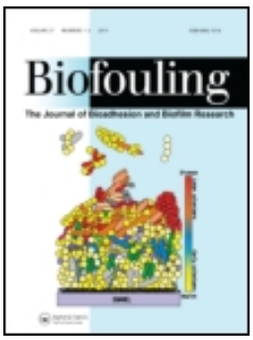

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AN EVALUATION OF BIOFILM DEVELOPMENT UTILIZING NON-DESTRUCTIVE ATTENUATED TOTAL REFLECTANCE FOURIER TRANSFORM INFRARED SPECTROSCOPY

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Chemical changes that occur within a microbial biofilm during development on a germanium internal reflection element (IRE) were monitored by attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR) for 188 h. The amount of protein detected at the IRE/medium interface increased throughout the course of the experiment. Extracellular polysaccharides were mainly produced during the initial stages of biofilm development. These results demonstrate that changes in metabolic activity of surface-associated bacteria during biofilm development on surfaces exposed to a flowing bulk aqueous phase can be evaluated by ATR/FT-IR.

KEY WORDS: Bacteria, extracellular polysaccharides, protein

INTRODUCTION

There is a growing awareness that microbial biofilms participate in a number of commercially-important processes. In addition to their well-known role in wastewater treatment, biofilms are also considered to be important in the fouling of heat exchange surfaces and in metal corrosion (Characklis & Cooksey, 1983). Our understanding of the mechanisms by which biofilm populations alter the properties of the surfaces with which they associate has been limited by difficulties encountered in sample acquisition. Sampling strategies developed for homogeneous suspensions of free-living microbial populations have proven inadequate for biofilm characterization. Consequently, biofilm characterization has been restricted, for the most part, to morphological descriptions (Lappin-Scott & Costerton, 1989). Microbial biofilms in most natural environments contain a diversity of physiologically-distinct microorganisms. The success of each population and the community as a whole depends upon the maintenance of the structural integrity of the biofilm (Hamilton & Characklis, 1989). Physical relationships among microbial cells are maintained by extracellular polymeric material which extends between the cells and the surfaces with which they associate. The extracellular polymers form a matrix which impedes the diffusion of molecules within the biofilm. Thus, the chemical conditions within the biofilm may be quite different from those in the overlying bulk fluid (Characklis *et al.*, 1990).

These features of surface-associated microbial populations challenge microbiologists to identify novel approaches to assess biofilm activities. For example, a higher level of understanding could be achieved by evaluating the chemical properties of a biofilm without physical disturbance of the biofilm. This requires that evaluation be carried out *in situ* with the biofilm in a hydrated state. Evaluation should also be

conducted in such a way that there is minimal chemical interference from the bulk liquid phase. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FT-IR) offers the opportunity to obtain useful chemical information on biofilms and meet these criteria.

A procedure is described in this paper which permits the development of a microbial biofilm on the surface of an internal reflection element (IRE) housed in a Circle cell. By acquiring ir spectra of the material at the boundary of the IRE and the bulk fluid at defined intervals, it was possible to obtain chemical information on the developing biofilm in a non-destructive manner.

MATERIALS AND METHODS

Organism and culture medium

The bacterium used in this study was isolated from a pit in a section of corroded copper tubing removed from a heat exchanger and has been given the designation CP-1. The medium used was a defined culture medium (DCM) consisting of: 0·1 g $MgSO_4$, 0·2 g K_2HPO_4, 0·2 g $NaNO_3$, 0·2 g sodium succinate, 0·5 g sodium citrate, 5 g sucrose, 10 mg pyridoxol hydrochloride, 20 mg thiamine-HCl, 10 mg asparagine, 10 mg L-lysine, 10 mg L-cysteine, 10 mg L-ornithine, 10 mg L-glutamic acid, 2·4 mg $CoCl_2 \cdot 6H_2O$, 2·5 mg $CuSO_4 \cdot 5H_2O$, 2·4 mg $Ca(NO_3)_2 \cdot 4H_2O$, 2·8 mg $FeSO_4 \cdot 7H_2O$, 1·7 mg $MnSO_4 \cdot 2H_2O$, and 2·9 mg $ZnSO_4 \cdot 7H_2O$, dissolved in 1 L of double distilled water. The pH was adjusted to 7·0 and sterilized by membrane filtration, (0·45 μm pore size, Millipore Corp.).

