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Role of Aerobic Bacteria and Their Extracellular Polymers in the Facilitation of Corrosion: Use of Fourier Transforming Infrared Spectroscopy and "Signature" Phospholipid Fatty Acid Analysis

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ABSTRACT

Insight into mechanisms by which microbes facilitate corrosion has been inhibited by the lack of methods to quantitatively define the biomass, community structure, and metabolic activity of microbial biofilms and their extracellular products. Chemical analysis of cellular components common to all cells provides measures of the total microbial biomass, whereas analysis of components restricted to subsets of the total community can give insights into the community structure of the biofilm. Rates of formation or turnover of these components measured after incubation with labeled precursors correlate with metabolic activities. These analyses are necessarily destructive and represent the average values of the surface from which the components were extracted. The technique of Fourier transforming infrared spectroscopy (FT/IR) provides a nondestructive technique for examining biofilms on the surface of metals on a scale approaching the size of microcolonies of bacteria. Chemical methods are utilized to validate FT/IR spectra and these results are correlated with electrochemical measurement of corrosion current density. This information is used to correlate accelerated corrosion of 304 stainless steel surfaces in sea water with the presence of two Vibrio species and their associated inorganic extracellular material. This exudate has spectral characteristics consistent with calcuim hydroxide containing an organic matrix. The increased corrosion current density is reversible with the removal of the extracellular accumulations. An aerobic

marine Pseudomonad increases the corrosion current density once the adherent biofilm elaborates polysaccaharide exopolymer which contains calcium hydroxide. Neither of these polymers accumulate iron or other metals. It appears that the inhomogeneous distribution of microbial biofilms in an extracellular polymer matrix containing calcium hydroxide on the metal surfaces appears to generate sites of different cathodic activity which induces the accelerated corrosion.

INTRODUCTION

The increasing necessity to recycle both fresh and saltwater with the almost inevitable accumulation of nutrient traces stimulates the formation of microfouling biofilms. These biofilms composed of microbes and their extracellular products can facilitate corrosion. The activities of microbial biofilms can induce corrosion by a number of mechanisms[1, 2]. The literature of microbially influenced corrosion (MIC) has been reviewed[2]. This literature deals primarily with the corrosion of steels by sulfate-reducing anaerobic bacteria and the corrosion of aluminum by hydrocarbon degrading organisms. These are clearly important problems that will be addressed in this symposium by other investigators.

The understanding of the mechanisms by which bacteria facilitate corrosion offers the greatest chance for rational control measures. Problems in demonstrating mechanisms of MIC have been complicated by the fact that the classical methods of microbiology that were so successful in the study of infectious disease (isolation and characterization of the pathogenic species) have proved of little use in the understanding of biofilm dynamics. Our laboratory has been involved in the development of methods for defining the biomass, community structure, nutritional status, and metabolic activities of mixed microbial consortia for several years. Components distributed generally in the cells such as the phospholipids serve as measures of biomass. Components or proportions of components that are restricted to subsets of the total microbial community can act as "signatures" of that subset and be utilized as measures of community structure. Measuring the rates of formation or turnover of these components after incubation with labeled precursors can give insight into the metabolic activities of the biofilms. Measuring the rates of formation and degradation of endogenous storage lipids gives insight into the nutritional status of the biofilm. The methodology, validation, and some applications of these techniques have been reviewed[3, 4, 5]. Support by the Office of Naval Research promoted the application of these techniques to studies of the role of biofilms in the facilitation of corrosion. Support by the Department of Defense University Research Instrumentation Program provided the Fourier transform infrared spectrometer (FT/IR) for the analysis of biofilms. FT/IR makes possible the monitoring of living biofilms nondestructively on scales approaching the size of the microbes themselves. This report will describe the interactions of aerobic microbes with metals exposed in aqueous environments.

EXPERIMENTAL

Coupons--Mild steel plates of approximately 156 cm^2 x 1 mm in thickness were sandblasted then polished with steel wool. Teflon film 2 um thick 306 cm^2 in surface area and commercial grade titanium foil 4 um thick and 240 cm^2 in surface area were also utilized. Each of the metal surfaces was cleaned by a lipid extraction, and autoclaved at 120°. This has been shown to remove traces of microbes from surfaces. Coupons of 304 stainless steel from Metal Samples, Inc. (Mumford, AL) 15.9 mm in diameter with a face polished with 600 grit powder were boiled in toluene for 5 min and rinsed with acetone to remove surface films.

