

JMM 00120

Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria-polymer mixtures and biofilms

Peter D. Nichols, J. Michael Henson, James B. Guckert, David E. Nivens and David C. White

Department of Biological Science, Florida State University, Tallahassee, FL 32306-3043 (U.S.A.)

(Received 1 November 1984) (Revised version received 21 March 1985) (Accepted 13 May 1985)

Summary

Fourier transform-infrared (FT-IR) spectroscopy has been used to rapidly and nondestructively analyze bacteria, bacteria-polymer mixtures, digester samples and microbial biofilms. Diffuse reflectance FT-IR (DRIFT) analysis of freeze-dried, powdered samples offered a means of obtaining structural information. The bacteria examined were divided into two groups. The first group was characterized by a dominant amide I band and the second group of organisms displayed an additional strong carbonyl stretch at $\sim 1740\text{ cm}^{-1}$. The differences illustrated by the subtraction spectra obtained for microbes of the two groups suggest that FT-IR spectroscopy can be utilized to recognize differences in microbial community structure. Calculation of specific band ratios has enabled the composition of bacteria and extracellular or intracellular storage product polymer mixtures to be determined for bacteria-gum arabic (amide I/carbohydrate C-O $\sim 1150\text{ cm}^{-1}$) and bacteria-poly- β -hydroxybutyrate (amide I/carbonyl $\sim 1740\text{ cm}^{-1}$). The key band ratios correlate with the compositions of the material and provide useful information for the application of FT-IR spectroscopy to environmental biofilm samples and for distinguishing bacteria grown under differing nutrient conditions. DRIFT spectra have been obtained for biofilms produced by *Vibrio natriegens* on stainless steel disks. Between 48 and 144 h, an increase in bands at ~ 1440 and 1090 cm^{-1} was seen in FT-IR spectra of the *V. natriegens* biofilm. DRIFT spectra of mixed culture effluents of anaerobic digesters show differences induced by shifts in input feedstocks. The use of flow-through attenuated total reflectance has permitted in situ real-time changes in biofilm formation to be monitored and provides a powerful tool for understanding the interactions within adherent microbial consortia.

Key words: *Amide I band - Bacteria - Digesters - Exopolymer - FT-IR - In situ analysis*

Introduction

Infrared (IR) spectroscopy is a nondestructive technique, requires minimal sample preparation, and allows the rapid characterization of structural features of complex, polymeric material. The IR portion of the spectrum is rich in information regarding the

vibrational and rotational motions of atoms in molecules. Specific IR absorptions can be assigned to particular types of covalent bonds, and modifications of these bonds by the local electronic environment can be detected in the details of the spectra [1-3].

One of the problems restricting the application of IR spectroscopy to environmental samples has been that the atomic interactions sensed in the IR portion of the spectrum are at relatively low energies. Thus, the detection of these interactions is relatively inefficient. This has precluded, until recently, the full use of IR analysis for complex materials isolated from the environment. Fourier transform-infrared (FT-IR) instrumentation has overcome previous problems associated with low transmittance heterogeneous samples. Multiple scanning and manipulation routines, including the capability to perform spectral subtractions, together with microbeam condensers allowing comparison of 10 μm diameter areas have further improved FT-IR spectroscopy as a rapid nondestructive analytical tool [4]. Recent applications of the FT-IR method for obtaining IR spectra include the study of blood protein interactions with surfaces in flow-through systems [5-7] and the study of coal structure and oxidation processes [8, 9].

Microbial attachment processes on germanium crystals have been monitored using FT-IR spectroscopy at selected time intervals [10]. The application of FT-IR to a number of surface studies has been reviewed by Jakobsen [11].

Analytical biochemical methods involving the identification of specific 'signature' or 'biological marker' lipids have been used to determine microbial biomass and community structure [12-15]. Such biochemical techniques, although they provide qualitative and quantitative insights into environmentally relevant problems, are destructive and time consuming. FT-IR analysis of microbial samples, including biofilms, represents a largely untried approach, which could complement and/or supplement existing analytical procedures for the characterization of such materials.

In this report FT-IR spectra of bacteria and related laboratory and field samples will be presented. Such data may be utilized to chemically catalogue bacteria and related environmental samples. The development of critical bands and band ratios for known mixtures of bacteria-exopolymer and pathogenic-nonpathogenic bacteria should enable application of FT-IR spectroscopy to the monitoring of environmental samples.

Materials and Methods

Bacteria

Lyophilized cells of the following bacteria were obtained from Sigma Chemical Company (St. Louis, MO): *Escherichia coli* strain B (ATCC 11303), *Bacillus subtilis* (ATCC 6633), *Pseudomonas fluorescens* (ATCC 1340), *Staphylococcus aureus* strain Newman D₂C, *Clostridium perfringens* (ATCC 13124). *Vibrio natriegens* and *V. anguillarum* were gifts of Dr. G. Geesey (Long Beach State University, Long Beach, CA) and were grown on Bacto marine broth 2216 (Difco). *Pseudomonas atlantica* was a gift of Dr. W. Corpe (Columbia University, New York, NY) and was grown in 1% (w/v) galactose with either 0.1% or 0.01% (w/v) proteose peptone (Difco) in 25 ppt artificial seawater, ASW, (Forty fathoms marine mix, Marine Enterprises, Towson, MD). *Methylobacterium organophilum* and *Methylosinus trichosporium* were gifts of Dr. R. Hanson (Gray Freshwater Biological Institute, Navarre, MN). *Nitrobacter winogradskyi* was a gift of Dr. E. Bock (University of Hamburg, FRG).

Sample preparation

Weighed amounts of bacteria, poly- β -hydroxybutyrate (PHB; gift of Dr. R. Lafferty, Technische Universität, Graz, Austria), gum arabic (Sigma Chemical Co.) and proteose peptone (Difco) were combined in various ratios (weight percent) for FT-IR analysis.

A biofilm was produced by *Vibrio natriegens* on stainless steel disks (1 cm diameter, Metal Associates, Munford, AL) placed in flasks of marine broth 2216. At various times, disks were removed, rinsed in 25 ppt ASW, and lyophilized. Disks were stored under vacuum over phosphorus pentoxide until examination.