Continuous culture of CP-1 in Micro Circle Cell

Micro-Circle cells (Spectra Tech, Stamford, CT) were rebored to an internal free volume of 176 μL with internal reflection element in place. Germanium (Ge) IREs were polished with alumina particles (0·05 μm diameter) and rinsed with distilled water before being inserted into the micro-Circle cells. The Circle cells containing the IREs were sterilized using ethylene oxide. Tubing and glassware was sterilized by autoclaving prior to connection to the Circle cells. Circle cells were positioned on an optical bench inside a Perkin Elmer Model 1800 Fourier transform infrared spectrometer equipped with a KBr beam splitter and a liquid N_2-cooled, medium range mercury-cadmium-telluride detector (5000–580 cm^{-1}). One Circle cell served as a reference while the other Circle cell received a bacterial inoculum. The tubing used to deliver sterile medium to the Circle cells was aseptically connected to the Circle cells by the use of Swagelok fittings (Fig. 1).

Culture medium (DCM) was pumped from a reservoir (500 mL) through the Circle cells with a model 375A Sage peristaltic pump (Orion Research Inc.) at a flow rate of 400 $\mu L \cdot h^{-1}$. The optical bench housing the Circle cells was purged with air that had been passed through a Balston air dryer (Model 75-50) to remove water vapor and carbon dioxide. The chamber was maintained at 25° ± 1°C during each experiment.

A deadleg positioned immediately upstream of one Circle cell functioned as a port for bacterial inoculation of the Circle cell and Ge IRE, while a medium break tube located upstream of the deadleg prevented contamination of the medium reservoir.

Collection of infrared spectra

Experiments were initiated by collecting spectra in each Circle cell before the introduction of DCM. Sterile DCM was then pumped through both reference and sample Circle

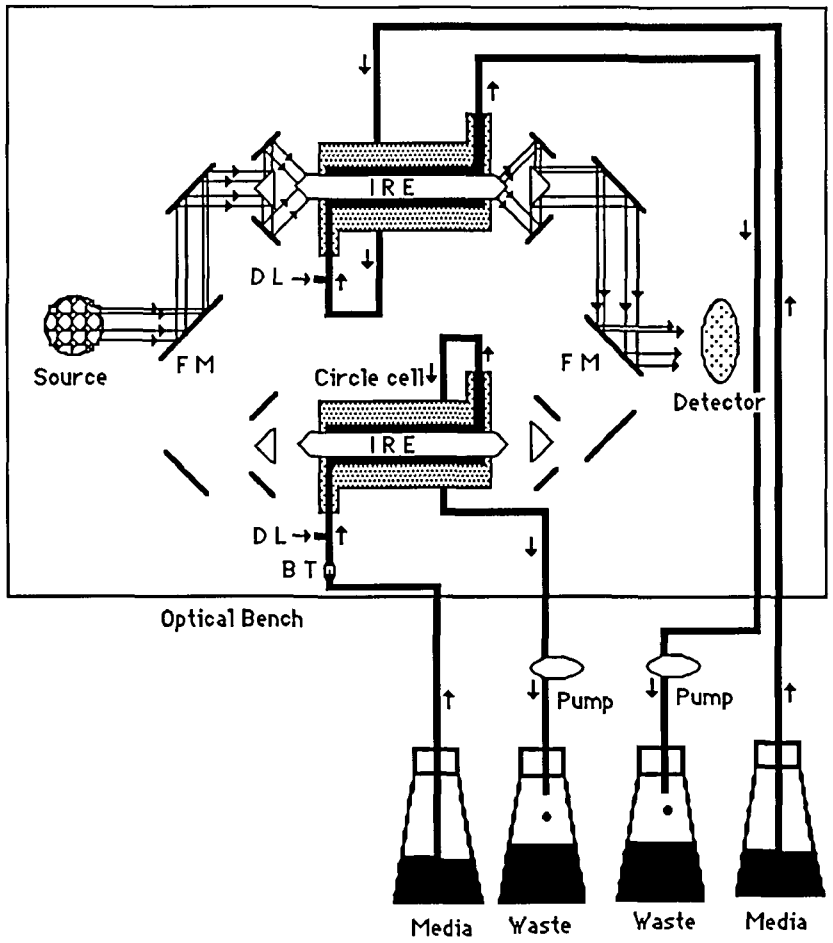

Fig. 1 Schematic diagram showing the position of the Circle cells on the optical bench of the FT-IR spectrometer, the flow of media to the Circle cells and the path of the infrared radiation through the Circle cells. IRE = internal reflection element, BT = Media break tube, DL = Dead leg, FP = Flip mirror.

cells, and spectra were collected in the double beam mode until stable absorbance values were obtained in both cells over the range 2000–1000 cm^{-1}. Once the absorbance values were stable over a 30 min period, the sample cell was inoculated with 0·3 mL of a 48 h bacterial culture that had been agitated at 100 rpm at 25°C.

