Role of Alginate and Its O Acetylation in Formation of *Pseudomonas aeruginosa* Microcolonies and Biofilms

DAVID E. NIVENS,¹ DENNIS E. OHMAN,^{2,3} JESSICA WILLIAMS,^{4,5} AND MICHAEL J. FRANKLIN^{4,5*}

Center for Environmental Biotechnology, University of Tennessee, Knoxville, Tennessee, 37996¹; Department of Microbiology and Immunology, Medical College of Virginia at Virginia Commonwealth University, Richmond, Virginia 23298²; McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249³; and Department of Microbiology⁴ and Center for Biofilm Engineering,⁵ Montana State University, Bozeman, Montana 59717

Received 16 June 2000/Accepted 26 October 2000

Attenuated total reflection/Fourier transform-infrared spectrometry (ATR/FT-IR) and scanning confocal laser microscopy (SCLM) were used to study the role of alginate and alginate structure in the attachment and growth of *Pseudomonas aeruginosa* on surfaces. Developing biofilms of the mucoid (alginate-producing) cystic fibrosis pulmonary isolate FRD1, as well as mucoid and nonmucoid mutant strains, were monitored by ATR/FT-IR for 44 and 88 h as IR absorbance bands in the region of 2,000 to 1,000 cm^{-1} . All strains produced biofilms that absorbed IR radiation near 1,650 cm^{-1} (amide I), 1,550 cm^{-1} (amide II), 1,240 cm^{-1} (P=O stretching, C—O—C stretching, and/or amide III vibrations), 1,100 to 1,000 cm^{-1} (C—OH and P—O stretching) 1,450 cm^{-1} , and 1,400 cm^{-1} . The FRD1 biofilms produced spectra with an increase in relative absorbance at 1,060 cm^{-1} (C—OH stretching of alginate) and 1,250 cm^{-1} (C—O stretching of the O-acetyl group in alginate), as compared to biofilms of nonmucoid mutant strains. Dehydration of an 88-h FRD1 biofilm revealed other IR bands that were also found in the spectrum of purified FRD1 alginate. These results provide evidence that alginate was present within the FRD1 biofilms and at greater relative concentrations at depths exceeding 1 μm , the analysis range for the ATR/FT-IR technique. After 88 h, biofilms of the nonmucoid strains produced amide II absorbances that were six to eight times as intense as those of the mucoid FRD1 parent strain. However, the cell densities in biofilms were similar, suggesting that FRD1 formed biofilms with most cells at depths that exceeded the analysis range of the ATR/FT-IR technique. SCLM analysis confirmed this result, demonstrating that nonmucoid strains formed densely packed biofilms that were generally less than 6 μm in depth. In contrast, FRD1 produced microcolonies that were approximately 40 μm in depth. An *algJ* mutant strain that produced alginate lacking O-acetyl groups gave an amide II signal approximately fivefold weaker than that of FRD1 and produced small microcolonies. After 44 h, the *algJ* mutant switched to the nonmucoid phenotype and formed uniform biofilms, similar to biofilms produced by the nonmucoid strains. These results demonstrate that alginate, although not required for *P. aeruginosa* biofilm development, plays a role in the biofilm structure and may act as intercellular material, required for formation of thicker three-dimensional biofilms. The results also demonstrate the importance of alginate O acetylation in *P. aeruginosa* biofilm architecture.

Many species of bacteria produce extracellular polymers that may facilitate nonspecific adhesion to surfaces and provide the framework for biofilms (7). Alginate is an extracellular polysaccharide produced by a variety of gram-negative bacteria including *Azotobacter vinelandii*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* (11, 19, 22, 23, 44). In chronic pulmonary infections of cystic fibrosis (CF) patients, alginate acts as a virulence factor by encapsulating the *P. aeruginosa* cells. Alginate provides the bacteria with selective advantages for colonization of the pulmonary tissue, through increased resistance to opsonization and phagocytic engulfment (1, 48, 50) as well as through increased protection from toxic oxygen radicals (31, 51). Alginate likely does not play a role in the specific adhesion of *P. aeruginosa* to pulmonary tissue. However, it may play a role in formation of the bacterial microcolonies that have been observed in vivo (29).

CF patients are initially colonized with nonmucoid strains of *P. aeruginosa* that produce little or no alginate. However, over

time the majority of isolates from chronic pulmonary infections display a mucoid phenotype, indicative of the hyperproduction of alginate (24, 42). The mechanism for the overexpression of alginate is complex and requires several regulatory proteins that act in a hierarchical regulatory cascade (55). The top of the regulatory hierarchy is mediated by an alternative sigma factor, σ^{22} , encoded by *algT* (also designated *algU*), located at 68 min on the *P. aeruginosa* chromosome (10, 13, 28, 34). *P. aeruginosa* isolates from sources other than pulmonary tissues usually display the nonmucoid phenotype. The nonmucoid phenotype of these isolates is due to control of σ^{22} by the anti-sigma factor MucA or MucB (21, 33, 35, 56). The genes for these negative regulators lie on the same operon as *algT(U)*. Pulmonary isolates of *P. aeruginosa* from CF patients often have mutations in *mucA* (33). Mutations in this negative regulator of σ^{22} result in increased expression of *algT*, by an autoregulatory mechanism (10), and increased expression of the alginate biosynthetic operon (21, 47), which is controlled by the *algD* promoter (9). Therefore, mutations in *mucA* result in hyperproduction of *P. aeruginosa* alginate in CF pulmonary isolates. Upon growth of mucoid CF isolates on laboratory medium, the strains rapidly switch to the nonmucoid pheno-