Effluent was removed from thermophilic digesters 24 h after feeding. The digesters received 16 g of Bermuda grass:cattle feed (Seminole Brands) (3:1) daily. In addition to the daily feed some digesters were continuously infused with sodium propionate, (10 $\mu\text{mol/ml/day}$), sodium butyrate (15 $\mu\text{mol/ml/day}$), or sodium nitrate (10 $\mu\text{mol/ml/day}$). The effluent was lyophilized and frozen until examination.

All solid samples were ground for 1 min (Wig-L-Bug, Spectra Tech, Inc., Stamford, CT), transferred to a 13 \times 2 mm sample cup and leveled without compression using a spatula prior to FT-IR analysis.

Fourier transform-infrared spectroscopy

Data collection parameters reported below are the information recommended by a subcommittee of the Coblenz Society to be included with published spectra from computerized IR instruments [16].

Spectral collection, processing and transformation

All spectra of powdered solids and for biofilms on metal surfaces were interpreted based on the Kulbelka-Munk (K-M) analysis [17] used as an approximation of Beer's Law for reflectance spectroscopy [18]. The spectra obtained for the flow cell experiments are reported in absorbance units. Spectra were collected on a Nicolet 60SX FT-IR equipped with a liquid-nitrogen cooled, high-sensitivity, mercury:cadmium:tellurium detector (range = 5500-710 cm^{-1}), a mid-IR Globar source, and a KBr beamsplitter (Nicolet Instrument Corp., Madison, WI). Interferograms were zero-filled and apodized by the Haap-Genzel function prior to the Fourier transformation utilizing Nicolet SX software (TMON version 1.5).

Diffuse reflectance (DRIFT)

Powdered bacteria, bacteria-polymer mixtures, digester samples and stainless steel disks were evaluated with a DRIFT accessory (Spectra Tech, Inc., Stamford, CT). Each sample scan resulted in a single-sided interferogram of 4096 data points which provided a resolution of 4 cm^{-1} . Signal averaging of 500 scans per sample with a medium correlation (correlation factor, 8) required 2.5 min of total measurement time. All resulting spectra were ratioed to the appropriate background spectrum. Replicate spectra were collected for all samples and mixtures analysed by DRIFT. Standard deviations for calculated band ratios (obtained using intensities at peak maxima) were in the range 2-12%.

Determination of the specular reflectance contribution for biofilms on stainless steel

disks analyzed in the drift accessory has not been performed. The sample chamber was evacuated for two minutes and purged with nitrogen for two minutes prior to sample or background data collection. Spectra showed near identical baselines and are plotted uncorrected.

Attenuated total reflectance (ATR)

The ATR flow cell (Harrick Scientific Corp., Ossining, NY) was set up in the micro-beam chamber on the 60SX optical bench (linear condensation, 15). The internal reflection element was germanium metal ($50 \times 10 \times 2$ mm, refractive index, 4.01, Harrick Sci. Corp.) with an angle of incidence $\theta = 45^\circ$ which results in 25 internal reflections through the element [19]. Assuming a refractive index for the biofilm of 1.33, that of seawater, the depth of penetration (d_p) of the infrared energy into the biofilm with respect to the wavelength of light (λ) is: $d_p = 0.0638 \cdot \lambda$ [20]. This results in a d_p for the amide I band (1650 cm^{-1}) of 385 nm, and 583 nm for the C-O stretch of carbohydrates (1090 cm^{-1}). Each sample scan resulted in a double-sided interferogram of 4096 data points which provided a resolution of 8 cm^{-1} . Signal averaging of 100 scans per sample with an intermediate correlation (correlation factor, 2) required 30 s of total measurement time. All resulting spectra were ratioed to a background spectrum of the dry ATR cell. Prior to water subtraction, the baseline drift due to the dependence of d_p on the light wavelength was corrected. Water backgrounds were spectra of reverse osmosis deionized water (RODW) flowing through the ATR cell. Backgrounds were chosen that had been flowing for the same time through the ATR cell as the sample spectra. The RODW spectra were processed in the same manner as the sample prior to subtraction. The sample chamber and optical bench were under a constant nitrogen purge throughout the analysis.

ATR flow cell experiments

Natural seawater (NSW) was collected in an acid-rinsed Nalgene container from the Florida State University Marine Laboratory (Turkey Point, FL) settling system. To minimize bottle effects [21], 20 l of NSW was collected, transported back to the main laboratory, and connected to the flow cell within 3 h of collection. Prior to analysis the NSW aseptically passed through a $0.4 \mu\text{m}$ Nuclepore filter.

After collection of the dry ATR cell background, NSW flow began (approximately 10 ml/min, Technicon proportioning pump, Chauncey, NY). Spectra were collected at 5 min intervals.

Results

Bacteria

A range of bacteria have been analyzed by DRIFT spectroscopy and representative spectra are illustrated in Fig. 1. Spectra obtained for *Pseudomonas fluorescens*, *Desulfovibrio gigas*, *Staphylococcus aureus*, *Clostridium perfringens*, *Escherichia coli*, *Methylobacterium organophilum* (grown on methane) and *Methylosinus trichosporium* (grown on methane) together with a number of other sulphate-reducing bacteria analyzed are generally similar. Subtle variations in peak ratios were apparent, however, in many of