Materials. Glass-distilled solvents and reagents were purchased from Burdick and Jackson (Muskegon, MI), Sigma Chemical Co (St. Louis, MO), and Applied Science, Inc. (Deerfield, IL). Chloroform was freshly glass-distilled before use.

Medium--In experiments with the marine organisms the cleaned coupons were placed in marine broth 2216 (Difco Products, Detroit, MI) made to 3.74% ( ), boiled, and filtered through 0.2 um pore filters (Millipore) before autoclaving for growth of the Vibrios. Pseudomonas atlantica was grown in media made from sea salts (Instant Ocean, Aquarium Systems Inc., East Lake, OH) 250 g/l containing 1% galactose and 0.5% proteose peptone (Difco Laboratories, Detroit, MI). Coupons were placed in 300 ml Erlenmeyer flasks, 100 ml medium was added, and the flasks were capped with stainless steel caps. The flasks were autoclaved at 121°C for 20 min. Sterile galactose was added after the medium cooled. In some experiments the disks were fixed to the sides of the culture flasks at the water line and the flasks were incubated on a rotary shaker so the disks were periodically submerged.

Organisms--Cultures of Vibrio natriegens and Vibrio anguillarum were the gifts of Dr. Rita R. Colwell of e University of Maryland, College Station, MD, and Pseudomonas atlantica strain T6c was the gift of W. A. Corpe of Columbia University, New York City. Cultures were inoculated using 1 ml from organisms grown for 18 h (stationary phase organisms). Stocks were maintained on agar slants at 5°C. Culture purity was followed microscopically and by Gram stain reactions.

Incubation--Flasks inoculated with the marine bacteria were shaken at 23°C (room temperature) at 80 rpm on a gyratory shaker (New Brunswick Scientific Co., NJ). At the times indicated, the disks were recovered from the flasks and were rinsed once with filter sterilized (0.2 um pore size), artificial seawater (Forty Fathoms, Marine Enterprises, MD) at a concentration of 25 parts/thousand.

The iron bacteria were cultured in tap water from the site of the cor led water pipes without added components.

Analysis The analytical scheme utilized for specimens is diagrammed in Fig. 1. Numbers refer to the literature citations for the methods.

Corrosion current density--Disks from the control and inoculated flasks were placed in the K105 flat specimen holder (E. G. and G. Princeton Applied Research Co., NJ) using 2 high density graphite counter electrodes and a saturated calomel reference electrode with the E. G. and G. model 350A corrosion measurement system. Each measurement was performed in 25 parts/thousand artificial seawater with the first disk being scanned from $E_{corrosion}$ to -500 mV cathodically to obtain the cathodic Tafel constant (β_c). The second disk was scanned from $E_{corrosion}$ - 10 mV to + 300 mV to obtain the anodic Tafel constant (β_a). Data from the second disk was then utilized to measure the polarization resistance (R_p) by analyzing from $E_{corrosion}$ -5 mV to $E_{corrosion}$ +5 mV. $I_{corrosion}$ was then calculated as $\beta_a\beta_c/2.3 \times R_p (\beta_a + \beta_c)$[6].

FT/IR measurement--Disks were stored over P_2O_5 in evacuated desiccators until examined. The disks were then placed in the diffuse reflectance accessory (Spectra Tech. Inc, Stamford CT) in the Nicolet 60SX FT/IR (Nicolet Instruments Inc., Madison, WI).

Each sample resulted in a single-sided interferogram of 4069 data points which gave a resoluton of 4 cm^{-1}. Signal averaging of 500 scans per sample required 2.5 min of total measurement time. The resulting spectra were ratioed to the appropriate background spectrum. In these experiments, the sample chamber was evacuated for 2 min and purged with dry nitrogen for 2 min prior to obtaining the spectra.

The spectra were interpreted based on Kubelka-Munk (K-M) analysis[7] used as an approximation of Beer's law for reflectance spectroscopy[8]. The liquid-nitrogen cooled, mercury-cadmium-tellurium detector (range 5500 to 710 cm^{-1}), a mid range IR globar source, and KBr beam splitter were utilized with the