Following inoculation, spectra were collected in real time from both Circle cells in the double beam and single beam modes. In the double beam mode, a synchronized oscillating mirror directed the ir beam back and forth between the 2 Circle cells to allow interferograms to be taken alternately from the sample and reference beams. Both interferograms were stored and continuously averaged in the RAM memory of the computer which controlled the spectrometer. The sample spectrum was ratioed to a recently-collected reference spectrum. A double beam spectrum consisted of 50 cycles, with each cycle comprising 6 scans of the sample Circle cell (S) and 4 scans of the reference Circle cell (R), in the order 3 S, 2 R, 2 S, 2 R, 1 S. In the single beam mode a

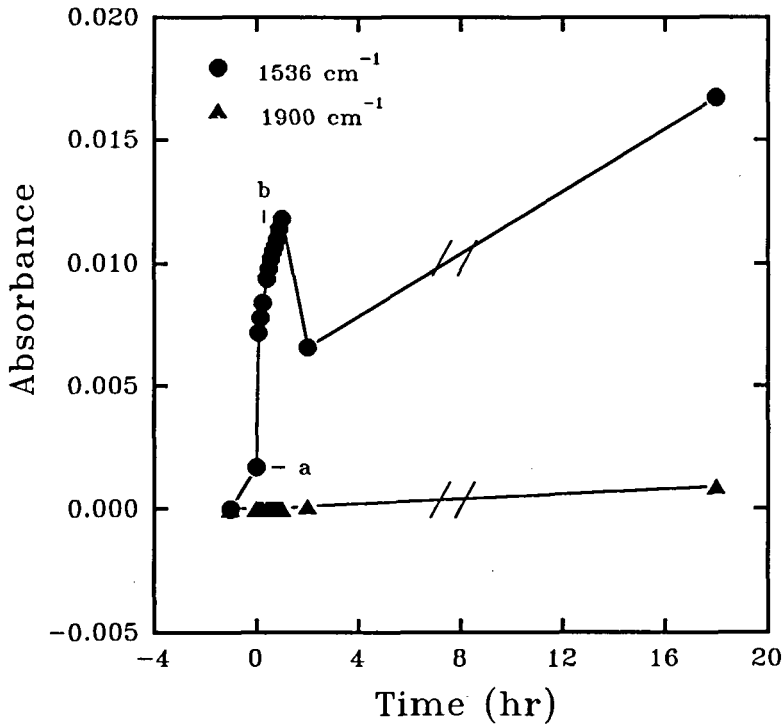

Fig. 2 Effect of inoculation of Circle cell containing a Ge IRE with a culture of CP-1 on ir absorbance at 1536 cm^{-1} (amide II) and 1900 cm^{-1} (region where bacteria or their products do not absorb). Inoculum introduced to Circle cell preconditioned with sterile, flowing culture medium at t = 0 h (a) followed by flowing sterile culture medium at t = 0·45 h (b). Flow rate of inoculum and culture medium through Circle cell was 400 $\mu L \cdot h^{-1}$.

spectrum comprised 50 averaged interferograms from either the reference or sample Circle cell. All single beam spectra obtained were ratioed against a spectrum of sterile DCM, obtained prior to the inoculation of bacteria to the sample Circle cell.

All interferograms were double-sided and apodized with a weak Beer-Norton function prior to the fast Fourier transformation using the Model 1800 computer software. Scans were taken over the range of 4000–700 cm^{-1} with an interval of 1 cm^{-1} and a nominal resolution of 4·00 cm^{-1}. The interferometer OPD mirror velocity was set at 3·00 cm sec^{-1} and the Jacquinot stop employed was 6. To compensate for a slightly sloping baseline, all spectra were flattened over the range 2000–900 cm^{-1} using the Model 1800 computer software, "Linear Program" which was specifically designed for ATR spectra.