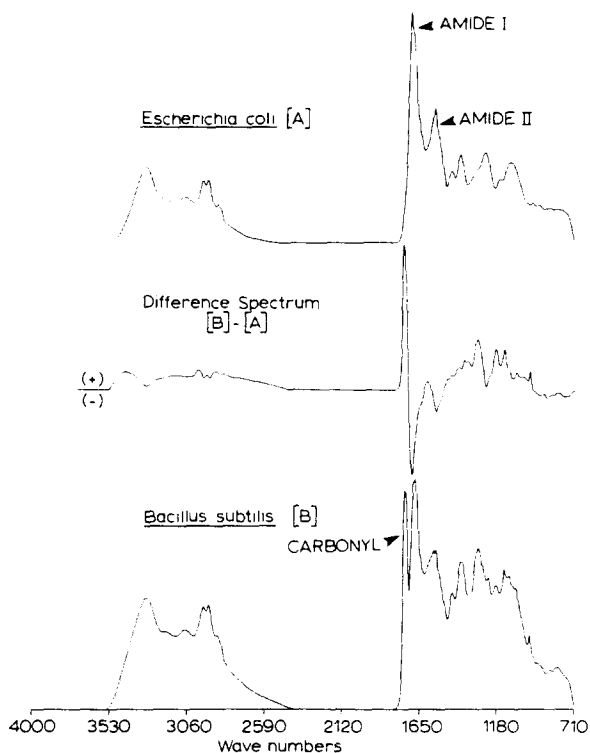


Fig. 1. FT-IR spectra ($4000\text{--}710\text{ cm}^{-1}$) of representative group I (*E. coli*) and group II (*B. subtilis*) organisms showing dominant amide I and II bands (group I) and an additional carbonyl stretch at 1740 cm^{-1} (group II). The middle spectrum is the difference spectrum obtained from a one-to-one subtraction of the two organisms.

these organisms. A representative spectrum of this group of microbes is shown in Fig. 1 (upper spectrum). Strong amide I (between 1690 and 1650 cm^{-1}) and amide II (1550 cm^{-1}) bands are present in all these organisms. Other prominent bands are present at 3300 cm^{-1} (O-H stretch), 2950 cm^{-1} (C-H stretch), 1450 , 1250 and 1090 cm^{-1} .

A number of other microbes analyzed, *Bacillus subtilis* (Fig. 1, lower spectrum), *Methylobacterium organophilum* and *Nitrobacter winogradskyi*, showed spectral features distinguishing them from the organisms listed above: The FT-IR spectrum of *B. subtilis* contained, in addition to the spectral bands noted above, an enlarged O-H stretch and a large carbonyl peak centered at $\sim 1740\text{ cm}^{-1}$. *M. organophilum* when grown on methanol contained, in addition to the strong amide I and II bands present when grown on methane, a carbonyl stretch at 1740 cm^{-1} . The carbonyl stretch was the largest peak detected in the spectrum of *N. winogradskyi* and additional spectral features, not present in other microbes studied, were also observed.

Bacteria-polymer mixtures

The DRIFT spectra obtained for mixtures of gum arabic and *E. coli* are shown in

Fig. 2. The major peak for the spectrum of *E. coli* is the protein amide I band, whilst the dominant peak in the spectrum of gum arabic is the carbohydrate derived C-O stretch centered at $\sim 1150\text{ cm}^{-1}$. Logarithmic plots of the carbohydrate to amide I (Fig. 2) and amide II bands and the relative proportion of gum arabic reveal relationships between these parameters giving correlation coefficients (r^2) of 0.97 and 0.97 for the two five-point lines; probability (p) of no true correlation < 0.001 . A similar trend was observed for prepared mixtures of gum arabic and proteose peptone, the latter being rich in proteinaceous material (unpublished data).

The major band present in the FT-IR spectrum of PHB is the carbonyl band centered at 1750 cm^{-1} . A plot of the band ratio of the PHB carbonyl stretch to the amide I band for prepared mixtures of PHB with *B. subtilis* is illustrated in Fig. 3. The relationships which exist between the carbonyl and the amide I and II band ratios and the proportion of PHB present are described by correlation coefficients (r^2) of 0.96 and

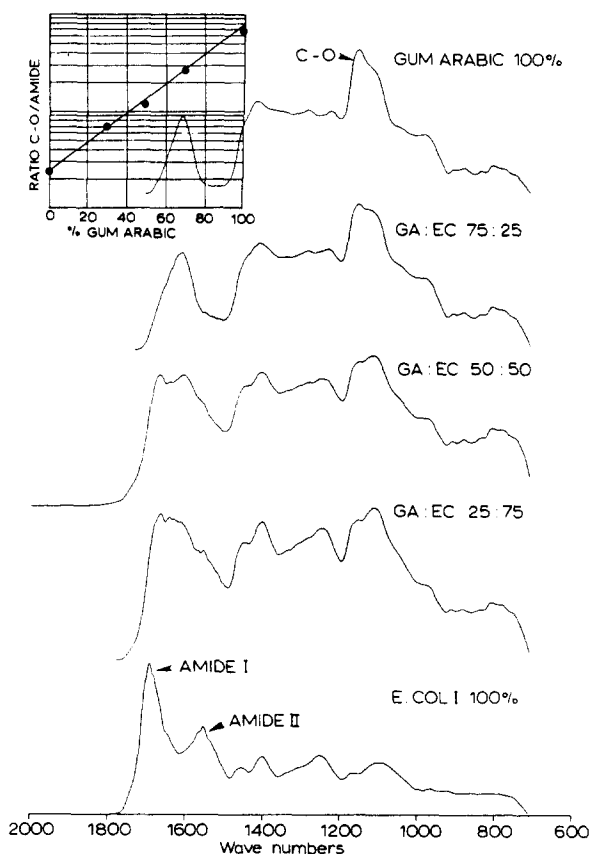


Fig. 2. Comparison of the FT-IR spectra of mixtures of gum arabic and *E. coli* and the ratio of carbohydrate C-O ($\sim 1150\text{ cm}^{-1}$) to amide I ($\sim 1680\text{ cm}^{-1}$) bands as a function of the proportion (weight percent) of gum arabic in the mixtures.