* Corresponding author. Mailing address: Department of Microbiology, Montana State University, Bozeman, MT 59717. Phone: (406) 994-2420. Fax: (406) 994-4926. E-mail: umbfm@montana.edu.

FIG. 1. Use of ATR/FT-IR for biofilm analysis. An IR beam reflects within an IR-transparent substance (germanium) termed an IRE. The reflection generates a field of radiation (evanescent field) in the medium outside of the IRE. The intensity of the evanescent field decays exponentially to zero within approximately $1\ \mu\text{m}$ of the IRE. Molecules of cellular biomass or extracellular polymers within the evanescent field absorb the IR radiation, thereby producing IR absorption spectra. Molecules outside of the evanescent field are not detected. Experimentally, opposing sides of the IRE are sealed with two stainless steel plates with O rings to create flow channels.

type. This switching is often the result of suppressor mutations at the *algT* locus (10, 46).

The structure of alginate from CF isolates is a linear polymer of D-mannuronic acid (M) and its C5 epimer, guluronic acid (G), linked by β 1-4 glycosidic bonds (11, 18). *P. aeruginosa* alginates are found not as repeating disaccharides but as random blocks of MM residues and MG residues (25, 26). The alginate produced by CF isolates, including *P. aeruginosa* FRD1, is O acetylated at the C-2 and/or the C-3 positions of the mannuronic acid residues (8, 16, 52). Most of the biosynthetic genes for alginate are located in an operon at 34 min on the *P. aeruginosa* chromosome (4, 5). The *algD* gene, which encodes GDP-mannose dehydrogenase, is the first gene in the biosynthetic operon (9). A Tn501 transposon insertion in *algD* resulted in the nonmucoid phenotype, due to the lack of GDP-mannose dehydrogenase and to the polar effect of the transposon insertion on the downstream alginate biosynthetic genes (4, 45). Genes for the structural modification of alginate also lie on the alginate biosynthetic operon. The products of *algI*, *algJ*, and *algF* are required for the addition of O-acetyl groups to the alginate polymer, and mutations in *algI*, *algJ*, or *algF* resulted in production of an alginate polymer that was not O acetylated (16, 17, 49). Since O acetylation affects the physical properties of alginate, including viscosity, interaction with calcium ions, and the reaction with the mannuronan epimerase and mannuronan lyase (15, 45, 53), alginate O acetylation may affect the ability of *P. aeruginosa* to form biofilms in vivo.

To provide chemical and structural information on living bacterial biofilms, nondestructive analytical and microscopic methodologies have been developed (40). Attenuated total

reflection/Fourier transform-infrared spectroscopy (ATR/FT-IR) is a technique that has provided information about microorganisms and extracellular polymers at solid-liquid interfaces (39, 41). With the ATR/FT-IR technique, an IR spectrometer provides IR radiation that is reflected within an IR-transparent substance (e.g., germanium) termed an internal reflection element (IRE) (Fig. 1). Under certain conditions in which the outside medium has a lower refractive index than the IRE, the reflection generates a field of radiation in the medium outside of the IRE, termed an evanescent field. The intensity of this external evanescent field decays exponentially to zero within approximately $1\ \mu\text{m}$ of the IRE. Molecules of cellular biomass or extracellular polymers within the evanescent field absorb the IR radiation, thereby producing an IR absorbance spectrum. Molecules outside of the evanescent field are not detected (Fig. 1). Therefore, ATR/FT-IR can provide unique spectral information on the innermost portion of biofilms near the liquid-substratum interface without disturbance of the biofilm.

Another technique used to study living biofilm is scanning confocal laser microscopy (SCLM) (30). SCLM allows optical sectioning of biofilms and three-dimensional reconstruction of the SCLM images. The SCLM technique reduces the out-of-focus haze that is produced in light microscopy, resulting from the depth of the focal plain through the biofilm.