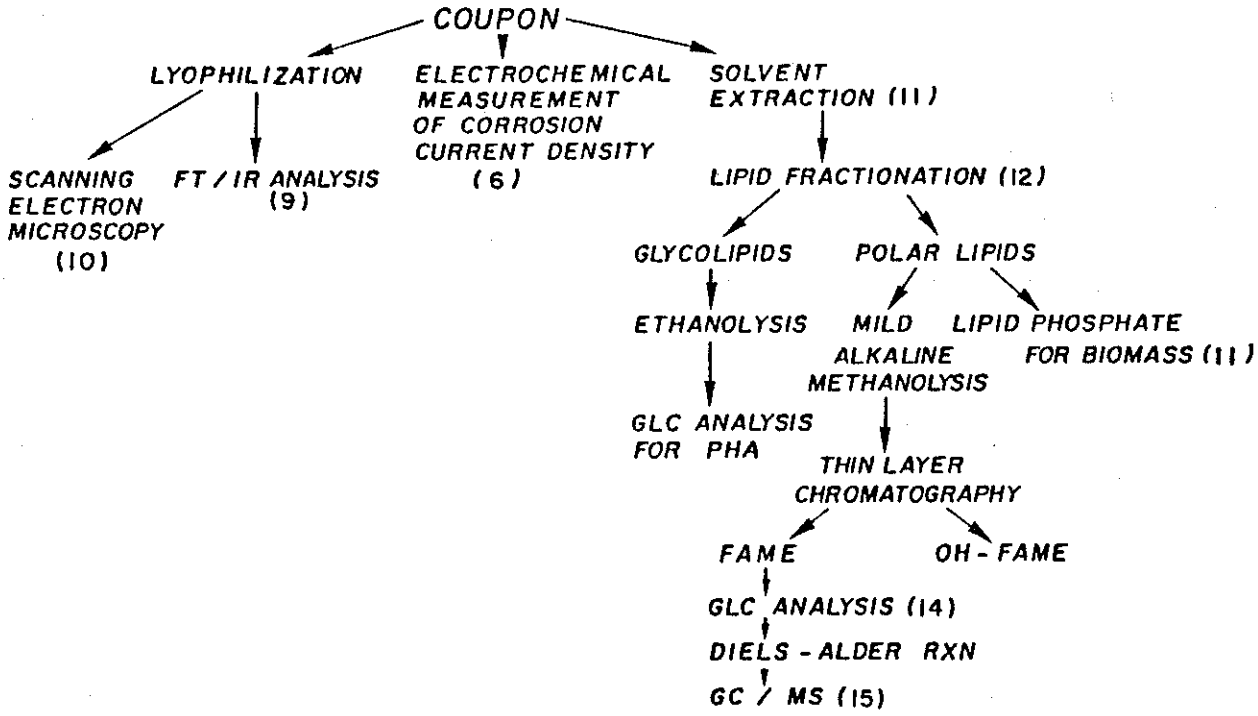

Figure 1. Diagram of the analytical scheme utilized in this study. The numbers refer to literature citations.

Nicolet 60 SX FT/IR. Interferograms were zero-filled and apodized by the Haap-Genzel function prior to the fast Fourier transformation using Nicolet SX software (TMON version 1.5). Spectra showed nearly identical baselines and are plotted uncorrected. Multiplication factors for the Kubelka-Munk absorbance plotted on the abscissa are given in the illustrations of the spectra. Other details of the FT/IR analysis system are given in a report of applications to microbial biofilms [9].

Scanning electron microscopy-- Coupons were fixed in glutaraldehyde, lyophilized, sputter coated and examined with the JEOL 100-CX STEM microscope [10]. Energy Dispersive X-ray analysis was performed by J. T. Fellers using the Tracor-Northern energy dispersive X-ray accessory at the Florida State University STEM facility.

Extraction of the lipids. The lipids were extracted from coupons in a single-phase chloroform-methanol extraction [11]. The lipids were concentrated by rotary evaporation and under a stream of nitrogen and stored under nitrogen at -20°C.

Fractionation of the lipids. The lipids were fractionated on columns of silicic acid (Unisil, 100-200 mesh, Clarkson Chemical Co., Williamsport, PA) prepared in Pasteur pipettes with glass wool plugs. The columns were packed with a chloroform slurry containing 0.4 g silicic acid and the lipids applied to the columns in chloroform. The neutral lipids were eluted with 10 ml of chloroform; the glycolipids which contain the PHA were eluted with 10 ml of acetone; and the polar lipids were eluted with 10 ml of methanol. The recovery was quantitative [12].