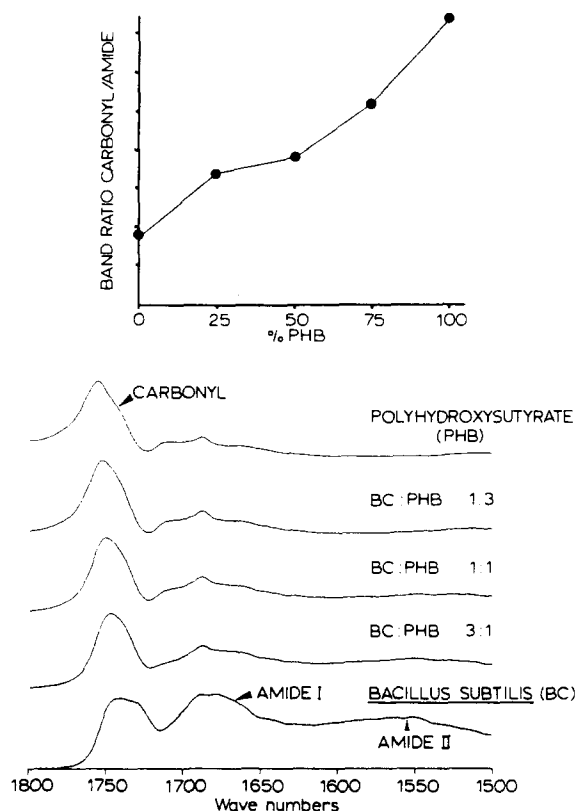


Fig. 3. Comparison of the FT-IR spectra of mixtures of poly- β -hydroxybutyrate (PHB) and *Bacillus subtilis* and the ratio of PHB carbonyl stretch ($\sim 1740\text{ cm}^{-1}$) to amide I ($\sim 1680\text{ cm}^{-1}$) bands as a function of the proportion (weight percent) of PHB in the mixtures.

0.78, respectively, for the five-point lines with probabilities of no true correlation of less than 0.001.

FT-IR spectra of a polymer sample and for cell material harvested in stationary phase at levels of 0.1% and 0.01% proteose peptone in the growth medium are presented in Fig. 4. The spectrum obtained for *P. atlantica* exopolymer is similar in appearance to the IR spectrum of gum arabic with the C-O stretch at $\sim 1150\text{ cm}^{-1}$ being the dominant band. The IR spectrum of *P. atlantica* cell material obtained from the 0.1% proteose peptone medium is characterized by significantly stronger bands at 1150 cm^{-1} (C-O stretch) and 3350 cm^{-1} (O-H stretch) than observed for the cells harvested from medium containing 0.01% proteose peptone.

Biofilms on stainless steel surfaces

FT-IR spectra of stainless steel disks removed from batch cultures of *Vibrio natriegens*, *V. anguillarum* or *P. atlantica* are presented in Fig. 5. For disks exposed to *V. natriegens*, after an incubation period of 48 h, a prominent amide I band ($\sim 1650\text{ cm}^{-1}$)

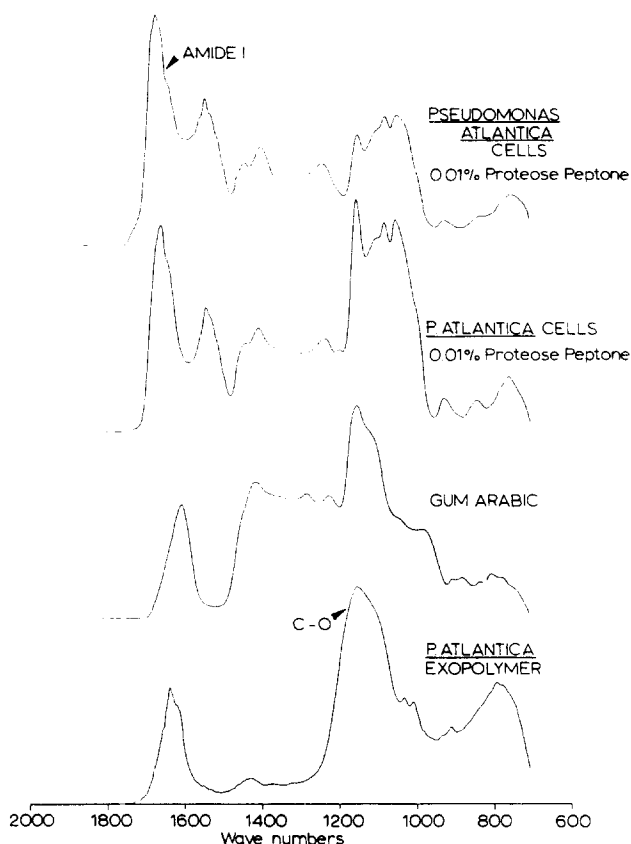


Fig. 4. FT-IR spectra of *Pseudomonas atlantica* cells grown under different growth conditions and exopolymer produced by *P. atlantica*.

was observed. At subsequent sampling points in the experiment, bands centered at 1790, 1490, 1440, 1080 and 850 cm^{-1} were detected in the spectra in addition to the amide I band. The two dominant peaks present after 96 and 144 h of the *V. natriegens* experiment were the bands centered at ~ 1440 and 850 cm^{-1} (Fig. 5). A similar result was obtained for *V. anguillarum*. For *P. atlantica* the early appearance of an amide I band was followed by the occurrence of a peak centered at ~ 1150 cm^{-1} (Fig. 5, upper spectrum).

Digester material

FT-IR spectra were recorded for samples of digester feed and effluent obtained from control and propionate-, butyrate- and nitrate-amended digesters (Fig. 6). Major bands in the DRIFT spectra of carbohydrate-rich digester feed were those at 3400 cm^{-1} (O-H stretch), 2950 cm^{-1} (aliphatic C-H stretch), 1730, 1630, 1370, 1240, 1160 and 1120 cm^{-1} (Fig. 6). Material removed from the control digester is characterized by a relatively

