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Monitoring microbial adhesion and biofilm formation by attenuated total reflection/Fourier transform infrared spectroscopy

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Summary

A major problem in accurately defining bacterial adhesion mechanisms and processes occurring in biofilms on surfaces is the lack of techniques that nondestructively provide on-line information about the microorganisms, their extracellular polymers, and metabolites. The attenuated total reflectance (ATR) technique of Fourier transform infrared spectroscopy (FT-IR) is ideally suited to monitor molecular interactions at the solution/internal reflection element (IRE) interface, and we report its application to biofilm research. Two methodologies were utilized to obtain the ATR/FT-IR spectra of living *Caulobacter crescentus* cells attached to germanium crystals. Initially, spectra of attached bacteria in high purity water produced molecular details of the attachment process without spectral interferences from components of the medium. A growth medium, utilized in the second method, allowed direct examination of the infrared absorption bands associated with the actively growing microorganisms on the surface of the IRE in the spectral region of 2000 to 1200 cm^{-1}. Using the amide II band as a marker for biofilm biomass, the detection limit was determined to be approximately 5×10^5 cells $\cdot cm^{-2}$. These results proved that the ATR-FT/IR methodologies can be utilized to provide chemical information from bacteria and bacterial products located within approximately 1 μm of the surface without spectral interferences due to components of the medium.

Key words: *Caulobacter*; Biofilm; Attenuated total reflection; Infrared

Introduction

In both natural and artificial aqueous environments such as lakes, streams, oceans, or water systems, microorganisms attach to solid surfaces or surface films. Such

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organisms utilize adsorbates and/or solutes as nutrients, excrete extracellular polymers and form biofilms. These gelatinous films are dynamic entities that can dramatically change the surface, interfacial and aqueous phase chemistry. Biofilms, beneficial in many ways, are responsible for the removal of pollutants from natural waters [1], the functioning of sewage treatment plants [2], and production of vitamins and short chain fatty acids in the human body. Biofouling can have deleterious effects in medicine and industry and has been implicated in malfunction of implanted medical devices [3–5]. Biofilms reduce heat transfer efficiency in heat exchange units [6], increase the viscous drag of ships, are the source of contamination in ultrapure water systems, and facilitate corrosion of metal structures [7]. Life within the biofilm may provide a competitive advantage over a planktonic existence, since biofilm microorganisms can utilize, in addition to liquid phase nutrients, adsorbed compounds, metabolites from other attached microorganisms, and surface degradation products. Biofilm microbes also are protected against antagonistic compounds such as biocides [8] and antibiotics [9].

Investigations into the interactions between microorganisms and solid surfaces have been limited by the lack of on-line techniques which can monitor the attachment process, biofilm development and biofilm chemistry. Most biochemical and microscopic procedures involve efforts to achieve quantitative removal of surface films and subsequent off-line, destructive analysis. Attenuated total reflection/Fourier trans-

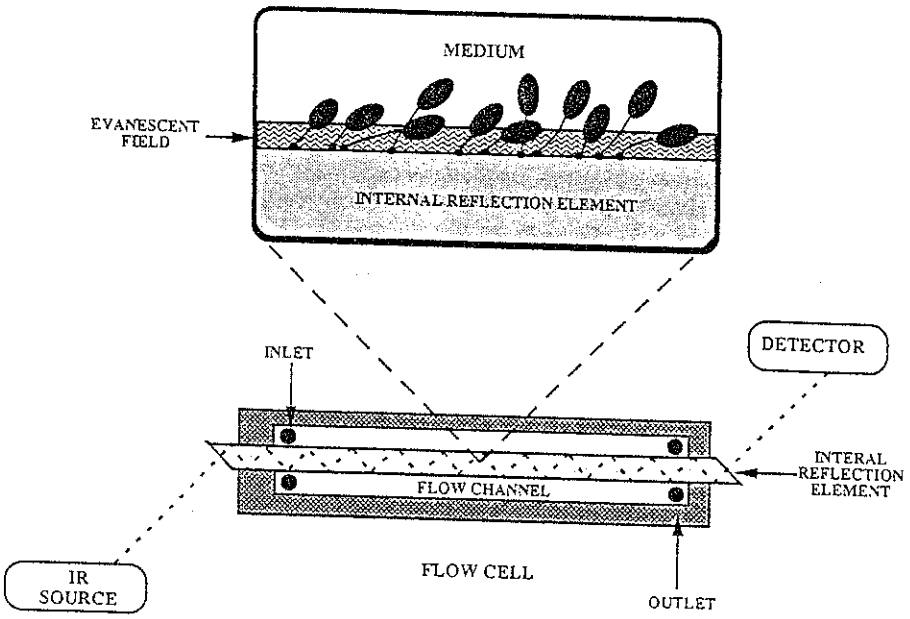

Fig. 1. Diagram showing the top view of the flow cell as well as an expanded conceptual view of the surface. The infrared radiation, represented by the dashed line, impinges on the internal reflection element (IRE) and is multiply reflected on the inside surface of the IRE creating a evanescent field on the outside of the IRE. The evanescent field can be absorbed by biofilm components which in turn produces an infrared absorbance spectrum.

form infrared spectroscopy (ATR/FT-IR) circumvents the necessity for quantitative removal by providing non-destructive analysis of biofilms. With this technique, infrared (IR) radiation is multiply reflected on the inner surface of an internal reflection element (IRE) [10]. At each reflection site a longitudinal wave of radiation penetrates from the IRE into the adjacent environment. The intensity of the radiation exponentially decays to zero within approximately one micrometer of the IRE's surface (Fig. 1). This radiation is termed an evanescent wave and can be absorbed by compounds near the surface, thus producing IR absorption bands.