Analysis of the lipids. The neutral lipid fraction was analyzed for TG by gas-liquid chromatography (GLC). Poly beta-hydroxyalkanoate (PHA) was analyzed by GLC after acid ethanolysis [13]

The polar lipids were first subjected to mild alkaline methanolysis

which yields ester-linked fatty acid methyl esters (FAME)[11]. The water soluble fraction of the mild alkaline methanolysis was dried, digested with perchloric acid and the phosphate analyzed colorimetrically[11]. This is a measure of the diacyl phospholipid content. The FAME were purified by thin layer chromatography and analyzed by capillary GLC[14]. The structures were assigned to the FAME on the basis of retention time compared to standards, the results of hydrogenations, and the fragmentation patterns after gas chromatograhy/mass spectrometry (GC/MS)[14]. The configuration and position of the double bonds of monoenoic fatty acids was determined from the elution time from the capillary column in comparison with standards and by GC/MS fragmentography of the Diels-Alder adducts formed by reaction with 5,5-dimethoxy-1,2,3,4-tetrachlorocyclopentadiene[15].

Nomenclature of the FAME. Fatty acids are designated as the number of carbon atoms in the aliphatic chain:the number of double Bonds with the position of the double bond nearest the aliphatic (w) end of the molecule. The configuration as c for cis and t for trans follows. Suffixes a,i, br, and cy indicate anteiso-branching, iso-branching, branching and cyclopropane rings are present in the molecule.

RESULTS AND DISCUSSION

Vibrio natriegens and Vibrio anguillarum. Growth of these non-sulfate reducing marine Vibrios on 304 stainless steel coupons induces accelerated corrosion[16]. The growth of Vibrio natriegens increased the corrosion current density 19-fold over the control in a 6 day period. The corrosion current density began to increase when colonies of organisms with extracellular excretions were detectable on the surface of the coupon by microscopy with epifluorescent illumination and scanning electron microscopy (Figure 2). The presence of the bacteria in the film could be detected by the increase in the amide I trench at ~1660 cm^{-1}. This is clearly demonstrated in the infrared spectrum of E. coli which forms no significant exopolymers (Figure 3, bottom spectrum). The most rapid increase in corrosion current density correlated with the formation of an extracellular material as seen microscopically. The appearance of extracellular material correlated with a great increase in the infrared absorbance at ~1440 cm^{-1}. In the period between 72 and 144 hours of bacterial growth there is a great increase in the absorbance at ~1440 cm^{-1} compared to the absorbance at ~1660 cm^{-1} (Figure 4, top and middle spectrum). This organism forms very similar extracellular material when associated with the surface of aluminum, titanium, teflon, or stainless steel (compare the middle and lower spectrum of Figure 3).

Removing the biofilm as monitored by the FT/IR decreased the corrosion current density. Sonication and washing removed more bacterial cells than extracellular material (2.7-fold greater decrease in ~1660 cm^{-1} compared to ~1440 cm^{-1}). Lipid extraction with additional sonication induced a further 43-fold decrease in absorbance at ~1440 cm^{-1} with a total removal of the bacterial protein absorbance at ~1660 cm^{-1} and a 10-fold decrease in corrosion current density[16].

The related bacterium Vibrio anguillarum also induces increased corrosion current density when grown on the surface of 304 stainless steel. After 144 hours it forms a smaller proportion of extracellular material as evidenced in the decreased ratio of exopolymer absorbance ~1440 cm^{-1} to bacterial protein ~1660 cm^{-1} when compared with V. natriegens (compare the middle spectrum of Figure 3 and Figure 4). The corrosion current density correlates with the ~1440 cm^{-1} absorbance (V. anguillarum= 666 nA/cm^2 compared to 2900 nA/cm^2 for V. natriegens).

The similarity of the infrared spectra suggests the extracellular accumulation associated with the growth of the two marine Vibrios is calcium hydroxide. The accumulation is not pure calcium hydroxide but possibly is

Figure 2. Micrographs of Vibrio natriegens colonizing 304 stainless steel surfaces stained with acridine orange and exposed to epifluorescent illumination at 400X after 48 hours of incubation (top panel). Scanning electron micrographs of the biofilm generated by Vibrio natriegens grown on 304 stainless steel disks for 144 hours (bottom panel) at magnification of 2000 (A-left) and 6000 (B-right).

modified by an organic matrix as indicated by the perturbations in the major infrared absorbancies (Figure 5). Applications of authentic calcium hydroxide (without the organic matrix, as in Figure 5, upper panel) onto 304 stainless steel did not induce the high current densities seen with the extracellular accumulation by the bacteria. Analysis by STEM showed only calcium and chloride were present so it is clearly not accumulating metals. The most likely mechanism for facilitation of corrosion for the two marine Vibrios seems to be the generation of areas of differing cathodic activity. These areas of differing cathodic activity may well be the result of the heterogeneous distribution of the bacteria and exocellular polymers clearly illustrated in the photomicrographs (Figure 2). This work also strongly suggests that accumulations of materials elaborated by the bacteria can facilitate corrosion. Elegant experiments reported by Brenda Little (17, and as reported in this volume) using galvanically coupled electrodes in chambers separated by a membrane of pore size that excludes bacteria in order to compare sterile and infected surfaces suggest that exopolymer could facilitate corrosion.