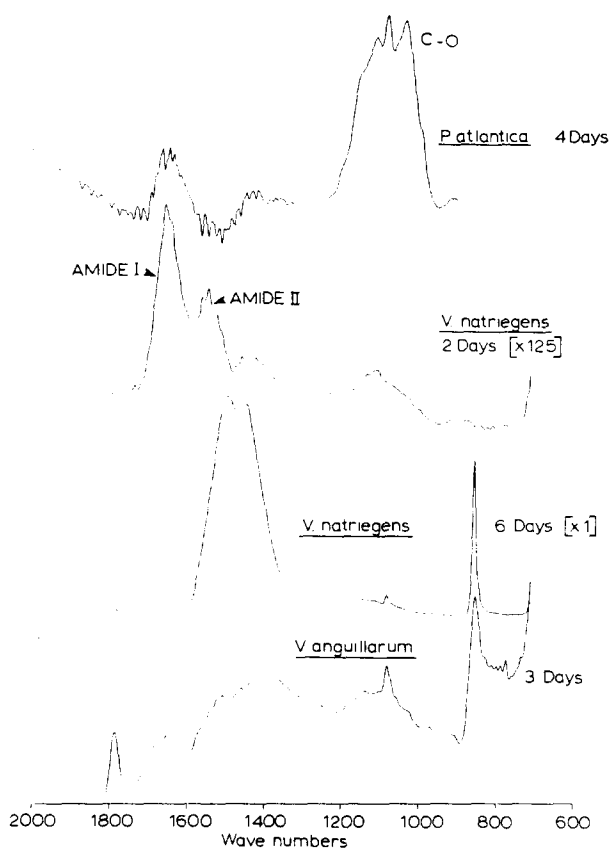


Fig. 5. Comparison of FT-IR spectra of the surfaces of stainless steel disks removed from batch cultures of *Vibrio natriegens*, *V. anguillarum* and *P. atlantica*. Parenthesized numbers for *V. natriegens* indicate scaling factors for quantitative comparison of the two spectra.

smaller O-H stretch together with higher relative K-M intensities at 1660 and 1510 cm^{-1} . A close similarity is apparent between the spectra obtained for the propionate- and butyrate-amended digester effluents (Fig. 6). Bands centered at 1600 and 1415 cm^{-1} , characteristic of the asymmetrical and symmetrical stretching bands, respectively, of the carboxylate anion were the major additional bands present in these two samples. A higher relative K-M intensity at $\sim 1660 \text{ cm}^{-1}$ (amide I), when compared with the digester feed material, is also apparent in these spectra.

The band centered at 1440 cm^{-1} is seen at a higher relative reflectance in the nitrate-amended digester products when compared to the control and feed derived samples (Fig. 6). As was noted for the other digester samples a smaller relative K-M intensity occurred at 3400 cm^{-1} (O-H stretch).

ATR flow cell experiments

Figure 7 shows the hourly biofilm accumulation at the germanium surface. There is

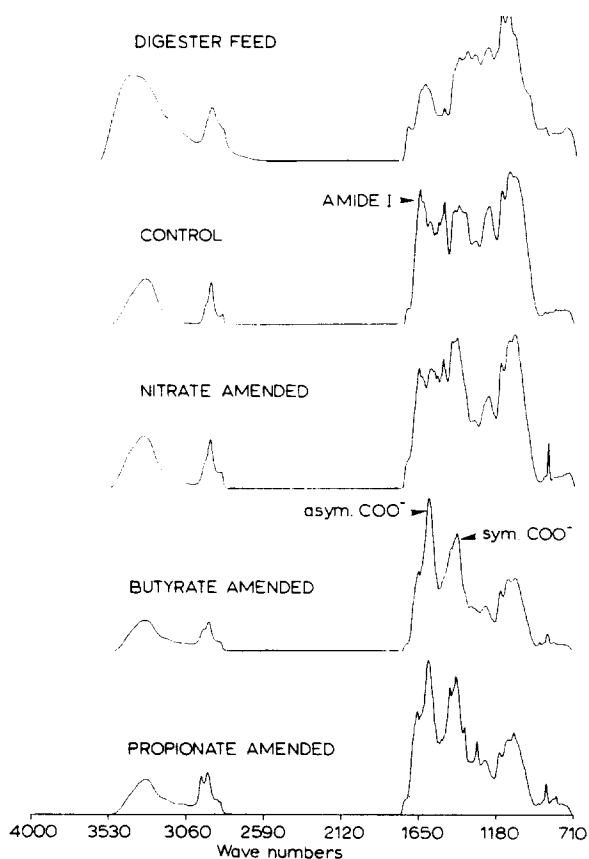


Fig. 6. Comparison of FT-IR spectra of digester feed and material removed from control and propionate-, butyrate- and nitrate-amended digesters.

an accumulation of material with an intensity centered at 1100 cm^{-1} , interpreted as the C-O stretch of carbohydrate. RODW controls show no accumulation of carbohydrate after 8 h.

Discussion

General considerations

Bacterial cell material and the cell-polymer mixtures can be analyzed by more conventional IR techniques such as KBr pellet transmittance and absorbance spectroscopy. The reflectance technique was the method we selected for analysis of biofilms and other material which can not be rapidly and quantitatively removed from the surfaces to which they are attached. Although it has been shown that the best DRIFT spectra of organic samples are obtained by grinding the sample with about 20-times their weight in KCl for 1–3 min, identifiable spectra have, however, been obtained of neat samples [22].

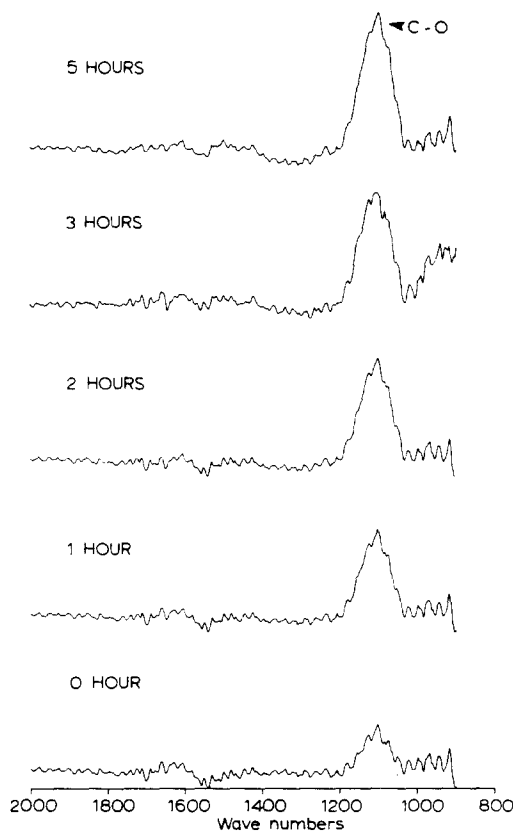


Fig. 7. FT-IR spectra ($2000\text{--}940\text{ cm}^{-1}$) showing hourly biofilm accumulation at the germanium surface of the ATR crystal. The spectra of the bulk phase NSW (0 hour) was obtained immediately following the filling of the flow cell. All spectra are baseline corrected, water subtracted and plotted on the same ordinate axis.