In this study, the role of alginate in biofilm development was investigated by on-line examination of *P. aeruginosa* colonization of surfaces, using ATR/FT-IR and SCLM. Two mucoid and three nonmucoid *P. aeruginosa* strains were examined here, including the mucoid CF pulmonary isolate FRD1 (43),

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype ^a	Source/reference
<i>E. coli</i> HB101	<i>proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20</i>	This laboratory
<i>P. aeruginosa</i>		
FRD1	Cystic fibrosis isolate, <i>mucA22</i> (Alg ⁺)	43
FRD2	<i>mucA22 algT18</i> (Alg ⁻)	14
FRD440	<i>mucA22 hisI algT::Tn501</i> (Alg ⁻)	14
FRD1131	<i>mucA22 algD::Tn501-33</i> (Alg ⁻)	45
FRD1153	<i>mucA22 algJ5</i> (Alg ⁺ , lacks O acetylation)	17
Plasmids		
pRK2013	ColE1-Tra(RK2) ⁺ Km ^r	12
pMF36	<i>P_{trc}</i> -expression vector pKK233-2 with <i>oriV_{SF} oriT Ap^r</i>	15
pMF230	pMF36 with <i>mut2</i> GFP	This study

^a Abbreviations for phenotypes: Alg⁺, alginate overproduction; Alg⁻, nonmucoid; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; and Tra⁺, transfer by conjugation.

which has the *mucA22* allele and overproduces O-acetylated alginate. The nonmucoid strains examined were derivatives of FRD1 and included an *algT18* spontaneous nonmucoid mutant; FRD440, an *algT* Tn501 mutant; and FRD1131, an *algD* Tn501 mutant. To determine the role of alginate O acetylation in biofilm formation, a mucoid O-acetylation-deficient *algJ* mutant was analyzed by ATR/FT-IR and SCLM.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* and *P. aeruginosa* were maintained on L agar (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of Bacto Agar per liter). A 1:1 mixture of *Pseudomonas* isolation agar (Difco) and L agar was used to select for *P. aeruginosa* following matings with *E. coli*. Antibiotics were used at the following concentrations (per milliliter): ampicillin at 100 µg and carbenicillin at 300 µg. The medium for the flow system (1× biofilm medium) contained per liter: 0.09 mM sodium glutamate, 0.5 mM glycerol, 0.02 mM MgSO₄, 0.15 mM NaH₂PO₄, 0.34 mM K₂HPO₄, and 145 mM NaCl. The pH of the medium was adjusted to 7.0. The medium for *P. aeruginosa* FRD440 also contained histidine at a concentration of 0.02 mM. When bacteria containing plasmid pMF230 were analyzed, 50 µg of carbenicillin per ml was added to the 1× biofilm medium.

DNA manipulations. General DNA manipulations were performed as described previously (32). Restriction endonucleases were purchased from Boehringer Mannheim. Triparental matings were used to mobilize plasmids from *E. coli* to *P. aeruginosa*, using the conjugative helper plasmid pRK2013 (12). Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer.

To visualize bacteria with SCLM, a plasmid that constitutively expressed the green fluorescent protein (GFP) was introduced into each of the *P. aeruginosa* strains. The gene for the GFP containing the *mut2* mutation (6) was amplified by PCR from plasmid pBCgfp (54). The PCR primers used in the amplification were as follows: GFPSal3, 5'-GCGCGTCGACAGGAGAAGAAAAATGAGTAAAGGAGAAGA-3'; and GFPHind4, 5'-GTACCTGGAATTCACGAAGCTTATTGTATAGTTTCATCC-3'. The primers were designed to introduce a *P. aeruginosa* ribosomal binding site upstream of *gfp*. The PCR product was digested with *SalI* and *HindIII* and ligated into pUC19. The *XbaI* and *HindIII* fragment from pUC19, containing the GFP *mut2* mutation, was ligated into vector pMF36 (16) behind the *trc* promoter, forming plasmid pMF230. pMF230 contained an *oriT* site and the stable replication fragment and therefore could be mobilized into *P. aeruginosa* by triparental mating, where it was maintained. Since pMF230 does not contain the *lacI* repressor, *gfp* was expressed constitutively.

Flow systems. The flow system for the ATR/FT-IR experiments had three separate flow cells and was designed for use with a multichannel IR spectrometer (41). Specifically, each flow system contained a medium reservoir(s); three pumps, each with two pump heads; silicon tubing including inoculation tubes; three flow cells; and three waste reservoirs. Each flow cell (Harrick Scientific

Corp., Ossining, N.Y.) consisted of two flow plates and a germanium (Ge) IRE that was 50 mm by 10 mm by 2 mm, with the entrance and exit windows cut at 45° angles. Each flow plate contained an O-ring groove with a Viton O ring and a flow-channel groove with an inlet and an outlet (Fig. 1). A flow cell was assembled by mounting flow plates on each side of the IRE, creating sealed flow channels on each side of the IRE. Titanium masks were inserted between the O ring and the IRE, thereby minimizing the O-ring interaction with the evanescent field. The volume of each flow channel was approximately 0.4 ml. The flow cells were assembled, leak tested, and sterilized with ethylene oxide gas. The remainder of the flow system was sterilized by autoclaving.

The flow systems for the SCLM experiments consisted of a medium reservoir, a pump, silicon tubing, a flow cell, and a waste reservoir. The flow cell contained a polycarbonate support with inlet and outlet ports. A glass coverslip was sealed onto the polycarbonate support with a Viton gasket. The biofilms that formed on the glass coverslip were examined by SCLM. Both the ATR/FT-IR and SCLM flow cells were maintained at 37(+1)°C during biofilm formation.