Pseudomonas atlantica. P. atlantica is a gram-negative aerobic

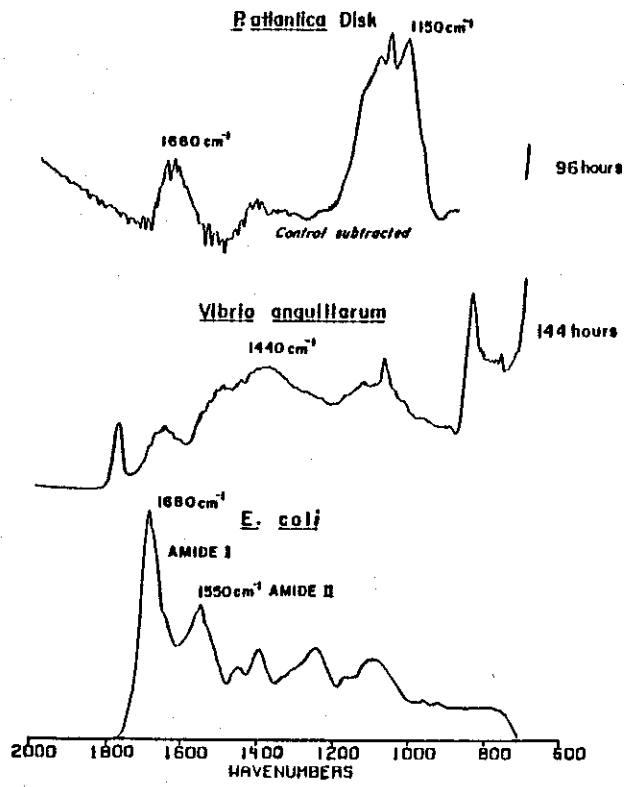

Figure 3. Fourier transform infrared spectra (FT/IR) of biofilms induced by the colonization of V. natriegens (top spectrum) and V. anguillarum (middle spectrum) on 304 stainless steel surfaces. The top panel shows the difference spectrum between the sterile control and the biofilm generated in the presence of V. natriegens. The spectrum of lyophilized E. coli is given in the bottom panel for comparison.

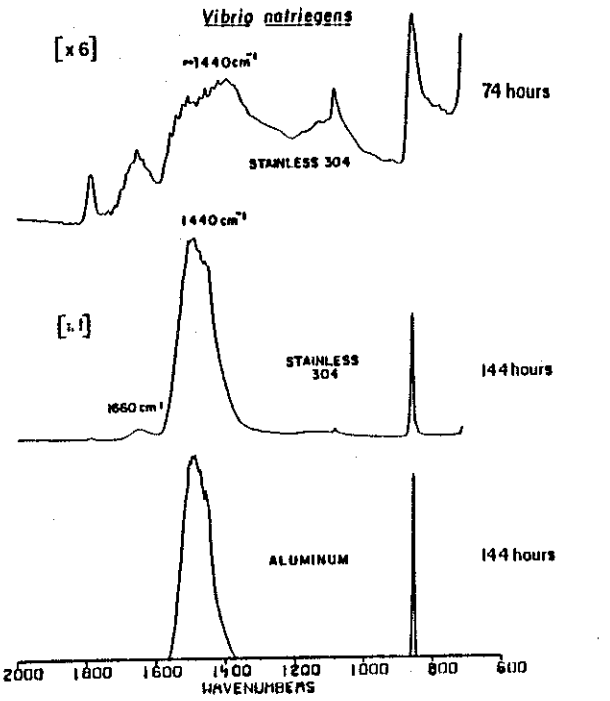

Figure 4. Fourier transform infrared spectra (FT/IR) of biofilms formed by V. natriegens on the surface of 304 stainless steel after incubation for 72 hours (top panel) and 144 hours (middle panel) compared to the biofilm formed by this organism on aluminum foil in 144 hours (bottom panel). values indicate the multiplication factor of 6 for the abscissa of the top panel compared to the middle spectra.

bacteria that forms large amounts of a uronic acid containing polysaccharide exopolymer when metabolically stressed [18]. Organisms like this are readily isolated from estuarine sediments and rapidly attach to surfaces in contact with seawater. It is highly likely that the secretion of acidic polysaccharide exopolymers is a critical step in the irreversible attachment of these types of organisms to surfaces exposed to seawater [19]. Mechanically or chemically disturbing biofilms on simulated condenser tubes exposed to rapidly flowing seawater greatly increased the formation of these uronic acid containing exopolymers [20].