RESULTS

Double beam spectra acquired from the sample and reference Circle cells over the course of the experiment yielded a stable baseline over the mid-ir range as reflected by the absorbance value at 1900 cm^{-1} in which no absorbance peaks due to the presence of the bacteria were expected (Fig. 2). This result indicates that any fluctuations in ir beam

energy, optical path and sampling area were the same for both reference and sample beams. Under these conditions, the instrument provided an ir spectrum that reflected only those changes contributed by bacteria at the interface of the bulk liquid phase and the surface of the Ge IRE in the inoculated Circle cell.

Sequential acquisition of double beam spectra following introduction of the bacterial inoculum at the deadleg indicated that the intensity of an absorption peak at 1536 cm^{-1} reached a value of 0·014 absorbance units within 1 hour of inoculation (Fig. 2). This band was assigned to the Amide II band of protein associated with the bacterial cells in the inoculum. As the inoculum was replaced with a continuous flow of sterile culture medium through the Circle cell, the intensity of the protein band decreased to 0·008 absorbance units within 2 h of inoculation, demonstrating that the bulk of the bacteria added to the Circle cell had passed through as a slug. That some of the bacteria present in the slug inoculum attached to and colonized the IRE was indicated by the persistence and subsequent increase in intensity of the characteristic bacterial protein absorption bands in spectra acquired after 18 h, long after the slug inoculum had passed through the Circle cell and replaced with flowing culture medium (Fig. 2).

A double beam spectrum acquired 26 h after inoculation exhibited increased absorbance values for Amide I (1626 cm^{-1}) and Amide II (1536 cm^{-1}) over those obtained after 2 h, suggesting that bacterial cells were replicating on and colonizing the IRE surface (Fig. 3). A single beam spectrum of the sample cell acquired within a few minutes of the double beam spectrum yielded absorption band intensities that were similar to those obtained in the double beam spectrum (Fig. 4). In this instance, the single beam spectrum was ratioed against a background spectrum collected in the same cell just prior to bacterial inoculation (in the presence of flowing, sterile culture medium). These results indicate that there were no significant changes in the ir spectrum of the reference cell during the 26 h period that the cell was exposed to flowing, sterile culture medium. This conclusion is supported by the single beam spectrum obtained from the reference cell after 26 h. The ratioed spectra (obtained by ratioing the spectrum collected at 26 h with a background spectrum obtained in the same cell just prior to inoculation) shows very little change in absorbance (Fig. 4). Even after 188 h of exposure to flowing, sterile medium, the single beam spectrum from the reference cell deviated no more than 0·0015 Absorbance Units from the background spectrum obtained at the beginning of the experiment (data not shown). These results suggest that, in this particular system, single beam spectra ratioed against a background spectrum obtained prior to inoculation provided spectral information as accurate as that obtained using double beam mode.

Bacterial growth and biofilm formation on the IRE in the sample Circle cell was monitored for 188 h using single beam mode (Fig. 5). Between 26 and 188 h, the intensity of the Amide II (1536 cm^{-1}) and Amide III (1274 cm^{-1}) protein bands increased steadily by an order of magnitude (Table 1). Absorption bands contributed by bacteria and bacterial products such as polysaccharide at 1055 cm^{-1} also increased over time but not to the extent demonstrated by protein (Table 1).

Changes in the concentration of various compounds in the developing biofilm were evaluated by comparing the area of their absorption peaks with that of protein (Amide II). Some products (yet to be identified) such as those responsible for the absorption bands at 1488 and 1355 cm^{-1} decreased in concentration relative to protein during biofilm maturation (Table 2). Other products which contributed absorbance at 1157 and 1055 cm^{-1} increased in concentration relative to protein during the early stages of biofilm development and then decreased during biofilm maturation.