Inoculation and endpoint analyses. Prior to injection, each strain was incubated in multiple flasks containing 10× biofilm medium for 16 to 20 h at 37°C. When the cell density reached approximately 10⁷ cells/ml, as determined by absorption/scattering (optical density) at 610 nm and previously determined growth curves, the cultures were diluted with an appropriate amount of sterile 0.85% NaCl solution to make a 10⁶-cells/ml solution. Prior to inoculation, sterile water was pumped through each flow channel to obtain background spectra. After the background spectra stabilized, sterile 1× medium was pumped through the system to condition the IRE. Injection tubing connected to the outlet tubing of each flow cell was filled with 5 ml of an inoculum. The flow cells were inoculated by reversing the direction of flow, to pump the inoculation medium into each flow channel. Once inoculated, the pumps were stopped for 20 min to allow cell attachment. Following inoculation, sterile 1× biofilm medium was pumped into the flow cells at a rate of 1.2 ml/min for 44, 64, or 88 h.

Plating on L agar was used to determine the number of cells on the surfaces at the end of 44-h and certain 88-h experiments. Crystals with attached biofilms were placed in glass dishes, and biofilms were removed with a Teflon policeman. Each solution of the cells was added to a test tube with glass beads, vortexed for 5 min, and plated on L agar. Mucoid colonies were differentiated from nonmucoid colonies by visual inspection.

At the end of a few 88-h experiments, the IRE was carefully removed from the flow cell and air dried in a desiccator. Once dried, the IRE with the attached biofilm was placed into a sample holder and the sample spectrum was determined. The IRE was immediately cleaned and analyzed to collect a background spectrum.

Spectrometer and data processing. The ATR/FT-IR experiments were performed with a customized Analect FT/IR (Orbital Analect, Pomona, Calif.) spectrometer specifically designed for biofilm monitoring (41). This instrument was composed of three optical channels and used two carousels of mirrors to transfer the IR light to and from each flow cell. The instrument had a mercury-cadmium-telluride detector for increased sensitivity and a refractively scanning interferometer for temperature stability. All raw data were collected at a resolution of 4 cm⁻¹. Each stored interferogram, an average of 256 scans, was Fourier processed with a Mertz apodization function (27). The resultant sample and water reference single-beam spectra were divided by a system background, single-beam spectrum, producing transmission spectra that were then converted to absorption spectra. Water absorbance bands were then interactively subtracted from each spectrum to produce a biofilm spectrum (20). In most cases, the final absorbance spectra were baseline corrected.

SCLM. Confocal images were collected using a Leica TCS-NT confocal scanning laser microscope equipped with an argon 488-nm laser, 500-nm dichroic beam splitter, and 525/50-nm filter block. This configuration provided optimal signal analysis from the GFP *mut2* mutation. An ×10 or ×40 oil immersion lens was used to collect 32 to 64 stacks of images, depending on the thickness of the biofilm. The images were taken with 1,024 by 1,024 bit resolution in the *x* and *y* plane. Three-dimensional reconstruction of the images was performed using a maximum projection of the stack.

RESULTS

Spectral analysis of biofilms formed by mucoid, nonmucoid, and O-acetylation-deficient strains of *P. aeruginosa*. ATR/FT-IR was used to generate IR spectra from the portion of *P. aeruginosa* biofilms developing within the approximately 1 µm of germanium surfaces. Figure 2 shows three-dimensional dia-

A. Alg⁺ O-Ac⁺ **B. Alg⁻** **C. Alg⁺ O-Ac⁻**

FIG. 2. Interfacial IR absorption spectra of attachment/growth of the mucoid CF isolate *P. aeruginosa* FRD1 and *alg* mutant strains plotted at 4-h intervals. Note scale changes to enhance the spectral features (panel B is fivefold greater than panels A and C). In all experiments, an inoculum of 10^6 cells/ml was introduced into the flow system at time zero. After 20 min, the sterile medium was pumped through the flow cell to stimulate biofilm development. The fluctuations near $1,640\text{ cm}^{-1}$ were attributed to the subtraction of a water band. (A) These spectra show the IR bands indicative of *P. aeruginosa* FRD1 biomass that absorbed near $1,650\text{ cm}^{-1}$ (amide I), $1,550\text{ cm}^{-1}$ (amide II), $1,250\text{ cm}^{-1}$ (P=O stretching, C—O—C stretching, and/or amide III vibration), $1,090\text{ cm}^{-1}$ (P—O and C—OH stretching), $1,060\text{ cm}^{-1}$ (C—OH stretching of alginate), $1,450\text{ cm}^{-1}$, and $1,400\text{ cm}^{-1}$ (due in part to C—H deformations and symmetric stretching of carboxylates, respectively). The bands associated with FRD1 biomass were not detected until 24 h. (B) Interfacial IR absorption spectra of biofilms formed by nonmucoid strain *P. aeruginosa* FRD440. The IR bands indicative of biomass were detected at 8 h. The bands in the FRD440 spectra were similar to bands from the spectra of the mucoid *P. aeruginosa*