The growth of P. atlantica on 304 stainless steel disks for 5 days increased the corrosion current density from 94 (56) nA/cm^2 in the control to 264 (183) nA/cm^2. The presence of P. atlantica on the stainless steel surface decreased $E_{corrosion}$ from -158 (44) mV to -213 (25) mV in this same 5 day incubation. The FT/IR can be utilized to directly demonstrate that the bacteria and its exopolymer are present on the stainless steel disks. The polymer produced by P. atlantica can be readily purified. Comparison of the infrared spectra of the lyophilized organisms and the isolated polymer shows the purification significantly decreases the proportion of the absorbance corresponding to the amide I

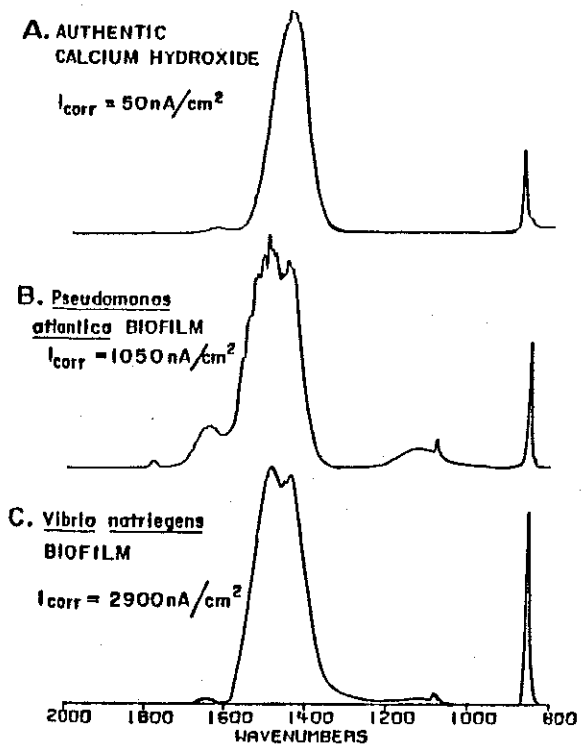

Figure 5. Fourier transforming infrared spectra (FT/IR) of authentic calcium hydroxide (upper spectrum), biofilm of P. atlantica on 304 stainless steel after vigorous aeration of the culture (middle spectrum), and extracellular exudation of V. natriegens (lower spectrum).

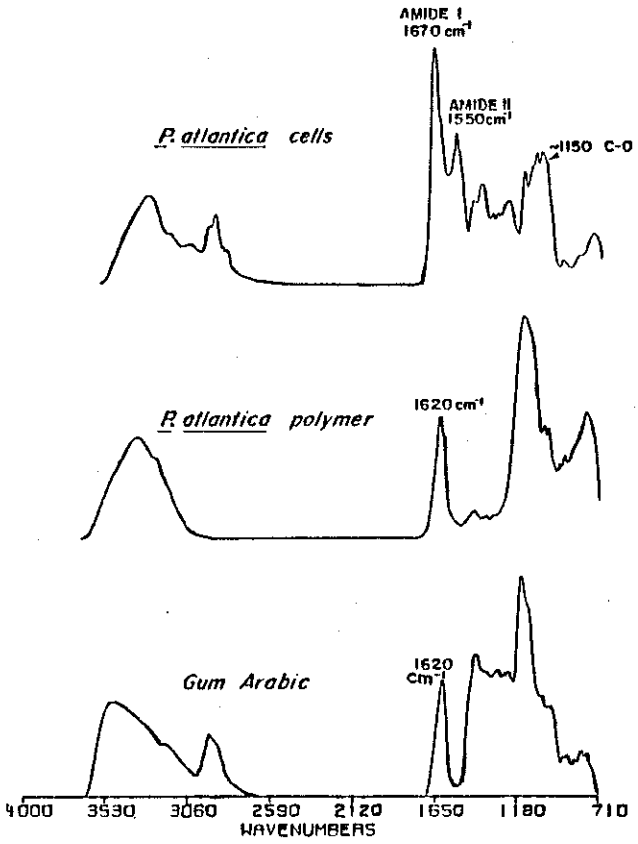

Figure 6. Fourier transforming infrared spectra (FT/IR) of lyophilized Pseudomonas atlantica cells (top spectrum) and purified exopolysaccharide polymer (middle spectrum) compared to gum arabic polymer (lower spectrum) measured using the diffuse reflectance mode.