ATR/FT-IR spectroscopy has been applied to study biological systems [11] and examine the adsorption of macromolecules such as proteins [12] onto surfaces of IREs or coated IREs. In microbiology, FT-IR analysis has been used to study lyophilized bacteria, aqueous suspensions of bacterial cells, bacteria/polymer mixtures and dried biofilms, and ATR has been used to monitor the attachment of organic solutes in particle-free natural seawater to clean germanium IREs [13,14]. In one of the original ATR/FT-IR biofilm studies, dried biofilms and organic conditioning layers adsorbed to germanium IREs were examined [15]. In addition, ATR/FT-IR has been used to monitor the utilization of pyruvic acid by *Escherichia coli* in fermentation medium [16] and microbially influenced corrosion of copper films coated on germanium IREs [17]. In other studies, cell suspensions of different bacterial strains were applied to IREs, and statistical differences between strains were demonstrated [18,19]. The ATR-FT/IR technique has monitored the attachment of zoospores, but these studies did not examine organisms under growth conditions [20]. Spectra of biofilms obtained using ATR/FT-IR-have been presented [21,22]. However, these studies do not detail the importance of adsorption of medium components which can interfere with ATR/FT-IR spectra.

In this study, the ATR-FT/IR technique provided non-destructive monitoring of the direct interaction between living *Caulobacter crescentus* cells, their polymers, and germanium crystals by eliminating and discerning bands due to nutrients which can adsorb and form abiotic films on the IREs. *C. crescentus* was selected as a test organism because it is found in many natural environments, can grow and attach in oligotrophic environments and has a defined attachment mechanism [23–25]. Moreover, several studies have been done characterizing surface polysaccharides of *C. crescentus*, including the surface lipopolysaccharide, exopolysaccharides and the adhesion holdfast organelle [23–27]. In the present study, the ATR/FT-IR spectra of these bacteria revealed molecular details of the attachment process and the development of biofilms in their fully hydrated state.

Materials and Methods

Bacterial strain

Caulobacter crescentus CB2A was used in these studies [28]. Stock cultures were maintained on medium containing 0.1% (wt/volume) yeast extract, 0.2% (w/v) proteose peptone, 0.8 mM $MgSO_4$, and 1.6% agar in 18 MΩ/cm water. The medium was titrated to pH 7.2.

FT/IR analysis

The ATR/FT-IR analyses were performed in a flow cell (Harrick Scientific Corp., Ossining, NY) with a parallelogram germanium IRE, 50 mm × 10 mm × 2 mm and an entrance window cut at a 45° angle, wedged between two flow channel plates and sealed with Viton o-rings. The volume of each flow channel was 0.4 ml. Aluminum foil masks were used to eliminate spectral interference from the o-rings. The flow cells were sterilized with ethylene oxide.

Single sided interferograms were collected at 4 cm^{-1} (256 scans) resolution at a retardation rate of 1.57 cm/s by a Nicolet 60SX (Madison, WI) FT-IR spectrometer equipped with a water cooled glowbar source, a Michelson interferometer, KBr beam splitter and a mercury:cadmium:telluride (MCT) detector. The spectrometer was purged with air that had CO_2 and water vapor removed. All interferograms were Fourier processed using phase correction and a Happ-Genzel apodization function [29].

Air spectra were collected prior to each experiment and served as both an assurance of temperature stability and proper mirror and flow cell alignment. The stable air spectrum was used as the system background. Water-reference spectra were obtained by pumping sterile high purity water through the ATR flow cell for 3–4 h. After stable conditions were achieved, the cell suspension (adhesion experiments) or nutrients and growing bacterial cells were pumped though the flow cell. The ratios of the Fourier processed water and biofilm spectra to the Fourier processed stable air background spectrum were converted to absorbance spectra. Since water absorbs IR radiation, water absorption bands must be removed from all medium and biofilm spectra by interactive subtraction [30]. Water vapor subtraction and baseline correction were performed when necessary.

Microbial adhesion under conditions of limited growth

Cells were grown in medium containing 0.1% (wt/v) yeast extract, 0.2% (w/v) proteose peptone, and 0.8 mM $MgSO_4$ and harvested in early log phase (0.2 absorbance units [AU] at 660 nm) by centrifugation (20 min, 8000 × *g*). The pellet was rinsed three times and suspended in sterile high purity water (Burdick and Jackson, Muskegon, MI) to 0.1 AU at 660 nm. The suspension was passed through the ATR flow cell at a flow rate of 0.5 ml/min^{-1}. After 3 h, loosely adhering bacteria were washed from the crystal surface by pumping sterile deionized water through the ATR flow cell. The IRE was then stained with 0.27 mM acridine orange and the number of attached cells was determined by an epi-fluorescence microscopic counting procedure. The experiment was performed four times.

As a control experiment, bacterial suspensions were prepared and re-centrifuged. The supernatant was filtered into a sterile reservoir, pumped through the ATR flow cell and the spectra recorded. This experiment was performed twice.

Biofilm development during growth

The medium for monitoring biofilm development was prepared using 18 $M\Omega \cdot cm$ water and contained the following ingredients: 1.0 mM KH_2PO_4, 0.28 mM glucose, 0.24 mM NH_4Cl, 0.051 mM $MgSO_4$, 0.017 mM $CaCl_2$ and 0.25% (v/v) Wolfe's mineral solution [31]. All chemicals were reagent grade. The medium was titrated to

pH 7.2. In addition, the media were sterilized by filtration (0.2 μm pore size).

All nutrients of the growth medium were analyzed by the ATR/FT-IR technique. The concentrations of the nutrients were selected based on their ability to sustain growth while minimizing spectral interferences resulting from the adsorption of IR active nutrients onto the IREs. Sterile solutions of each nutrient were prepared at different concentrations and pumped through the flow cell at a flow rate of 0.5 ml/min for 24 h to ascertain the effect of adsorption. After single component analysis was completed, the growth medium was prepared, sterilized, pumped through the ATR flow cell and the spectra recorded.