grams plotting IR spectra from $2,000$ to $1,000\text{ cm}^{-1}$ (x and y axes) at 4-h intervals (z axis). In all experiments, a solution of approximately 10^6 cells/ml was inoculated into each flow cell, allowing bacterial contact with the opposing surfaces of a germanium IRE and initial adsorption of the *P. aeruginosa* cells. The first spectrum in each plot was collected during inoculation. For each strain, no IR bands resulting from the cells in the inoculum were observed. Baseline fluctuations in the region near $1,640\text{ cm}^{-1}$ are artifacts resulting from subtraction of a water band. After 20 min, sterile medium was pumped through the flow cells to stimulate biofilm development. In control experiments that analyzed sterile medium, only small increases in absorbances near $1,640$ (water subtraction artifact), $1,260$, and $1,080\text{ cm}^{-1}$ were detected. A band at $1,260\text{ cm}^{-1}$ was spurious and possibly resulted from the flow cell O ring. The absorbance at $1,080\text{ cm}^{-1}$ was typically less than 2 milliabsorbance units (mAU) after 44 h and likely resulted from the adsorption of inorganic phosphate to the germanium crystal (39).

The IR spectra of the developing biofilm formed by the mucoid CF pulmonary isolate, FRD1, is shown in Fig. 2A. For the first 24 h, spectra generated during biofilm development contained fluctuations similar to those observed with sterile medium, with the exception of a second component in the $1,100$ to $1,000$ region that was initially detected at $1,060\text{ cm}^{-1}$ in the 16-h spectra. After 24 h, IR bands indicative of biomass, as previously defined (36–39), were detected near $1,650\text{ cm}^{-1}$ (amide I), $1,550\text{ cm}^{-1}$ (amide II), $1,450\text{ cm}^{-1}$ (due in part to C—H deformation), $1,400\text{ cm}^{-1}$ (due in part to symmetric stretch for the carboxylate ion), and $1,250\text{ cm}^{-1}$ (P=O and C—O—C stretching and/or amide III) and in the $1,100$ - to $1,000\text{-cm}^{-1}$ region (P—O and C—OH stretching). The lack of these IR bands prior to 24 h indicates that not enough biomass was present in the analysis region to produce a signal. After initial detection, all IR bands associated with the FRD1 biofilms increased proportionally with time, signifying an accumulation of biomass within the analysis region.

Figure 2B shows the three-dimensional diagram plotting the IR spectra of a developing biofilm formed by FRD440, a nonmucoid *algT::Tn501* mutant of FRD1. As with the FRD1 cells, no IR bands indicative of biomass were detected in the inoculum. Spectra collected after 8 h produced IR bands indicative of biomass. Compared to mucoid strain FRD1, nonmucoid strain FRD440 produced a biofilm with an interfacial biomass that appeared to be fivefold greater; thus, the scale in Fig. 2B was expanded fivefold. Biofilm spectra tended to increase in intensity with time and remained remarkably similar for the duration of the experiment. Biofilms formed by two other nonmucoid strains, FRD2, a spontaneous *algT* mutant, and FRD1131, an *algD::Tn501* mutant, gave a time course of IR spectra with relative characteristics similar to spectra produced by FRD440 *algT::Tn501* biofilms (data not shown); however, spectra of FRD1131 *algD::Tn501* biofilms had weaker initial

FRD1, except for the absence of the band at $1,060\text{ cm}^{-1}$. (C) Interfacial attachment/growth profile of mucoid *P. aeruginosa* FRD1153 *algJ*, a strain that lacked alginate O acetylation. At approximately 32 h, all of the bands indicative of biomass were detected, including the band at $1,060\text{ cm}^{-1}$.

FIG. 3. Spectra of biofilms formed by mucoid and nonmucoid strains of *P. aeruginosa* following dehydration. Each biofilm was cultured for 88 h and then dried in a desiccator and analyzed. The drying process collapsed the biofilm onto the surface of the IRE. (A) Dehydrated FRD1 biofilm showing IR bands associated with the cells: 1,650 cm^{-1} (amide I), 1,545 cm^{-1} (amide II), 1,450 cm^{-1} (C—H deformations), 1,405 cm^{-1} (in part due to symmetric stretching of the carboxylate ions), 1,250 cm^{-1} (P=O stretching, C—O—C stretching, and/or amide III), and the 1,100- to 1,000- cm^{-1} region (P—O and C—OH stretching). IR bands associated with the alginate were also detected: 1,735 cm^{-1} (C=O stretching of esters), 1,615 cm^{-1} (asymmetric stretching of the carboxylate ion), 1,405 cm^{-1} and 1,375 cm^{-1} (symmetric stretching of the carboxylate ion), 1,250 cm^{-1} (C—O—C for the ester), and 1,060 cm^{-1} (C—OH stretching of alcohols). (B) Spectrum of dried mucoid FRD1153 showing the lack of prominent alginate bands. The final three spectra show the IR bands associated with the dried nonmucoid strains FRD2 (C), FRD1131 (D), and FRD440 (E).