In this study, in order to keep sample preparation time to a minimum and because analysis of environmental microbial biofilms would not be facilitated by removal of the material from surfaces and mixing it with a nonabsorbing matrix, powdered solids were analyzed by DRIFT as neat solids.

Bacteria

The bacteria analyzed in this study by FT-IR spectroscopy can be divided into two groups. The first group is characterized by a dominant amide I band (Fig. 1). Similar spectroscopic features have been previously reported in transmittance spectra obtained for *E. coli* [10]. A significantly higher signal to noise ratio (S/N) is observed with the FT-IR spectrometric technique. The higher S/N ratio of these spectra enables spectral manipulation procedures, including the use of spectral expansion and subtraction routines, to be performed more readily and with more precision. The dominance of the amide I band and to a lesser extent the amide II band in the bacterial IR spectra is

consistent with the high proteinaceous content of such material. The position of the amide I maxima obtained for unmixed neat bacterial samples was in a number of cases at a higher wavenumber than is usually reported for proteinaceous material [11]. This observation is probably due to some form of matrix effect. Protein typically represents approximately 60% (dry weight basis) of the bacterial cellular material [23]. Methods have been recently reported using FT-IR spectroscopy for the determination of protein and moisture content of wheat [24, 25]. Similar procedures could be applied to bacterial samples.

The second group of bacteria all contained a carbonyl stretch at just below 1740 cm^{-1} (Fig. 1, lower spectrum). The wavenumber maximum of this peak corresponds to that observed for PHB mixed with bacterial cells (Fig. 3). The shift of the PHB carbonyl maximum (1752 cm^{-1}) to a lower wavenumber (1740 cm^{-1}) when mixed with protein rich bacterial cells may be due to intermolecular hydrogen bonding occurring between the two materials. A similar phenomenon was observed for mixtures of *E. coli* and gum arabic. The amide I band shifted to a lower wavenumber upon the addition of gum arabic. These shifts may be due to the matrix phenomenon noted above. A difference spectrum (middle spectrum, Fig. 1) obtained by the subtraction of a representative group I bacterium (*E. coli*) from a group II bacterium (*B. subtilis*) illustrates the potential for FT-IR spectroscopy to enhance differences between two organisms. These data suggest that the potential also exists for these differences to be quantified and that such techniques may be applicable to the recognition of differences in microbial community structure.

Bacteria-exopolymer mixture

Examination of the FT-IR spectra for mixtures of bacteria and gum arabic (Fig. 2) shows that key IR band ratios are correlated with the composition of the material. Further analyses involving the measurement of carbon and nitrogen present in *E. coli*-gum arabic mixtures and in extracellular polymer (ECP) produced under different culture conditions by *P. atlantica* using an elemental analyzer have indicated that the IR band ratios can also be correlated with the nitrogen content of the sample (unpublished data). The ratio of the bacterial protein amide I and II bands to carbohydrate C-O stretch ($\sim 1150\text{ cm}^{-1}$), based on the data presented here for bacteria-gum arabic mixtures, may be useful in estimating the proportions of both bacteria and extracellular polymer present in environmental biofilms.

Comparison of the C-O/amide I band ratios of (i) *P. atlantica* exopolymer, (ii) *P. atlantica* cells grown with 0.1% proteose peptone, and (iii) *P. atlantica* cells grown with 0.01% proteose peptone (Fig. 4) with data reported above for prepared standard mixtures of gum arabic-*E. coli* (Figs. 2 and 3) indicates that the three samples contain about 80, 50 and 20%, respectively, of carbohydrate-rich material. The production of exopolymer rich in polysaccharide by *P. atlantica* grown with 0.5% peptone has been documented [26], and our data indicate that in addition to exopolymer, material attached more intimately to the cells is also present. FT-IR spectra obtained for *P. atlantica* exopolymer produced under a variety of growth conditions in our laboratory show markedly different C-O/amide I and II band ratios (unpublished data). FT-IR spectroscopy offers a rapid means to monitor polymer composition prior to the use of complex,

time-consuming biochemical procedures which identify individual components present in extracellular and cell wall polysaccharide polymers from environmental biofilm samples [27, 28].

Bacteria-PHB mixtures

The relationships observed for the PHB carbonyl stretch/amide I (Fig. 3) and amide II band ratios also show that FT-IR spectroscopy can be used to quantify bacteria/PHB mixtures. The data presented here for mixtures of bacteria and PHB polymers provides useful information necessary for the application of FT-IR spectroscopy to distinguish bacteria grown under nutrient limiting conditions resulting in accumulation of PHB [29]. During starvation the PHB polymer serves as a carbon and energy source [30]. The analysis of PHB polymer has been classically performed by gravimetric methods [31] or by spectrophotometric analysis of *trans*-crotonic acid produced by hydrolysis and dehydration of the polymer [32]. These methods are insensitive and subject to errors due to contamination. Analysis of the constituent β -hydroxy acids by gas chromatography (GC) has been documented [29, 33], including a recent report which showed that in addition to β -hydroxybutyric acid a number of longer chain hydroxy acids were present in the polymer [29]. The rapid, nondestructive analysis involving minimal sample preparation afforded by FT-IR spectroscopy offers a useful method which can complement existing procedures used for the analysis of environmental samples containing PHB. Further studies are planned to correlated data obtained from the FT-IR method with data provided by GC methodology.

Stainless steel surfaces

Corrosion studies being undertaken in our laboratory are directed towards determining the role of microorganisms in the processes involved. An EG&G Model 350A corrosion measurement console is being utilized to measure electrochemical parameters of stainless steel disks placed in both pure culture and environmental situations. The fact that IR spectra of the bacterial cellular and extracellular material could be easily obtained indicated that DRIFT spectroscopy of the metal disk surfaces may represent one technique to study changes occurring at the metal-water interface and to detect the presence of bacteria and biofilms.

The metal disks removed from batch cultures of *Vibrio natriegens* were analyzed by DRIFT spectroscopy prior to epifluorescence or scanning electron microscopy. The appearance of a strong amide I band together with an amide II band at 48 h (Fig. 5) parallels the formation of large bacterial colonies as noted by epifluorescence microscopy [34].