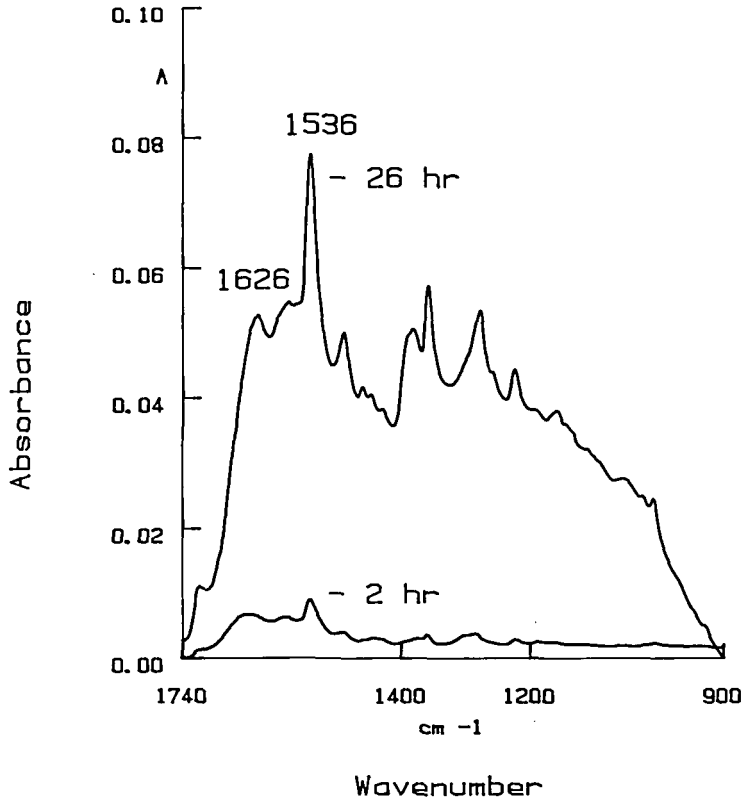

Fig. 3 Double beam spectra taken 2 h and 26 h after inoculation of CP-1 to the sample Circle cell.

DISCUSSION

Application of internal reflection spectroscopy to biofilm characterization developed from work using multiple attenuated internal reflection (MAIR) infrared spectroscopy to characterize the nature of the conditioning film that adsorbed to clean surfaces following submersion in natural waters (Baier & Loeb, 1971; Loeb & Neihof, 1975). Baier (1973) and DePalma and Baier (1978) demonstrated that a thin, proteinaceous film typically 100–200 Å in thickness appeared within the first 10 min of exposure to natural waters and caused the first modification of the initial surface condition. In natural waters, the source of this material was suggested to be humic substances present at part per million concentrations. It is this organic conditioning film rather than the original surface that microorganisms adhere to during colonization (Costerton *et al.*, 1978; Baier, 1980).

Infrared spectroscopy has been used to detect and characterize microorganisms from various habitats. Materials adsorbing to Ge internal reflection plates (IRP) exposed to warm, flowing, sub-surface ocean water for periods ranging from 24 to 144 h were examined by removing the IRP from the flow and collecting an absorbance spectrum of the materials associated with the dried surface. Changes in the spectrum of plates

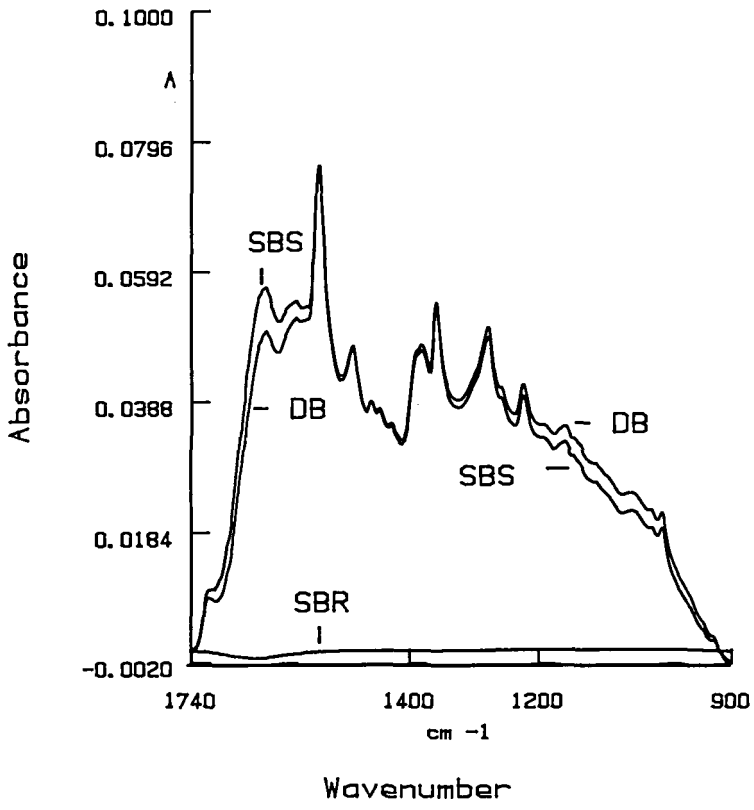

Fig. 4 Spectra taken 26 h after sample Circle cell was inoculated with CP-1. DB = Double beam spectrum, SBS = Single beam spectrum of sample Circle cell, SBR = Single beam spectrum of reference Circle cell.

collected at different times were reported to be due to the presence of bacteria on the Ge surface (Goupil *et al.*, 1980).