and II stretch regions [representing the bacterial proteins [9]] while increasing the absorbance at ~1150 cm^{-1} corresponding to the C-O stretch of the carbohydrate polymers (Figure 6). The spectrum of the bacterial polymer resembles the gum arabic polymer. Examination of the biofilms on the control and coupons incubated with P. atlantica shows the distinctive pattern of the organism and its polymer on the disk surface (Figure 6, top spectrum). In this spectrum the absorbance of the biofilm formed on the control disks has been subtracted.

Preliminary experiments have suggested that the purified polymer alone can increase the corrosion current density if applied to the stainless steel surface. However when disks were attached to the sides of flasks at the water line and the medium periodically submerged the surface as the flasks were rotated, a dense biofilm was formed (Figure 7). Under these conditions the corrosion current density increased 10-fold from the control values of 94 (56) nA/cm^2 to values over 1064 nA/cm^2. The biofilm showed an initial uniform bacterial colonization that was followed by the elaboration of extracellular polymer around microcolonies. The increase in corrosion density coincided with the elaboration of extracellular polymer. The FT/IR spectrum of the corrosion facilitating biofilm of P. atlantica shows a prominent 1440 cm^{-1} typical of calcium hydroxide along with the carbohydrate C-O stretch at ~1150 cm^{-1} and the amide I of the proteins at ~1650 cm^{-1} (Figure 5).

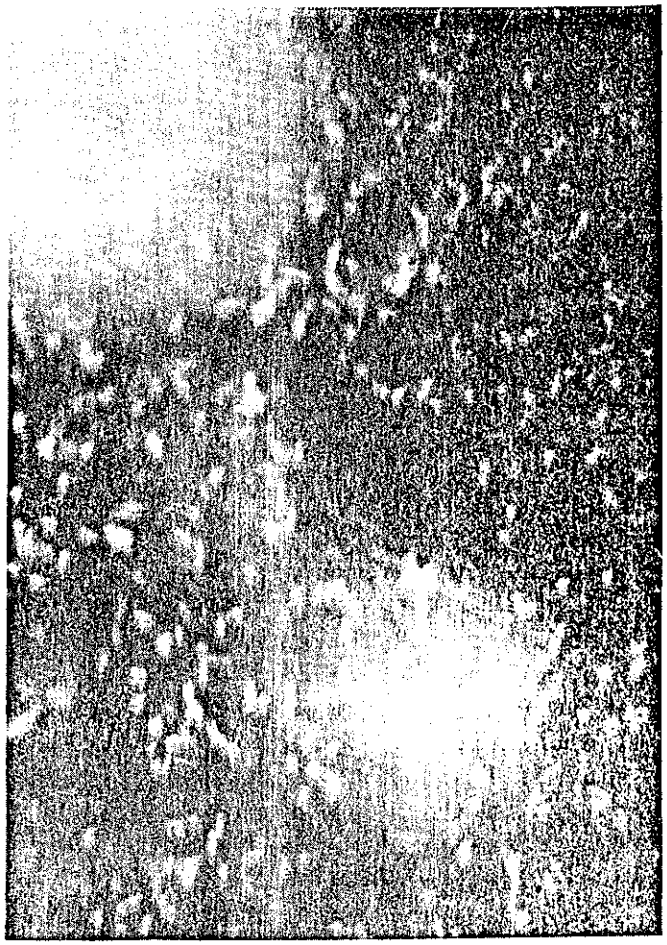

Figure 7. Micrograph of P. atlantica stained with acridine orange and exposed to epifluorescent illumination at 400X after 24 hours of vigorous aeration (upper panel) and after 72 hours of vigorous aeration.

This study demonstrates the power of the FT/IR spectrophotometer in defining the chemical composition of biofilms that is fast, sensitive, non-destructive and capable of examining areas 25 um in diameter [9]. The FT/IR can be utilized with the attenuated total reflectance cell (ATR) to observe a living biofilm for its accumulation of PHA or extracellular polymer as well as the attachment of microbes to the germanium crystal [9]. One of us (G. G. G.) together with Teike Iwaoka and Peter Griffiths of the University of California at Riverside has actually monitored the loss of a copper film sputtered on the ATR crystal in the presence of distilled water--finally a direct measure of corrosion on the scale approaching bacterial size.