Preliminary studies reveal that an increase in corrosion current density and corrosion rate occurs between 48 and 144 h for stainless steel disks removed from batch cultures of *V. natriegens* [34]. The increase in corrosion rate corresponded to an increase in the bands at ~ 1440 and 850 cm^{-1} present in the FT-IR spectra obtained for the disk surfaces (Fig. 5). Although subtle differences in band intensities and peak maxima occur due, for example, to interaction between extracellular and cellular material, the spectrum obtained for the *V. natriegens* biofilm at 144 h is consistent with the presence of significant portions of calcium hydroxide. At this stage, the data obtained for *V. natriegens*,

V. anguillarum and for concurrent projects using the marine pseudomonad *P. atlantica* (Fig. 5) indicate that these bacteria can play a role in the corrosion process and that FT-IR spectroscopy can be utilized to monitor changes occurring on the surface involved.

Digester material

The dried sludge from the digesters sampled showed a significant decrease in the carbohydrate C-O to amide I band ratio relative to digester feed (Fig. 6). These data, when taken together with a decrease in O-H stretch (3400 cm^{-1}) relative to aliphatic C-H stretch (2920 cm^{-1}), are consistent with an overall loss of carbohydrate structure from digester sludge relative to feedstock. Near infrared reflectance (NIR) has been utilized previously in attempts to determine fat, protein and carbohydrate content in cocoa powder [35] and for the measurement of protein and moisture in wheat [24, 25]. Grasses such as the Bermuda grass used as feedstock in the digesters analyzed here are rich in carbohydrate material [36]. The use of FT-IR spectroscopy in this study extends the application of IR reflectance of plant material to include digester-amended material and thus readily enables the monitoring of carbohydrate structural content present in the digesters.

A number of other spectral features, in addition to the bands characteristic of proteinaceous and carbohydrate material are apparent in the IR spectra. The dried butyrate- and propionate-amended digester sludge both showed strongly absorbing bands at 1600 and 1415 cm^{-1} due to the asymmetric and symmetric stretching bands of the propionate or butyrate carboxylate anion.

The information provided by FT-IR spectroscopy, when coupled with community structure information obtained by detailed lipid and other biochemical analyses, should enable biomass transformations in anaerobic digesters to be better understood.

ATR flow cell experiments

These results (Fig. 7) show that FT-IR/ATR is the method of choice for in situ, real-time analysis of a developing biofilm and similar complex biological systems as previously suggested [37].

Our preliminary experiments suggest that the initial organic layer adsorbed onto the germanium surface from $0.4\text{ }\mu\text{m}$ filtered NSW is predominantly carbohydrate. Further work is planned to determine more about the chemistry, size class, composition and kinetics of adsorption of this film and the microbial interactions with it.

Conclusions

In this study, FT-IR spectroscopy has been used to analyze bacteria, related bacteria-polymer mixtures, digester samples and microbial biofilms. Subtraction spectra may offer the potential of a rapid means for identifying certain specific bacteria. Calculations of specific band ratios can enable the composition of bacteria-polymer mixtures to be determined. These data provide useful reference information which can be applied to studies of (i) biofilm structure where amide and carbohydrate bands are of key importance, and (ii) nutritional status where the monitoring of amide and carbonyl bands is

undertaken. The recognition of other critical bands and band ratios is feasible based on the data presented in this report and will enable further application of the FT-IR method for insight into biofilm composition and structure.

Acknowledgements

The FT-IR was purchased with grant N0014-83-G0166 from the Department of Defense, University Instrumentation program through the Office of Naval Research. This work was supported by contracts N0014-83-K0056 and N0014-82-C0404 from the Department of the Navy, Office of Naval Research, with supporting efforts from grants NCA2-IR235-401 from the NASA Ames University consortium, and NAG2-149 from the Advanced Life Support Office, National Aeronautics And Space Administration, and from a cooperative program between the Institute of Food and Agricultural Sciences of the University of Florida and the Gas Research Institute, entitled *Methane from Biomass and Waste*. The authors wish to thank the staff of the National Center for Biomedical Infrared Spectroscopy, Battelle-Columbus Laboratories, Columbus, OH, for expert advice, consultation and demonstration of ATR flow cell experiments. Dr. G. Geesey is thanked for his helpful comments during manuscript preparation. Melanie Trexler is thanked for her assistance with figure preparation.

References

- 1 Bellamy, L. T. (1958) *The Infrared Spectra of Complex Molecules*. John Wiley and Sons, New York.
- 2 Parker, F.S. (1971) *Application of Infrared Spectroscopy in Biochemistry, Biology and Medicine*. Plenum Press, New York.
- 3 Griffiths, P. R. (1975) *Chemical Infrared Fourier Transform Spectroscopy*. Wiley-Interscience, New York.
- 4 Griffiths, P. R. (1983) Fourier transform infrared spectroscopy. *Science* 222, 297-302.
- 5 Winters, S., Gendreau, R. M., Leininger, R. I. and Jakobsen, R. J. (1982) Fourier transform infrared spectroscopy of protein adsorption from whole blood: ex vivo sheep studies. *Appl. Spectrosc.* 36, 404-409.
- 6 Gendreau, R. M., Winters, S., Leininger, R. I., Fink, D., Hassler, C. R. and Jakobsen, R. J. (1981) Fourier transform infrared spectroscopy of protein adsorption from whole blood: ex vivo dog studies. *Appl. Spectrosc.* 35, 353-357.
- 7 Gendreau, R. M., Leininger, R. I. and Jakobsen, R. J. (1982) Molecular level studies of blood protein-materials interactions. In: *Biomaterials 1980* (Winter, G. D., Gibbons, D. F. and Plenck, H. Jr., eds.) pp. 415-421, John Wiley and Sons, New York.
- 8 Fuller, M. P., Hamadeh, I. S., Griffiths, P. R. and Lowenhaupt, D. E. (1982) Diffuse reflectance infrared spectroscopy of powdered coals. *Fuel* 61, 529-536.
- 9 Solomon, P. R. and Carangelo, R. M. (1982) FTIR analysis of coal. 1. Techniques and determination of hydroxyl concentrations. *Fuel* 61, 663-669.
- 10 Baier, R. E. (1980) Substrata influences on adhesion of microorganisms and their resultant new surface properties. In: *Adsorption of Microorganisms to Surfaces* (Bitton, G. and Marshall, K. C., Eds.) pp. 59-104, John Wiley and Sons, New York.
- 11 Jakobson, R. J. (1979) Application of FTIR to surface studies. In: *Fourier Transform Infrared Applications to Chemical Systems*, Vol. 2 (Ferraro, J. R. and Basile, L. J., Eds.) pp. 165-191, Academic Press, New York.
- 12 White, D. C. (1983) Analysis of microorganisms in terms of quantity and activity in natural environments. In: *Microbes in their Natural Environments* (Slater, J. H., Whittenbury, R. and Wimpenny, J. W. T., Eds.) Soc. Gen. Microbiol. Symp. 34, 37-66, Cambridge University Press, London.