Diffuse reflectance spectroscopy has been used to characterize lyophilized bacterial cells (Nichols *et al.*, 1985; Nivens *et al.*, 1986). Recently van Der Mei *et al.* (1989, 1990) used transmission spectroscopy to compare the absorption spectra of a number of oral streptococcal strains that had been dried and ground into a pellet with KBr.

While these approaches can yield some useful information on the chemical composition of bacterial cells, the approach is destructive in nature and susceptible to artifacts created during the dehydration steps. Recent studies in the authors' laboratory have revealed that dehydration of bacterial products adsorbed to surfaces results in (1) a significant increase in absorbance of compounds relative to that obtained from hydrated samples, (2) a change in the relative absorption intensities of different chemical species associated with the surface.

Using an open boat Circle cell containing a zinc selenide (ZnSe) IRE, FT-IR spectra of hydrated and dehydrated exopolymer from the film-forming bacterium *Alteromonas (Pseudomonas) atlantica* were compared. Dehydration resulted in an increase in the intensity of protein absorption bands relative to carbohydrate absorption bands

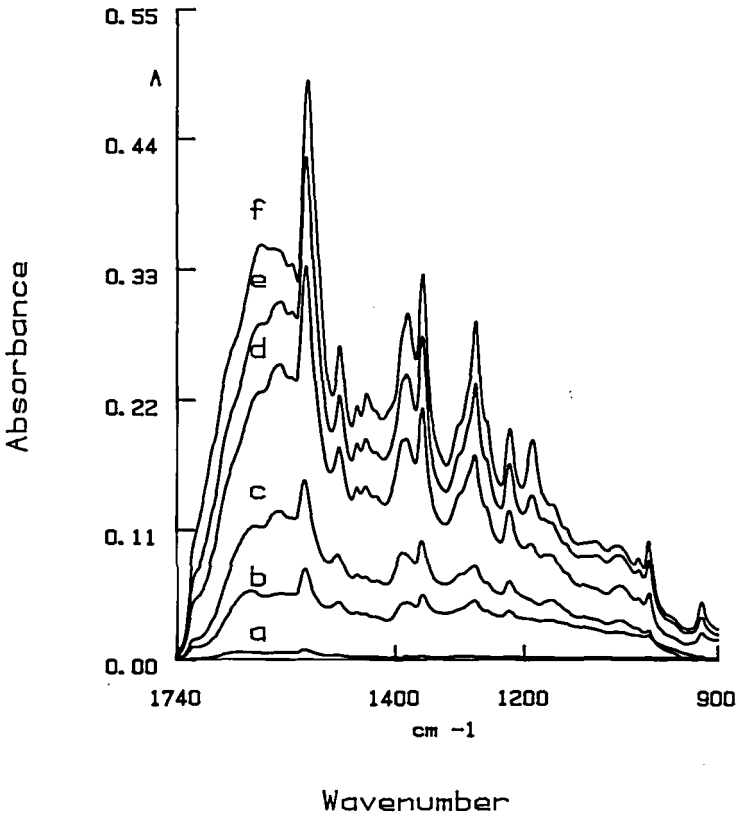

Fig. 5 Single beam spectra obtained at various intervals following the inoculation of the sample Circle cell with CP-1. a = 2 h, b = 26 h, c = 45 h, d = 90 h, e = 118 h, f = 188 h.