In order to confirm the composition of an abiotic film, a sterile solution of 10 mM K_2HPO_4 titrated to pH 7.2 was pumped through the ATR/FT-IR flow cell and adsorbed onto the IRE. Subsequently, solutions of 0.17 mM $CaCl_2$ or 0.1 mM $FeSO_4$ were pumped through the flow cell and the spectra obtained.

The flow system consisted of a medium reservoir, silicone tubing, a bioreactor, two pumps (Cole Parmer, Chicago, IL), the ATR flow cell and a waste reservoir (Fig. 2), and was sterilized by steam (20 psi, 120°C for 25 min). The bioreactor provided bacteria and medium to-the germanium IRE. The bioreactor consisted of an inlet drip tube, exit line, air line equipped with an air stone, and pressure-release line. The drip tube provided discontinuous flow to avoid back-contamination. The exit line kept the volume of medium in the bioreactor constant (350 ml) by connecting the exit line tube to a pump that was used to siphon medium from the bioreactor.

The bioreactor containing growth medium was inoculated with 3 ml of a 24 h *C. crescentus* culture. After a biofilm was observed on the glass surface of the bioreactor, the growth medium was pumped (Fig. 2, pump A) into and out of the bioreactor at a rate of 1.8–3.0 $ml \cdot min^{-1}$. These flow conditions resulted in 'wash-out' conditions, where the dilution rate was 0.3–0.5 h^{-1}. When the number of cells in the liquid phase

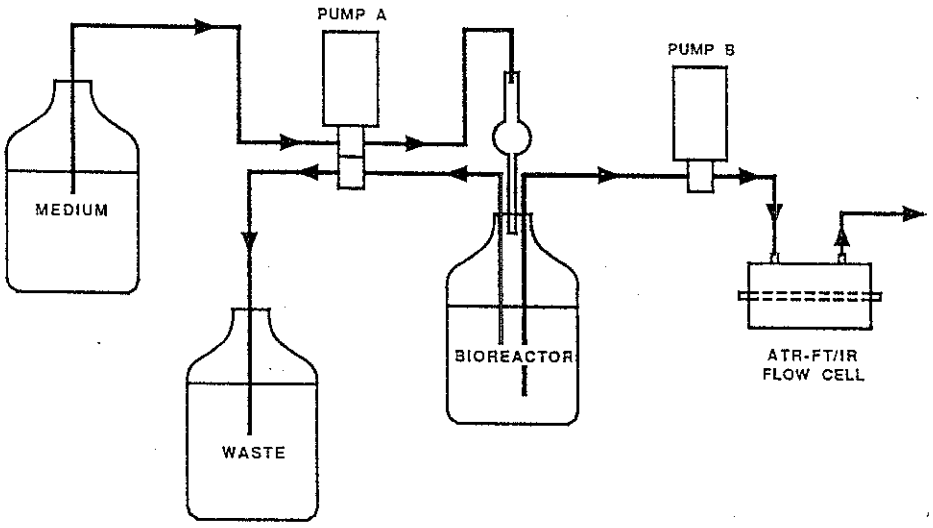

Fig. 2. Scheme presenting the flow system used in biofilm development studies. The bioreactor was used to provide growing microorganisms and nutrients to the surface of the internal reflection element.

became stable with time, the bioreactor was used to supply growing cells (fewer than 5 $\times$ 10^6 cells $\cdot$ ml^{-1}) and medium to the ATR flow cell. Once stable air and water spectra were stored, the medium and cells were pumped (Fig. 2, pump B) into the flow cell at a rate of 0.5 ml/min. and biofilm spectra were collected over time. At the end of each experiment, the number of cells per unit area was determined by the acridine orange straining and counting procedure (described below). The experiment was preformed six times using the same IRE and stopped at different absorbance values.

In order to determine the number of attached bacteria in the growth experiments, the bacteria were sonicated from the surface because the cell densities on the IRE were too large for direct microscopic enumeration. Specifically, bacteria attached to the IRE were removed by sonication for 10 s (Heat Systems-Ultrasonics Inc., Plainview, NY), transferred to a stained black filter (CoStar, Cambridge, MA, 0.2 μm pore size), and stained with 0.27 mM acridine orange solution [32]. The filter was placed on a microscope slide and the number of bacteria per unit area were counted. Twenty fields were counted for each sample. Examination of the IRE after sonication verified that the cells were quantitatively removed. The liquid phase microorganisms also were enumerated by this procedure by filtering a measured volume of liquid phase onto a black filter.

Scanning electron microscopy

Biofilm organisms attached to Ge IRE were fixed in 2.5 glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) for 15 min. The IRE was dehydrated with a

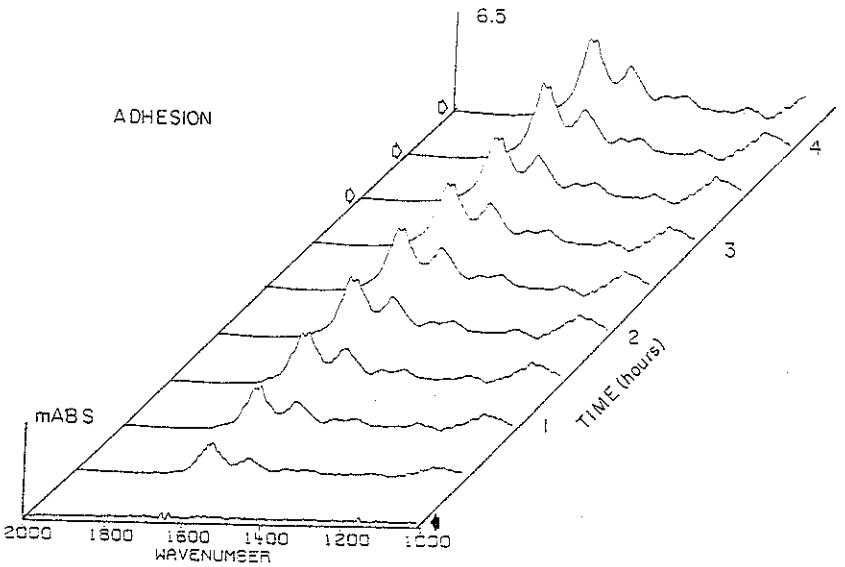

Fig. 3. Three dimensional plots of ATR/FT-IR adsorption spectra versus time monitoring the attachment of *C. crescentus* cells. The living bacteria were suspended in water and pumped through the ATR flow cell where they attached to the surface of the internal refection element. The first spectrum is a stable water background (dark arrow). The final three spectra (open arrows) were obtained by pumping water through the flow cell to wash away unattached bacteria.