- 13 Perry, G.J., Volkman, J.K., Johns, R.B. and Bavor, H.J. (1979) Fatty acids of bacterial origin in contemporary marine sediments. *Geochim. Cosmochim. Acta* 43, 1715-1725.
- 14 Gillan, F.T., Johns, R.B., Verheyen, T.V., Nichols, P.D., Esdaile, R.J. and Bavor, H.J. (1983) Monounsaturated fatty acids as specific bacterial markers in marine sediments. In: *Advances in Organic Geochemistry* (Bjoroy, M., Ed.) pp. 198-206, John Wiley and Sons, New York.
- 15 Parkes, R.J. and Taylor, J. (1983) The relationship between fatty acid distributions and bacterial respiratory types in contemporary marine sediments. *Estuarine, Coastal Shelf Sci.* 16, 173-189.
- 16 Grasselli, J.G., Griffiths, P.R. and Hannah, R.W. (1981) Criteria for presentation of spectra from computerized IR instruments. *Appl. Spectrosc.* 36, 87-91.
- 17 Kulbelka, P. and Munk, F. (1931) Ein Beitrag zur Optik der Farbanstriche. *Z. Tech. Phys.* 12, 593-601.
- 18 Hecht, H.G. (1980) Quantitative analysis of powder mixtures by diffuse reflectance. *Appl. Spectrosc.* 34, 161-164.
- 19 Harrick, N.J. (1967) *Internal Reflection Spectroscopy*. Wiley Interscience, New York.
- 20 Fink, D.J. and Gendreau, R.M. (1984) Quantitative surface studies of protein adsorption by infrared spectroscopy. I. Correction for bulk concentrations. *Anal. Biochem.* 139, 140-148.
- 21 Zobell, C.E. and Anderson, D.Q. (1936) Observations on the multiplication of bacteria in different volumes of stored seawater and the influence of oxygen tension and solid surfaces. *Biol. Bull.* 71, 324-342.
- 22 Fuller, M.P. and Griffiths, P.R. (1980) Infrared microsampling by diffuse reflectance Fourier transform spectrometry. *Appl. Spectrosc.* 34, 533-539.
- 23 Ingraham, J.L., Maaboe, O. and Neidhardt, F.T. (1983) *Growth of the Bacterial Cell*. Sinauer Associates Inc., Sunderland, MA.
- 24 Hruschka, W.R. and Norris, K.H. (1982) Least-squares curve fitting of near infrared spectra predicts protein and moisture content of ground wheat. *Appl. Spectrosc.* 36, 261-265.
- 25 Osborne, B.G., Douglas, S., Fearn, T. and Willis, K.H. (1982) The development of universal calibrations for measurement of protein and moisture in UK grown wheat by near-infrared reflectance analysis. *J. Sci. Food Agric.* 33, 736-740.
- 26 Corpe, W.A. (1970) An acid polysaccharide produced by a primary film-forming marine bacterium. *Dev. Ind. Microbiol.* 11, 402-412.
- 27 Fazio, S.A., Uhlinger, D.J., Parker, J.H. and White, D.C. (1982) Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. *Appl. Environ. Microbiol.* 43, 1151-1159.
- 28 Uhlinger, D.J. and White, D.C. (1983) Relationship between the physiological status and the formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. *Appl. Environ. Microbiol.* 45, 64-70.
- 29 Findlay, R.H. and White, D.C. (1983) Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus subtilis*. *Appl. Environ. Microbiol.* 45, 71-78.
- 30 Nickels, J.S., King, J.D. and White, D.C. (1979) Poly- β -hydroxybutyrate accumulation as a measure of unbalanced growth of estuarine detrital microbiota. *Appl. Environ. Microbiol.* 37, 459-465.
- 31 Lemoigne, M. (1926) Produits de deshydratation et de polymerisation de l'acid beta-oxybutyrique. *Bull. Soc. Chim. Biol.* 8, 770-782.
- 32 Law, J.H. and Slepecky, R.A. (1961) Assay of poly- β -hydroxybutyric acid. *J. Bacteriol.* 82, 33-36.
- 33 Braunegg, G., Sonnleitner, B. and Lafferty, R.M. (1978) A rapid gas chromatographic method for the determination of poly-beta-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol. Biotechnol.* 6, 20-37.
- 34 Nivens, D.E., Nichols, P.D., Henson, J.M., Geesey, G.G. and White, D.C. Reversible acceleration of corrosion of 304 stainless steel exposed to seawater induced by growth and secretions of the marine bacterium *V. natriegens*. Corrosion (submitted for publication).
- 35 Kaffra, K.J., Norris, K.H., Kulcsar, F. and Draskovits, I. (1982) Attempts to determine fat, protein, and carbohydrate content in cocoa powder by the NIR technique. *Acta Aliment.* 11, 271-288.
- 36 Dawes, C.J. and Lawrence, J.M. (1980) Seasonal changes in the proximate constituents of the seagrasses *Thalassia testudinum*, *Halodule wrightii*, and *Syringodium filiforme*. *Aquat. Bot.* 8, 371-380.
- 37 Gendreau, R.M. and Jakobsen, R.J. (1978) Fourier transform infrared techniques for studying complex biological systems. *Appl. Spectrosc.* 32, 326-328.