopy. The secret lies in the array processor computers that can perform Fourier transformations so rapidly that interference spectroscopy can be possible.

A summary of the use of FT/IR in microbial ecology has been published (22). The FT/IR examination by diffuse reflectance (DRIFT) of freeze-dried, powdered bacterial monocultures shows at least two major groups. The first group is characterized by a dominant amide I (between 1690 and 1650 cm^{-1}) and amide II (1550 cm^{-1}) bands found in *Escherichia coli, Pseudomonas fluorescens, Desulfovibrio gigas, Staphylococcus aureus, Clostridium perfringens, Methylobacterium organophilium,* and *Methylosinus trichosporium* (both the latter grown on methane). Subtle variations in peak ratios of several groups could be utilized to differentiate between the different species. The second major group of organisms contained an enlarged carbonyl band at 1740 cm^{-1}. This group included *Bacillus subtilis, Methylobacterium organophilium* (grown on methanol), and *Nitrobacter winogradskyi.*

These findings together with the powerful technique of subtraction of one spectrum from another suggest that DRIFT could be utilized to recognize differences in community structure. Preliminary experiments indicate that examination of planktonic microbiota on pre-extracted filteres by DRIFT can be correleted with a detailed examination of the lipid content.

Two measures have been identified as markers for the microbial nutritional status. The formation of PHB and the uronic acid-containing exopolysaccharide glycocalyx are responses to nutritional stress by bacteria (36). Both polymers can be detected with the FT/IR. The polymers, such as gum arabic like the glycocalyx produced by *P. atlantica* show a prominent absorbance at 1150 cm^{-1} for C-0 stretch. The logarithm of the ratio absorbance at C-0 stretch to amide I gives an excellent correlation with mixtures of *E. coli* and gum arabic (22). This analysis replaces a three week chemical tour-de-force involving GC/MS in the analysis of bacterial glycocalyx. The DRIFT spectrum of *E. coli* plus gum arabic and of *P. atlantica* induced to form polysaccharide glycocalyx are similar in appearance. Accumulations of PHB in bacteria or artificial mixtures of bacteria plus purified PHB show a linear correlation with the ratio of the carbonyl stretch at 1750 cm^{-1} to amide I. Using these recombination experiments as models, it proved possible to show DRIFT shifts in PHB and glycocalyx in the biofilms formed in anaerobic fermenters that were supplemented with various amendments (15). For example amendments with propionate or butyrate showed similar biofilms compared with the unsupplemented or the biofilm of the digester amended with nitrate.

With the DRIFT analysis it proved possible to demonstrate the reversible facilitation of corrosion of 306 stainless steel by the non-sulfate reducing marine bacterium *Vibrio natriegens* and its extracellular material on the surface (27). There was a 15-fold increase in the corrosion current density measured electrochemically from the Tafel constants and polarization resistance that correlated with the colonization of the stainless steel diskes by microcolonies of the bacteria. The colonization of the metal surface was detected both by direct microscopy after staining and epifluorescent illumination, scanning electron microscopy, and by an increase in the DRIFT absorbance at the amide I area centered at 1660 cm^{-1} corresponding to the bacterial protein. Maximum rates of corrosion were associated with the appearance of extracellular material with a spectral maximum centered at 1440 cm^{-1} similar to calcium hydroxide. Removing the biofilm, particularly the calcium hydroxide with its absorption at 1440 cm^{-1}, decreased the corrosion current density 10-fold. In this instance both the presence of a non-sulfate reducing bacterium and its extracellular products reversibly facilitated corrosion of stainless steel in seawater. Similar experiments showed that the obligate aerobe *Pseudomonas atlantica* significantly increased the corrosion current density when it secreted its extracellular carbohydrate glycocalyx which also contained the IR signature of calcium hydroxide (43).

When the FT/IR beam is passed through crystals of germanium or zinc selenide of the proper geometry it is possible to monitor the IR spectra of whatever exists in an area beyond the surface of the crystal in the pathway of the "evanescent" wave. Cells containing crystals, attenuated total reflectance cell (ATR) cells, make possible the examination of living biofilms that form on the surface. With the ATR cell it has proved possible to show that the carbohydrate-rich initial fouling polymer coats the germanium surface exposed to sterile seawater in about 13 hours (22) or to follow the effects of surface treatments and materials on the clotting sequence of a test system inserted into the blood stream of living

sheep (8). This is clearly the way to follow biofilm formation and possibly to potentially monitor fermentations continuously. (Shifts in microbial community structure and nutritional status in high solids anaerobic fermenters have been demonstrated with DRIFT of lyophillized films (15). Not only is the FT/IR non-destructive, rapid, and sensitive but it is possible to decrease the beam size to diameters approaching 10 μm in microscope atachments without corresponding losses in sensitivity. The 10-20 μm diameter is the scale of some of the microbial interactions of great interest.

An exciting collaboration has been established between the laboratories of G.Geesey and P. Griffiths. They showed that a circular ATR cell could be coated with copper and the uniformity of the coating assayed with the intensity of the strong water adsorbance at 1640 $(cm)^{-1}$ (17). The water adsorbance disappeared when the coating was thicker than the evanescent wave (> 5nm). Uronic acid containing exocellular polysaccharides added to cells coated with copper films attached to the metal as indicated by increases in the ratio of C-0 of the carbohydrates at 1050 $(cm)^{-1}$ /1640 $(cm)^{-1}$. The exopolymer resisted attempts at removal. With prolonged incubation increases in 1640 $(cm)^{-1}$ (increases in water in the area of the evanescent wave) correlated with the dissolution of copper from the film (10). This facilitiation of corrosion (metal loss) could also be demonstrated in coated ATR cells to which bacteria were added (9). This system provides for the first time methods to study the kinetics and chemistry of adhesion as well as a quantitative monitor of the facilitation of biological corrosion by bacteria und their extracellular products.

Development of the FT/IR offers a potentially rapid and non-destructive method to examine biofilms on the scale of the microbial consortia. The continued development of GC/MS methods provides the essential validation for IR signatures.

DIRFT and ATR-FT/IR particularly in the hands of imaginative investigators like Geesey and Griffiths offer a whole new vista for insight into the interactions of microbial consortia.

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