graded series of acetone:water solutions, air-dried, and post-fixed with osmium tetroxide vapor. Following evaporation of 100 A of gold palladium alloy to the specimen, it was observed using an ETEC Autoscan microscope.

Results

Microbial adhesion with limited growth

Bacteria were suspended in water to obtain spectra of attached microorganisms and to avoid spectral interferences due to the growth medium. Fig. 3 presents a three dimensional plot of the IR absorption spectra at half hour intervals. The stable water background spectrum collected at the start of the experiment is indicated by the dark arrow. The next six IR adsorption spectra monitored the attachment of *C. crescentus* suspended at $1.6 \pm 0.9 \times 10^7$ cells/ml. Only bacteria interacting with the evanescent wave are detected. The final three spectra were collected during a sterile water rinse used to remove any unattached microorganisms (Fig. 3, open arrows). Adhesion of *C. crescentus* to the IRE produced IR adsorption bands at 1648, 1550, 1454, 1397, 1341,

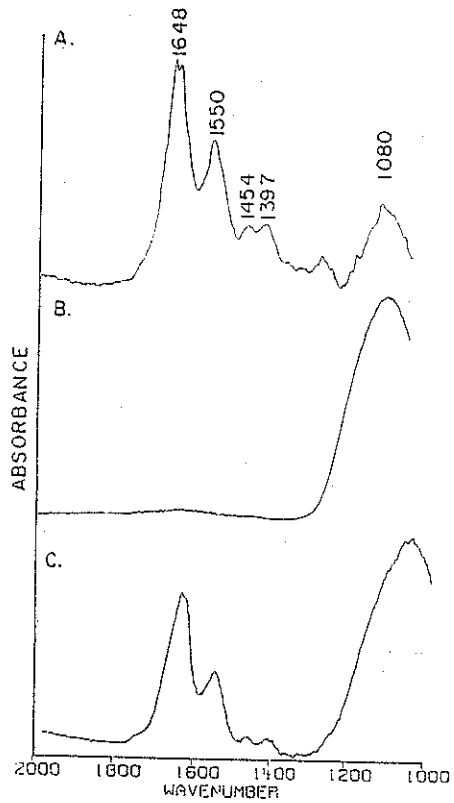

Fig. 4. ATR/FT-IR final spectra of attached *C. crescentus* cells without complication due to growth medium (suspended in water) [A], sterile growth medium [B], and a mature biofilm (developed in growth medium) [C].

1306, 1246 and 1080 cm^{-1} (Fig. 4A). The IR absorption bands at 1648, 1550, and 1306 cm^{-1} were attributed to amide I, amide II, and amide III vibrational modes respectively. The bands at 1454, 1397, 1246 and 1080 cm^{-1} were assigned to the C–H bend (scissoring), C–O stretch (symmetrical, carboxylate ion), the P=O stretch or