Table 1 Area of absorbance peaks at various intervals, following inoculation of the sample Circle cell with CP-1

Time (h)	Wavenumber (cm^{-1})										
	1536	1488	1444	1357	1274	1222	1187	1157	1055	1006	923
2	8·3	1·3	0·2	0·9	7·2	1·0	0·3	0·0	0·4	0·5	0·0
26	45·4	10·9	0·8	15·1	40·0	7·1	0·0	2·6	3·2	1·2	0·5
45	78·3	18·8	1·4	28·2	75·3	15·6	0·0	22·8	11·3	1·5	0·1
75	236·4	44·4	6·5	66·2	234·9	40·9	8·6	15·3	22·2	15·9	8·9
90	241·0	41·9	6·4	68·9	235·4	40·5	9·2	15·5	23·2	16·8	9·6
118	342·9	54·9	9·4	86·9	322·1	54·9	24·6	5·2	24·3	17·1	16·5
144	385·5	58·1	11·8	87·0	329·8	53·3	52·9	7·1	24·0	25·9	21·5
168	421·7	61·4	14·2	91·3	368·8	60·0	64·2	8·4	23·7	26·5	26·7
188	451·4	68·0	16·8	95·1	405·3	65·6	76·4	7·1	22·8	33·4	26·8

Table 2 Ratio of various absorbance peak areas to the area of the Amide II absorbance peak (1536 cm^{-1})

	Wavenumbers (cm^{-1}) ratioed					
Time (h)	*1488* 1535	*1357* 1536	*1274* 1536	*1187* 1536	*1157* 1536	*1055* 1536
26	0·24	0·33	0·88	ND	0·06	0·07
45	0·24	0·36	0·96	ND	0·30	0·14
70	0·18	0·28	0·99	0·04	0·06	0·09
90	0·17	0·29	0·98	0·04	0·02	0·10
118	0·16	0·25	0·94	0·07	0·02	0·07
144	0·15	0·23	0·86	0·14	0·02	0·06
168	0·15	0·21	0·87	0·15	0·02	0·06
188	0·15	0·21	0·90	0·17	0·02	0·05

ND = Not Detectable.

(Geesey & Bremer, 1990). Thus, for accurate information on the relative concentrations of chemical species adsorbed to surfaces in aqueous systems, it is important to obtain ir spectra under fully hydrated conditions. This approach also permits the evaluation of changes that occur on a submerged surface over time, an option which is not available when the surface is dehydrated prior to analysis.

The recent increase in capability of Fourier transform infrared (FT-IR) spectrometers and improvements in the design of liquid sampling cells for attenuated total reflection (ATR) spectroscopy now permits the acquisition of water-subtracted ir spectra of fully-hydrated material (Naumann, 1984; Braue & Panella, 1987a; Hopkinson *et al.*, 1987; Dousseau *et al.*, 1989). Water may be accurately subtracted from a spectrum by virtue of the high signal-to-noise ratio and computer manipulations achieved by these FT-IR instruments (Mattson *et al.*, 1975; Griffiths & de Haseth, 1985). Iwaoka *et al.* (1986) showed that the water from fully-hydrated acidic polysaccharide which had adsorbed to a Ge IRE positioned in a Circle cell could be subtracted with sufficient accuracy to detect contaminating (1%) levels of protein that were present.

The effective depth of penetration or sampling depth of the evanescent wave of ir radiation from the Ge IRE into the medium ranges from 0·33 μm at 1000 cm^{-1} to 0·67 μm at 2000 cm^{-1} (Harrick, 1967). Thus, the ATR spectra presented in this study reflect the vibrational energies of only those chemical species that accumulated in the 1 μm aqueous boundary layer adjacent to the surface of the Ge IRE.

Chemical species contributed by the culture medium to the conditioning film were efficiently eliminated from ATR spectra of bacterial biofilms that developed on the Ge IRE when either double beam or single beam sampling techniques were employed. Operating the instrument in the double beam mode had the advantage that a background (reference) spectrum was collected from a Circle cell that served as a sterile control within a few seconds of the time a spectrum was obtained from a Circle cell that received the bacterial inoculum. Because the two beams were closely matched, a double beam spectrum of good quality, with little trace of CO_2 or water vapor was obtained. Energy fluctuations and temperature changes that occurred in the instrument over the duration of the experiment were also eliminated from double beam spectra. Spectra acquired in this manner provided information that was virtually free of artifacts which often compromise results from long-term experiments conducted with single beam

instruments. The contributions of energy fluctuations and temperature changes to spectra acquired over the period of time required for biofilm development cannot be accurately determined with a single beam instrument.

Biofilm development was also accurately evaluated by operating the double beam spectrometer as two independent single beam instruments. Because spectra were collected sequentially from the inoculated and uninoculated Circle cells within a few minutes of each other over the duration of an experiment, contributions from the culture medium as well as fluctuations in temperature and energy output by the ir source to the biofilm spectrum were reproduced in the uninoculated control. Inherent differences between the 2 Circle cells were virtually eliminated by ratioing each spectrum against their respective reference spectrum collected at the beginning of the experiment (before introduction of the bacteria in the case of the inoculated Circle cell). This approach permitted greater flexibility of spectrum manipulation than could be achieved when the spectrometer was operated as a double beam instrument.