IR bands, indicating that less biomass was present in the analysis region during the initial biofilm development phase (described below).

Figure 2C shows the IR spectra associated with the development of a mucoid *P. aeruginosa* FRD1153 biofilm. Strain FRD1153 is an *algI* mutant strain that does not O acetylate alginate. During the initial 28 h, only the fluctuations consistent with sterile control experiments were observed. At approximately 32 h, IR bands associated with cellular biomass were detected. A second component in the 1,100- to 1,000- cm^{-1} region was also detected at 1,060 cm^{-1} in the spectrum obtained at 32 h. As with the other strains, the relative absorbances in each spectrum remained consistent with time. Compared with all other strains tested, the O-acetylation-deficient FRD1153 strain had the weakest absorbance bands, signifying poor attachment and/or surface-associated growth.

Spectral analysis demonstrates alginate within biofilms. Although spectra from all strains produced similar IR bands, spectra of mucoid strains contained a second band in the 1,100- to 1,000- cm^{-1} region of the spectrum (P—O and C—OH stretching region) that had much weaker relative absorbance in the spectra of the nonmucoid strains. To further characterize this and other spectral differences between the mucoid strains and the nonmucoid strains, 88-h biofilms of each strain were dehydrated and then analyzed by ATR/FT-IR (Fig. 3). Dehydration collapsed the biofilms, thereby increasing the amount

of biomass within the analysis region. As with the hydrated biofilms, the dehydrated FRD1 biofilms produced spectra that contained two bands in the 1,100- to 1,000- cm^{-1} region (Fig. 3A). However, in the spectrum of the dehydrated FRD1 biofilm, the 1,060- cm^{-1} band (now absorbing at 1,050 cm^{-1} due to dehydration) had greater relative absorbance than the 1,080- cm^{-1} band (shifted from 1,090 cm^{-1}). Furthermore, an additional band at 1,735 cm^{-1} appeared in the dehydrated FRD1 biofilm that was not readily discerned in the spectrum of the 88-h hydrated FRD1 biofilm. The 1,735- cm^{-1} band had relatively stronger absorbance than the band found in the spectra of the dehydrated O-acetylation-deficient strain (Fig. 3B) or in those of the nonmucoid strains (Fig. 3C to E). The spectrum for dehydrated FRD1 also showed pronounced increases in absorbance at 1,405 and 1,250 cm^{-1} relative to the nonmucoid strains. In addition, the 1,650- cm^{-1} band was broader in the FRD1 spectrum (Fig. 3A) than in the spectra from the other strains, revealing the presence of overlapped IR bands.

To determine if the spectral differences observed in the dehydrated FRD1 biofilms were due to alginate within the biofilm matrix, the spectrum produced following dehydration of FRD2 *algT18* was subtracted from spectrum produced by dehydrated FRD1 (Fig. 4A). The subtraction spectrum produced bands at 1,730 cm^{-1} (C=O stretching of esters), 1,615 cm^{-1} (asymmetric stretching of the carboxylate ion), 1,410 cm^{-1} , 1,375 cm^{-1} (symmetric stretching of the carboxylate ion), 1,250 cm^{-1} (C—O—C for the ester), and 1,060 cm^{-1} (C—OH stretching of alcohols). The same IR bands were

FIG. 4. (A) Spectral subtraction of dehydrated FRD1 biofilm and FRD2 biofilm. The band observed in the spectrum of the hydrated FRD1 biofilm at 1,060 cm^{-1} (C—OH stretching) was present in the subtraction spectra. Other IR bands observed in the subtraction spectrum were 1,615 cm^{-1} (asymmetric carboxylate stretching), 1,410 cm^{-1} (symmetric carboxylate stretching), 1,730 cm^{-1} (C=O stretching), and 1,250 cm^{-1} (C—O stretching). The IR bands at 1,730 and 1,250 were associated with the O-acetyl groups on the alginate that are linked to the mannuronate backbone by ester bonds. (B) The FT-IR spectrum of purified alginate from *P. aeruginosa* FRD1, showing bands similar to that observed from the FRD1-FRD2 subtraction spectrum. (C) The FT-IR spectrum of purified alginate from *P. aeruginosa* FRD1153 *algI*. This spectrum was similar to the spectrum of purified FRD1 alginate but lacked IR bands at 1,730 and 1,250 cm^{-1} that resulted from ester bonds of O-acetylated alginate.