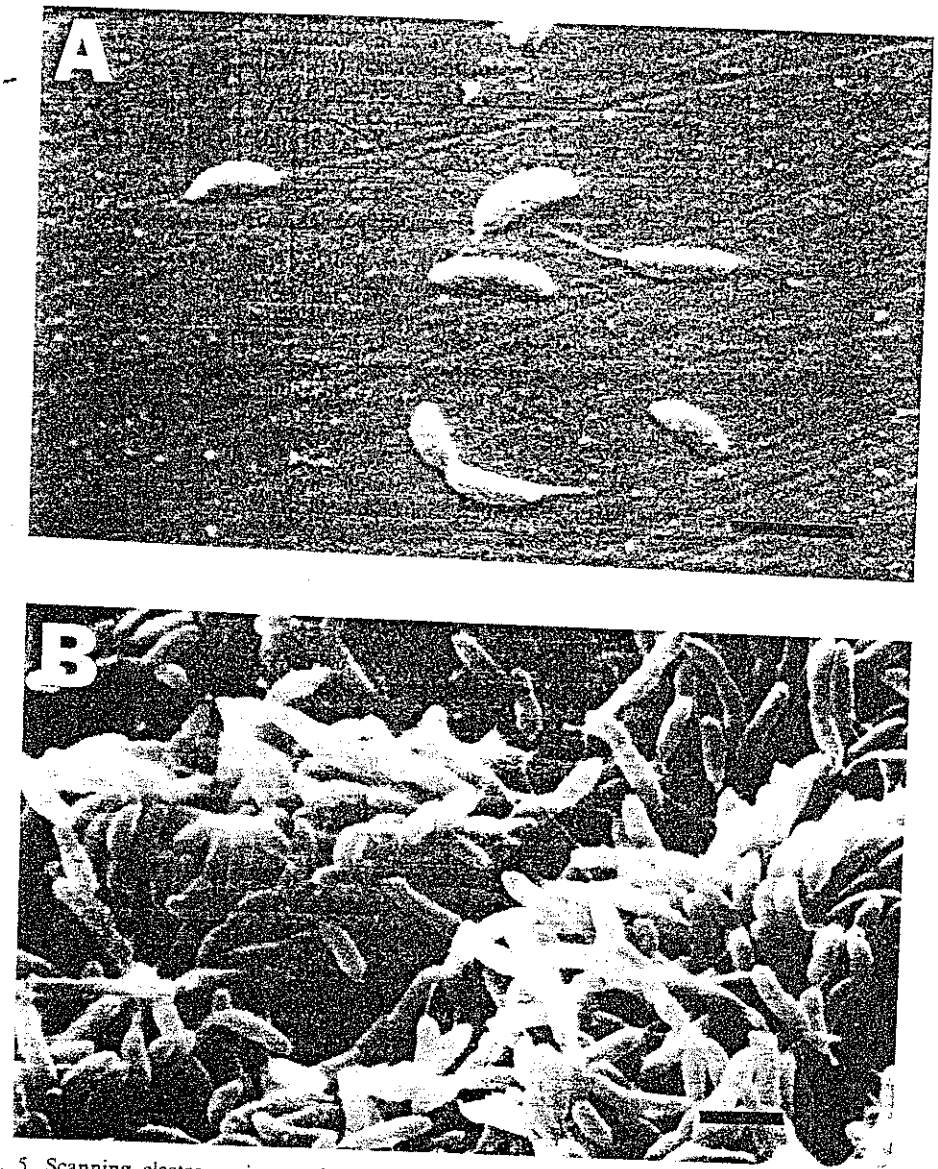

Fig. 5. Scanning electron micrographs of *C. crescentus* cells attached to a germanium crystal. (A) Micrograph showings attached bacteria from a 'bacterial adhesion' experiment. (B) Micrograph showing, mature biofilm bacteria from a 'biofilm development' experiment that produced longer stalks. The bacteria are attached to the crystal by a holdfast located at the distal end of the stalk. Bars, 2 μm.

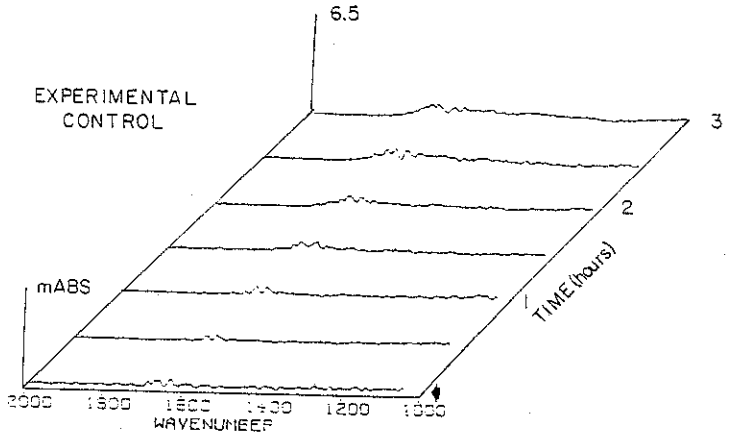

Fig. 6. A three dimensional plot of spectra versus time for a control experiment. The bacterial suspension was recentrifuged and the supernatant pumped through the ATR flow cell. The filled arrow represents the stable water background spectrum. All spectra are presented on the same scale as Fig. 3.

amide III and the C–O stretch (alcohols and carbohydrates) vibrational modes, respectively [13,14]. The intensity of all bands increased for the first 3 h. No differences were observed between water rinsed spectra and the final cell suspension spectrum demonstrating that most of the attached cells had firmly adhered. SEM examination of the IRE (Fig. 5A) showed that most of the attached *C. crescentus* cells produced stalks suggesting that the attachment was mediated by holdfast material.

Fig. 6 shows the ATR/FT-IR results of a control experiment plotting the spectra of the supernatant at 0.5 h intervals. The spectra are presented on the same scale as in Fig. 3. The filled arrow represents the stable water spectrum. The control experiments demonstrated that the IR bands detected in the cell suspension experiments (Fig. 3) resulted from attached *C. crescentus* cells and not from organic contamination caused by detached extracellular polymers, protein leakage resulting from osmotic shock, and/or water impurities.

Biofilm development experiments

Medium control experiments ascertained the effect of single medium components on the IR spectra. For example, at a glucose concentration of 1.2 mM, adsorbed glucose could be detected and produced bands at 1610, 1405, and 970 cm^{-1} interfering with the direct observation of the spectra of the initially attached bacteria (data not shown). At a glucose concentration of 0.28 mM, the amount of glucose that adsorbed onto the IRE was below the detection limit. At the concentrations utilized in the growth medium, analyses of other individual medium components did not produce IR absorption bands except 1 mM K_2HPO^4 buffered at pH 7.2. This solution produced a broad band at 1103 cm^{-1}. Despite this finding, the concentration of K_2HPO_4 was not lowered because phosphate limitation is linked to stalk elongation [33].

ATR/FT-IR analysis of the complete sterile growth medium detected an IR band not observed in the single component spectra. The band absorbed in the 1200–1000

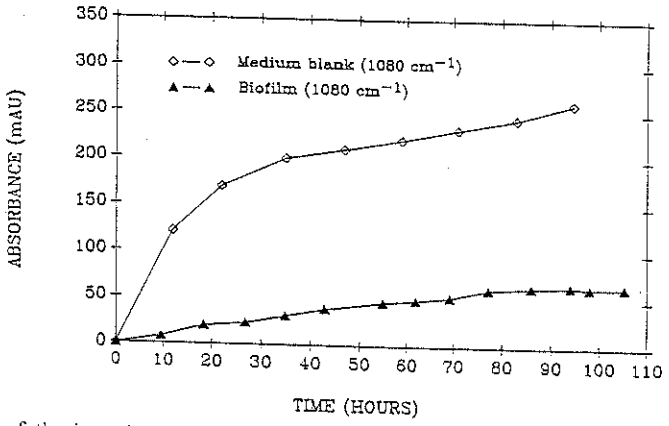

Fig. 7. A plot of the intensity of 1080 cm^{-1} band from a sterile control experiment and a biofilm experiment. The band was attributed to the P–O stretch and monitored the formation of an abiotic film. The bacteria decreased the rate of formation of this abiotic film.