Changes in the concentration of water and other culture medium components at the IRE surface during biofilm development can compromise interpretation of the biofilm spectrum when data is collected in either the double beam or single beam mode. While this phenomenon did not appear to be significant in the experiments described in this study (change of 0·0015 Absorbance Units for water (1640 cm^{-1}) over a 188 h period), it could interfere with interpretations of biofilm spectra acquired in other studies.

Using ATR/FT-IR, it was possible to detect the proteinaceous material associated with 10^6 cells·ml^{-1} in the slug inoculum. The Amide II absorbance detected shortly after introduction of the bacterial inoculum to the Circle cell was contributed by protein associated with bacterial cells and free protein derived from bacterial cells that accumulated at the Ge IRE surface. The amount of protein that remained adsorbed to the Ge surface after the slug inoculum had passed through the Circle cell was approximately 60% of that which was detected when the slug was present in the cell. These results suggest that bacterial proteins had an affinity for the Ge surface under the laminar flow conditions that were employed in this study. The importance of protein material at the IRE surface throughout biofilm development was demonstrated by strong absorption bands centered at 1638 cm^{-1} (Amide I), 1578 and 1536 cm^{-1} (Amide II) and 1275 cm^{-1} (Amide III) in all spectra collected after Circle cell inoculation. These assignments were based on results of Byler and Susi (1986) and Mantsch *et al.* (1986).

The increased complexity of the spectrum in the region adjacent to Amide I during biofilm development was at least partially due to the appearance of a component that absorbed at 1720 cm^{-1}. This absorbance band was assigned to the carbonyl stretch of ester linkages of lipid material based on previous work by Mantsch *et al.* (1986). The region from 1580 cm^{-1} to 1720 cm^{-1} of spectra collected after 118 h contained features of both lipid carboxylate and protein Amide I, complicating interpretation of their contributions to biofilm composition.

Beers Law states that "the absorbance of a component is proportional to the concentration of the component" and recently researchers have reported that intergrated peak area values give a good correlation with Beers Law over a large concentration range utilizing the Circle cell sampling technique (Braue & Pannella, 1987a,b). It is generally accepted that the Amide III region of the infrared spectrum (1274 cm^{-1}) contains information that pertains only to the secondary structure of proteins (Mantsch *et al.*, 1986). That the ratio of the areas of the absorbance peaks contributed by Amide II (1536 cm^{-1}) and Amide III (1274 cm^{-1}) remained constant over the duration of the experiment (Table 2) supports the application of Beers Law in these studies and

suggests that the Peak area of either of these absorption bands is a useful measure of protein concentration at the IRE surface. These results also suggest that the concentrations of other microbial metabolites at the IRE surface are reflected by the areas of their respective ir absorption peaks presented in Table 1.

The absorbance peak at 1055 cm^{-1} was assigned to the C-O stretch of pyranose subunits of bacterial polysaccharides (Gardella *et al.*, 1984; Geesey *et al.*, 1987). The results presented in Table 1 indicate that the concentration of polysaccharide increased at the Ge surface during the first 75 h of biofilm development and remained constant thereafter, even though protein concentration continued to increase. The highest polysaccharide/protein ratio occurred 45 h after inoculation of the surface with the bacteria (Table 2). These results suggest that, unlike protein, polysaccharide concentration at the base of the biofilm does not change during later stages of biofilm maturation.

Other products of surface-associated bacterial metabolism displayed time-dependent variations in concentration that were different from those of protein and polysaccharide. The unidentified metabolite contributing absorbance at 1187 cm^{-1}, which was observed in the bacterial inoculum, was not detected during the first 45 h of surface-associated bacterial growth but reappeared and increased in concentration thereafter.

These results demonstrate that changes in metabolic activity of surface-associated bacteria during biofilm development on surfaces exposed to a flowing bulk aqueous phase can be evaluated by ATR/FT-IR. This approach also provides information on the relative concentrations of metabolites that accumulate on the solid surface at the base of the biofilm. This information can be used to identify reactions that promote the deterioration of surfaces under microbial biofilms.

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