FIG. 5. Plot of amide II band absorbance ($1,550\text{ cm}^{-1}$) over time for mucoid and nonmucoid strains of *P. aeruginosa*. Open circles, FRD1; open diamonds, FRD1153 *algJ*; closed circles, FRD2 *algT18*; boxes, FRD440 *algT*:: Tn501-33; open triangles, FRD1131 *algD*::Tn501-31.

present in the IR spectrum of purified FRD1 alginate (Fig. 4B), demonstrating that alginate remained associated with the FRD1 biofilms. However, in the spectra of living hydrated FRD1 biofilms, the alginate bands were not only overlapped with the bands associated with the cells but relatively weaker than those produced with the dehydrated biofilms. This increase in the relative absorbance found after dehydration provides evidence that greater amounts of alginate were associated with the FRD1 cells located at depths greater than the ATR/FT-IR analysis range.

Similar experiments were performed on the *algJ* mutant *P. aeruginosa* FRD1153 and its purified alginate that lacked *O*-acetyl groups. The IR spectra of alginate purified from FRD1153 (Fig. 4C) did not have the ester bands at $1,730$ and $1,250\text{ cm}^{-1}$ that were found in the alginate of FRD1. Spectral subtractions of hydrated biofilms of FRD2 from hydrated biofilms FRD1153 produced poor quality spectra (data not shown), resulting from insufficient biomass in the FRD1153 biofilms. Subtraction of dehydrated biofilms also produced poor quality subtracted spectra, suggesting that insufficient quantities of alginate were associated with the 88-h FRD1153 biofilms. This was due to the fact that most of the FRD1153 cells switched to the nonmucoid phenotype by 88 h (see results below).

Spectral subtraction experiments for biofilms composed of the *algD* mutant FRD1131 and the *algT* mutants FRD440 and FRD2 produced only noisy spectra, with little or no signal, demonstrating little to no alginate within the biofilms of any of these nonmucoid strains.

Effect of alginate and *O* acetylation on biofilm growth dynamics. Biofilm dynamics are dependent on a number of different processes, including attachment of cells from the liquid

phase, growth of surface-associated cells, and detachment of the surface-associated cells. FT-IR was used to monitor the dynamics of biofilm formation transpiring within approximately $1\text{ }\mu\text{m}$ of the IRE, the analysis range of the attenuated total reflection sampling technique. The amide II band ($1,550\text{ cm}^{-1}$) was used as a marker for biomass since cellular proteins produce amide II absorbance and since the absorbance from water and alginate did not appreciably affect amide II absorbance. For each strain, the other bands in a spectrum remained generally proportional to amide II with time. With the amide II band, the ATR/FT-IR provided a unique view of biomass fluctuations occurring within the innermost portion of a biofilm. In comparison, endpoint analyses were performed at 44 and 88 h to determine the number of CFU associated with each biofilm for each strain.

For FRD1 biofilms, the amide II band was initially detected after 24 h and continued to increase until the absorbance reached 24 mAU at 88 h (Fig. 5). Between 24 and 64 h, the absorbance of the amide II band increased linearly at the rate of 0.5 mAU/h to 19 mAU (Table 2). Over the next 24 h, the amide II band increased only slightly to a maximum of 23 mAU, indicating that the biomass within the analysis region had somewhat stabilized. From 44 to 88 h, the cell density within the FRD1 biofilm increased 25-fold to $7.7 \times 10^8\text{ CFU/cm}^2$, with all of the colonies producing a mucoid phenotype at 44 h and 98% of the cells remaining mucoid at 88 h (Table 2). For comparison, the amide II absorbance increased only four-fold between 44 and 88 h, suggesting that the biofilm dynamics occurring within the analysis region were different from those transpiring within the entire biofilm.

With the *algT* mutant strains, FRD440 and FRD2, the amide II band showed a rapid increase over time. Both strains were

TABLE 2. CFU and interfacial growth rates

Strain	Genotype	Phenotype	Plate count data (CFU/cm ²) ^a		ATR/FT-IR (amide II absorbance)		
			44 h	88 h	Linear rate increase ^d (mAU/h)	Growth rate ^c [ln (1 + mAU)]/h	Doubling time ^e (h)
FRD1	<i>mucA22</i>	Mucoid	2.8 (0.9) × 10 ^{7b}	7.7 (0.7) × 10 ^{8b}	0.5 (0.95)	0.06 (0.94)	12
FRD2	<i>mucA22 algT18</i>	Nonmucoid	2.0 × 10 ⁸	6.6 (1.3) × 10 ⁸	4.6 (0.98)	0.35 (0.98)	2
FRD440	<i>mucA22 algT::Tn501-33</i>	Nonmucoid	2.6 (1.3) × 10 ⁸	4.1 (1.0) × 10 ⁸	5.4 (0.97)	0.35 (0.98)	2
FRD1131	<i>mucA22 algD::Tn501-31</i>	Nonmucoid	1.7 (0.8) × 10 ⁷	1.0 (0.5) × 10 ⁹	5.0 (0.99)	0.11 (0.98)	6
FRD1153	<i>mucA22 algJ3</i>	Mucoid (no O-acetylation)	4.6 (1.0) × 10 ^{6c}	6.6 (1.7) × 10 ^{8c}	3.2 (0.99)	0.11 (0.98)	6

^a CFU were determined by scraping the attached cells from the germanium IRE with a Teflon policeman and saline, transferring the cells to a test tube, and vortexing with glass beads for 5 min. The cells were serially diluted in sterile saline and plated on L agar. Standard deviations of three samples are indicated in parentheses.