cm^{-1} region of the spectrum with its maximum absorption at 1080 cm^{-1} (Fig. 4B). In addition, the band increased in intensity for more than 4 days (Fig. 7), indicative of a chemical reaction, not a simple adsorption process. The spectrum was similar to the spectrum of adsorbed K_2HPO_4, although the band in the medium spectra (1080 cm^{-1}) was more intense and shifted +23 cm^{-1} from the P–O stretch of the adsorbed phosphate (1103 cm^{-1}). Both Ca^{2+} and Fe^{2+} ions, present in the growth medium, precipitate PO_4^{3+} ions [34]. Results of the ATR/FT-IR analysis of 10 × solutions of K_2HPO_4 at pH 7.2 combined with $CaCl_2$ or $Fe(SO_4)_2$ showed that both Ca^{2+} and Fe^{2+} ions caused the maximum peak intensity of the P–O stretch of adsorbed PO_4^{3+} to shift to 1080 cm^{-1}. Thus, the 1080 cm^{-1} band resulted from the formation of a calcium-phosphate and/or iron-phosphate insoluble deposits on the surface of the IRE's. These results emphasize the importance of nutrient selection in ATR/FT-IR studies of biofilms.

Fig. 7 compares the intensity of the 1080 cm^{-1} band of a sterile medium control experiment and the intensity of the 1080 cm^{-1} band of a biofilm experiment. The results demonstrate that the addition of bacteria to the medium decreased the intensity of the 1080 cm^{-1} band. A plausible explanation of this result is that the bacteria can utilize the reactants as nutrients, thus reducing their interfacial concentration which in turn decreased the amount of the abiotic film being formed at a given time. Therefore, a simple subtraction of the sterile medium spectra from biofilm spectra at each time point would produce errant results.

The spectral window from 2000 to 1200 cm^{-1} was used to examine biological contribution to the biofilm spectrum, since no medium components absorbed radiation in this region. Fig. 8 plots the intensity of the 1648 (Amide I), 1550 (Amide II), 1454 (CH_2 scissor), 1397 (C–O stretch, carboxylate ion), and 1080 cm^{-1} (abiotic phosphate) bands versus time. An increase in intensity of all the bands was seen for approximately 70 h, followed by a static period where the 1648, 1550, 1454, and 1397 cm^{-1} bands leveled off. At 80 h, sterile medium was pumped through the flow cell causing a second increase in intensity of these bands probably resulting from

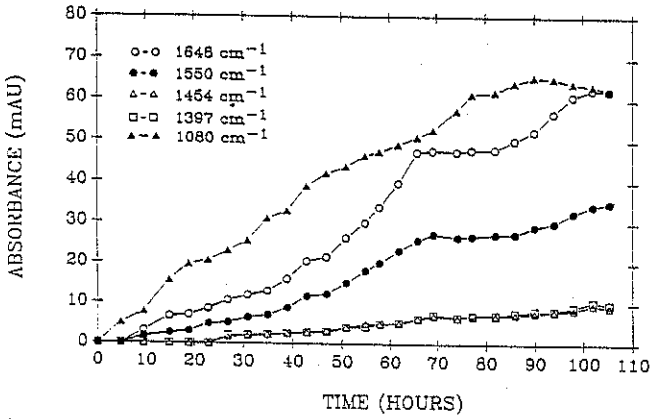

Fig. 8. Plot of the intensities of the infrared absorption bands from spectra of biofilms developing in growth medium: C–O and P–O stretch region at 1080 cm^{-1}, amide I band at 1648 cm^{-1}, amide II band at 1550 cm^{-1}, CH_2 scissoring band at 1454 cm^{-1}, C–O stretch carboxylate ion at 1397 cm^{-1}. At 85 h, the bioreactor was disconnected and sterile medium pumped into the flow cell to stimulate growth.

increased nutrient flux. At the end of this experiment, the cell density in the biofilm was determined to be 6.5×10^7 cells · cm^2. The number of bacteria in the liquid phase was monitored daily and determined to be less than 5×10^6 cells · ml^{-1}.

Similar experiments were performed for which the spectra were monitored with respect to time and the number of biofilm cells determined at the end of the experiment. A plot of amide II absorbance versus the number of biofilm bacteria at the end of each experiment is presented in Fig. 9. Amide II (N–H bending in proteins) was selected as a biomarker to monitor biofilm biomass since most bacteria are roughly 50% protein, and protein assays are commonly used to estimate biomass. The more intense amide I band was not utilized as a biomarker because it is more sensitive to water subtraction errors than the amide II band. The plot contains two

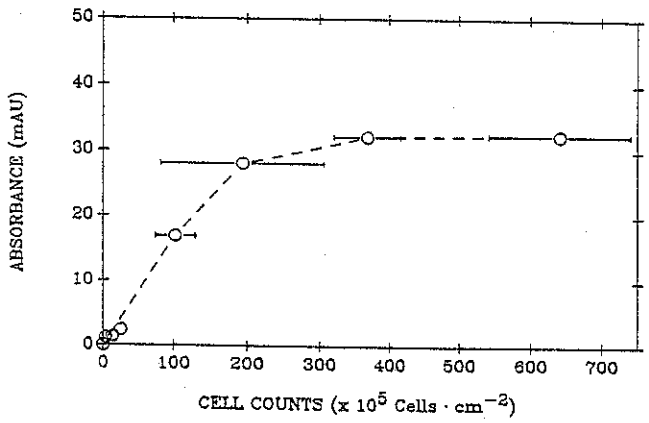

Fig. 9. Plot of amide II band versus the number of cells on the surface of the IRE at the end of each experiment.

regions of interest. In the region between the origin and 2×10^7 cells $\cdot$ cm^{-2}, the amide II band increased monotonically with the number of biofilm bacteria. In order to obtain an estimate of the detection limit, this region was assumed to be linear ($r^2 = 0.8933$). The detection limit was calculated by multiplying the sensitivity (slope of a calibration curve) by an absorbance value that is three times the root mean square of the background noise and was approximately 5×10^5 cells $\cdot$ cm^{-2}. In the region between 2×10^7 and 7×10^7 cells $\cdot$ cm^{-2}, the amide II band leveled off with increasing number of biofilm microorganisms.