Signature phospholipid fatty acid analysis. The facility with which specific types of bacteria can be recognized in complex biofilms can be illustrated using two aerobic microbes that are involved in MIC.

Iron bacteria. Black iron water pipes carrying treated municipal water were found to be severely corroded. The inside surfaces were heavily tuberculated with severe pitting corrosion beneath the tubercules. Extensive potentiodynamic corrosion tests directly on the pipes over a period of several months revealed an average linear resistance corrosion rate of 6 mpy on unfouled specimens.

Organisms were recovered from markedly corroded iron pipes exposed in this potable water supply system. The rusty tuberculated surface which significantly occluded the pipe diameter contained extractable phospholipids averaging 54 pmoles/cm^2. This is the equivalent of 10^6 organisms the size of Escherichia coli. The outside and ends of the pipes were thoroughly cleaned prior to the lipid extraction. The polar lipid fraction was recovered as illustrated in Figure 1 and the ester-linked fatty acids of the phospholipids analyzed as FAME. The results showed a pattern of short saturated, branched and monoenoic fatty acids. This analysis indicated what appears to be an absence of microeukaryotes such as algae, fungi, protozoa or micrometazoa in the contaminant community as shown by the absence of long chain alkyl and polyenoic fatty acids [21]. Second, the large proportion of cis-vaccenic acid (18:1w7c) in contrast to oleic acid (18:1w9c) and the presence of 18:1w7t suggest the community contains a high proportion of facultative heterotrophic gram-negative bacteria. The presence of cyclopropane 17:0 indicates that these gram-negative bacteria are most probably nutrient-stressed which can be a strong stimulus to the formation of exopolymers that seem to characterize these communities [22]. Anaerobic sulfate-reducing bacteria of the lactate-utilizing Desulfovibrio type are not a significant part of this community as indicated by the lack of i

17:1w7c and a 17:1w7c from the phospholipid ester-linked fatty acids[23] Neither are the acetate-utlizing Desulfobacter type sulfate-reducers as their major lipid signature 10 methyl 16:0 is not detected (N. Dowling, unpublished results from this laboratory). The presence of phospholipid ester-linked FAME that are hydroxylated is a distinctive property presently being exploited in an attempt to isolate and characterize these organisms. The OH-FAME represent 1.8% of the total FAME.

Raw water from this system was incubated aerobically with coupons of mild steel, teflon and titanium in thoroughly cleaned wide mouth jars. The jars included atmospheric headspace and were loosely capped with teflon lined lids. After 36 hours the extractable phospholipid phosphate of the teflon film, the titanium foil and the mild steel coupons was 1.5, 1.6, and 66.5 pmoles/cm^2 respectively.

Thiobacilli and corrosion of concrete. Another group of aerobic bacteria involved in corrosion are the autotrophic Thiobacilli which utilize reduced sulfur in the presence of air to form sulfuric acid. W. Sand and E. Bock of the Institute of General Botany, Department of Microbiology, University of Hamburg, West Germany, have described a test system in which concrete samples can be exposed to the actions of Thiobacilli[24]. In this system it has proved possible to accelerate the rate of degradation so that a 9 month exposure in the chamber corresponds to more than 5 years of exposure in a sewer system. With this test chamber it was possible to show that the rapid degradation correlates to the activity of Thiobacillus thiooxidans and not to other microorganisms. These workers have utilized this facility to design and test concrete for resistance to biogenic sulfuric acid attack[25].

In collaborative work we have been able to show that "signature lipids" characteristic of different Thiobacilli correlate with rates of degradation in both test chamber and in samples from the environment. Thiobacilli contain unusual ester-linked fatty acids in their phospholipids. Thiobacillus thiooxidans contains 19 and 17 carbon monoenoic fatty acids with the unsaturation located at the w9, w8, w7, and w6 positions (B. Kerger, unpublished data). The cyclopropane 19:0 which represented 88% of the phospholipid ester-linked fatty acids is extremely unusual. Further analysis of T. thiooxidans shows this component actually represents a series of isomers with the three membered ring in different positions. These fatty acid profiles are sufficently unusual so that they serve as excellent signatures to find these organisms in biofilms associated with this type of corrosion. These chemical analyses eliminate the need for time consuming most probable number (M.P.N.) culture tests and provide quantitative information as to the microbial associations involved in the corrosion.

Acknowledgements

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