Discussion

When interpreting ATR/FT-IR data from a biofilm study, it is important to understand how the intensity of the evanescent wave varies as a function of distance. The depth of penetration of the evanescent wave is defined as the distance from the surface where the intensity of the radiation decreases to e^{-1} (approximately 37%) of its intensity at the surface of the IRE, and can be determined by the following equation:

$$D_p = \lambda/2\pi n_1 (\sin^2\theta - (n_2/n_1)^2)^{1/2}$$

where λ is the wavelength of the radiation, n_1 is the refractive index of the IRE, n_2 is the refractive index of the medium in contact with the IRE, and θ is the angle of incidence [10]. For water ($n = 1.33$) in contact with a germanium crystal ($n = 4.0$) cut at a 45° angle and for the amide II band (1550 cm^{-1}), the depth of penetration is calculated to be 0.5 μm. Therefore, a majority of the IR absorptivity in each spectrum is obtained from microbial material located within 0.5 μm of the surface and only material within approximately 1 μm will be detected. As estimated from SEM results, an average *C. crescentus* cell has a width of 0.5 μm, a cell body length of 2 μm and a nonrigid stalk which ranges from 1 to 3 μm in length. Therefore, biofilm thickness, and cell orientation within a biofilm should be considered when interpreting data from ATR/FT-IR studies.

C. crescentus cells are prosthecate (stalked) microorganisms that have a biphasic life cycle. In the swarmer phase, the bacteria are flagellated and ideally swim until they contact a surface for attachment. Attachment is mediated by adhesive material called a holdfast. During the attached phase, a stalk forms with the holdfast localized at the distal end of the stalk [23,24].

C. crescentus cells do not attach to other *C. crescentus* cells [23] nor form colonies that protrude out of the evanescent field. Thus, it was initially hypothesized that the intensity of the bacterial IR absorption bands would increase as the number of cells interacting with the evanescent field increased. However, at a cell density greater than 2×10^7 cells $\cdot$ cm^{-2}, the amide II band leveled off suggesting that some bacteria or some portion of the attached microbes were not being detected or alternatively the amount of protein per cell has decreased.

As the surface cell density increases, bacterial growth may become limited by the nutrient flux to individual cells within the biofilm. Cell shrinkage, and subsequently less protein per cell, is a common response to nutrient limitation [35]. However, in *C.*

crescentus, limited nutrient availability promotes stalk development [33]. Therefore, at surface densities greater than 2×10^7 cells $\cdot$ cm^{-2}, the stalk of some cells may elongate extending the cell body toward the bulk phase which in turn would increase the flux of nutrients to the cell, but move the cell body away from the most intense portion of the evanescent field. In this case the intensity of the amide II band would not be proportional to the number of cells attached to the surface. SEM micrographs supported this hypothesis by demonstrating that elongated stalks were associated with mature *C. crescentus* biofilms (Fig. 5B).

The IR absorption bands in the biofilm spectra result mostly from classes of macromolecules produced by all bacteria. Although specific macromolecules within a class differ widely in their function, their IR spectra produce a similar overall appearance. For example, absorption from amide linkages in all proteins produced the amide I (1690–1640 cm^{-1}), amide II (1570–1515 cm^{-1}) and amide III (1300–1200 cm^{-1}) band. Amide I absorption bands are caused by carbonyl stretching modes, while amide II and amide III bands result from the interaction between a N–H bending (trans to the carbonyl group) and C–N stretching modes of secondary amides. Free carboxylate ions (1397 cm^{-1} band) are probably associated with amino acids, proteins and acidic polysaccharides. Carbohydrates and alcohols found in the nucleic acids, the cell envelope and/or extracellular polymers contributed to the bands in the C–O stretch region (1300–1000 cm^{-1}). Phosphorus, usually in the form of organic phosphate, constitutes approximately 3% of the composition of a microbial cell. The P=O stretch absorbs IR radiation near 1250 cm^{-1} and P–O absorbs radiation at 1100–1000 cm^{-1} which overlaps the C–O region.

Spectral details such as differences in relative absorbance and/or in peak position may indicate changes in amount of a specific macromolecule. For instance, in protein studies, small differences in the amide I and amide III bands have been used to distinguish α-helix structures from β-sheet conformations in proteins [36]. Ultimately, IR spectroscopy combined with resolution enhancement techniques may prove useful as a microbial species identification tool [19].

The ATR/FT-IR spectra of *C. crescentus* biofilms revealed small differences in relative intensities between biofilms obtained from cells attached from high purity water (Fig. 4A) and those from the growth medium (Fig. 4C). These differences may be related to the stalk and holdfast organelle. There were indications that during biofilm formation most of the attached *C. crescentus* cells had matured to stalked form and stalk elongation occurred. The stalk is simply an extension of the cell envelope containing similar outer membranes, peptidoglycan, cytoplasmic membrane and internal lipid membrane [25]. Since the most intense portion of the evanescent field is at the surface of the IRE, the differences noted may be due to an increased proportion of holdfast material in the evanescent field for the cells attached from growth medium. Thus, the decreased intensity of amide III, the carboxylate ion and CH_2 wag bands (relative to the amide II band) may reveal clues to the chemical nature of the holdfast and stalk. The exact composition of the holdfast is unknown. This technique may be useful for comparing holdfast organelles from different *Caulobacter* strains.

These results highlight the technique's ability to monitor surface recruitment phenomena of microorganisms and subsequent biofilm events. The described meth-

ods minimized or eliminated media effects which can complicate the IR spectra. This work also provided evidence that only portions of the biofilms near the surface are detected. Therefore, the ATR/FT-IR technique is an excellent tool to monitor the formation of biofilms and probe the surface contact layer of biofilms. In biofilm research, the surface contact layer is important because this layer secures the biofilm to the substratum. In general, any attempt to inactivate the viability of and/or remove the biofilm must involve treatment of bacteria living within the surface contact layer. Future work will utilize the ATR/FT-IR technique to study the physiological responses of microorganisms within this region to liquid phase perturbations.

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Monitoring microbial adhesion and biofilm formation by attenuated total reflection/Fourier transform infrared spectroscopy

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Summary

A major problem in accurately defining bacterial adhesion mechanisms and processes occurring in biofilms on surfaces is the lack of techniques that nondestructively provide on-line information about the microorganisms, their extracellular polymers, and metabolites. The attenuated total reflectance (ATR) technique of Fourier transform infrared spectroscopy (FT-IR) is ideally suited to monitor molecular interactions at the solution/internal reflection element (IRE) interface, and we report its application to biofilm research. Two methodologies were utilized to obtain the ATR/FT-IR spectra of living *Caulobacter crescentus* cells attached to germanium crystals. Initially, spectra of attached bacteria in high purity water produced molecular details of the attachment process without spectral interferences from components of the medium. A growth medium, utilized in the second method, allowed direct examination of the infrared absorption bands associated with the actively growing microorganisms on the surface of the IRE in the spectral region of 2000 to 1200 cm^{-1}. Using the amide II band as a marker for biofilm biomass, the detection limit was determined to be approximately 5×10^5 cells $\cdot cm^{-2}$. These results proved that the ATR-FT/IR methodologies can be utilized to provide chemical information from bacteria and bacterial products located within approximately 1 μm of the surface without spectral interferences due to components of the medium.

Key words: *Caulobacter*; Biofilm; Attenuated total reflection; Infrared

Introduction

In both natural and artificial aqueous environments such as lakes, streams, oceans, or water systems, microorganisms attach to solid surfaces or surface films. Such

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organisms utilize adsorbates and/or solutes as nutrients, excrete extracellular polymers and form biofilms. These gelatinous films are dynamic entities that can dramatically change the surface, interfacial and aqueous phase chemistry. Biofilms, beneficial in many ways, are responsible for the removal of pollutants from natural waters [1], the functioning of sewage treatment plants [2], and production of vitamins and short chain fatty acids in the human body. Biofouling can have deleterious effects in medicine and industry and has been implicated in malfunction of implanted medical devices [3–5]. Biofilms reduce heat transfer efficiency in heat exchange units [6], increase the viscous drag of ships, are the source of contamination in ultrapure water systems, and facilitate corrosion of metal structures [7]. Life within the biofilm may provide a competitive advantage over a planktonic existence, since biofilm microorganisms can utilize, in addition to liquid phase nutrients, adsorbed compounds, metabolites from other attached microorganisms, and surface degradation products. Biofilm microbes also are protected against antagonistic compounds such as biocides [8] and antibiotics [9].

Investigations into the interactions between microorganisms and solid surfaces have been limited by the lack of on-line techniques which can monitor the attachment process, biofilm development and biofilm chemistry. Most biochemical and microscopic procedures involve efforts to achieve quantitative removal of surface films and subsequent off-line, destructive analysis. Attenuated total reflection/Fourier trans-

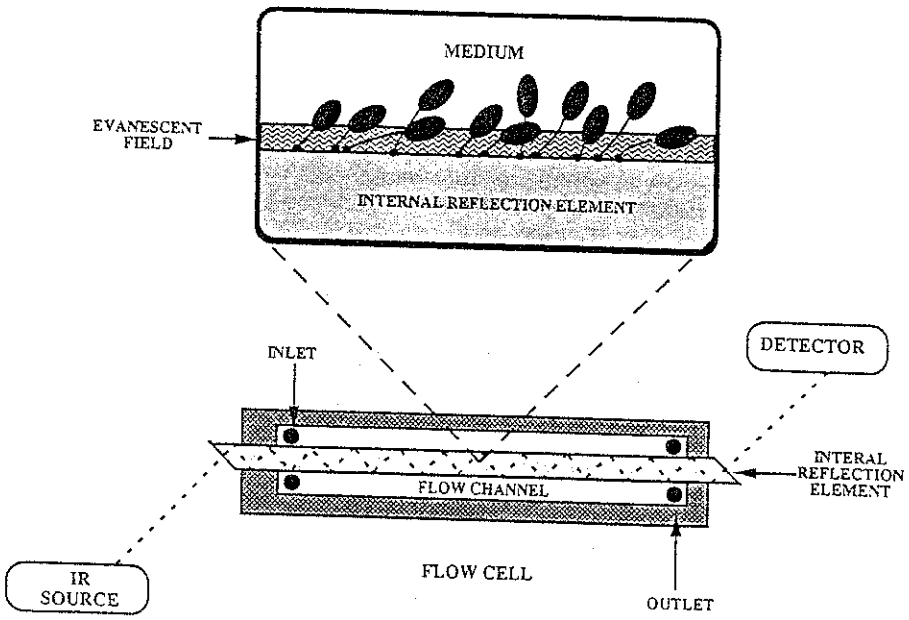

Fig. 1. Diagram showing the top view of the flow cell as well as an expanded conceptual view of the surface. The infrared radiation, represented by the dashed line, impinges on the internal reflection element (IRE) and is multiply reflected on the inside surface of the IRE creating a evanescent field on the outside of the IRE. The evanescent field can be absorbed by biofilm components which in turn produces an infrared absorbance spectrum.