^b 100% of FRD1 colonies were mucoid at 44 h; 98% of FRD1 colonies were mucoid at 88 h.

^c 100% of FRD1153 colonies were mucoid at 44 h; 35% of FRD1153 colonies were mucoid at 88 h.

^d Linear rates of increase were calculated from amide II absorbances between 32 and 64 h for FRD1, 8 and 32 h for FRD2 and FRD440, 40 and 52 h for FRD1131, and 60 and 88 h for FRD1153. *r*² values for linear regressions are indicated in parentheses.

^e Growth rates and doubling times were calculated between 24 and 60 h for FRD1, 4 and 16 h for FRD2 and FRD440, 16 and 52 h for FRD1131, and 40 and 88 h for FRD1153. The growth rate refers to the cells within the analysis range of the ATR-FTIR, within 1 µm of the IRE surface.

initially detected at approximately 8 h and showed a rapid increase until 32 h, after which the absorbance of the amide II for FRD440 and FRD2 stabilized near 150 and 140 mAU, respectively (Table 2). These final absorbance values were approximately sixfold stronger than those for FRD1. The rate of increase of the amide II band was 5.4 mAU/h for FRD440 and 4.6 mAU/h for FRD2, approximately 10 times greater than the rate of increase for the mucoid FRD1 biofilms (Table 2). Interestingly, even though the final absorbances and the rates of increase for the amide II band were different, the total numbers of cells in the biofilms were similar (Table 2). This suggested that most of the FRD1 cells were outside the analysis range of the ATR/FT-IR, as compared to cells of the nonmucoid strains.

In planktonic cultures, using 10× biofilm medium, the generation times of all strains in this study were similar, with doubling times of 2.4 h for FRD1 and 2.0 h for the *algJ* mutant, FRD1153. For the nonmucoid strains, the doubling times in planktonic culture were 2.0 and 2.8 h for the *algT* mutants, FRD2 and FRD440, respectively, and 2.0 h for the *algD* mutant, FRD1131. During biofilm growth, the amide II data were used to calculate growth rates in the biofilms by using a logarithmic plot. Assuming that at some time during the experiment the biomass within the ATR/FT-IR analysis region accumulates at an exponential rate, the biomass accumulation rate can be determined by plotting the natural logarithm of the amide II absorbance versus time during exponential growth. The slope represents the rate at which biomass within the analysis region accumulated exponentially. The biomass accumulation rate within the evanescent field was approximately fivefold less for FRD1 than for the *algT* mutant strains (Table 2). The biomass doubling time, calculated by dividing the ln (2) by the interfacial growth rate, was 12 h for FRD1, six times greater than the generation times for the *algT* mutants (Table 2). Since the numbers of cells in the biofilms at 88 h were approximately the same for the mucoid and nonmucoid strains, these results suggest that most of the cell division and accumulation of biomass for the FRD1 cells occurred outside the analysis range of the evanescent field (approximately 1 µm from the surface), as compared to cells of the nonmucoid strains.

The *algD* mutant, FRD1131, had a sigmoid-shaped response similar to *algT* mutants but showed an increased lag time in the

response of the amide II band (Fig. 5). Following initial detection at 20 h, the amide II band increased at a rate of 5.0 mAU/h and had a maximum amide II absorbance of 157 mAU, which was similar to the results obtained with the *algT* mutant strains. At 44 h, the number of FRD1131 cells was 20-fold less than the numbers of both *algT* mutant strains (Table 2), providing evidence that the lag resulted in fewer attached cells. At 88 h, the plate count was 10⁹ CFU/cm², only slightly higher than those for mucoid FRD1 and the *algT* mutant strains. The growth rate within the analysis region was threefold less than that for the *algT* mutants, indicating that biofilm dynamics may be different for this strain (Table 2).

The *algJ* mutant strain, FRD1153, showed the longest delay in the initiation of biofilm development, ranging from 32 to 40 h (Fig. 5). The cell density of FRD1153 was 4.6 × 10⁶ cells/cm² at 44 h (Table 2), less than all other FRD strains. FRD1153 showed a 3.8-mAU/h rate of increase in amide II absorbance after approximately 64 h. The amide II absorbance of FRD1153 ultimately surpassed the absorbance of FRD1 biofilms, indicating that the biomass within the analysis range was greater than that of the parent strain, FRD1. The final cell density of FRD1153 was 6.6 × 10⁸ cells/cm² at 88 h, similar to all other strains tested (Table 2). However, as determined by plate counts and colony morphology analysis, 65% of the cells had switched to the nonmucoid phenotype by 88 h, and therefore FRD1153 phenotypically resembled nonmucoid FRD2 cells more than the original FRD1153 strain.

SCLM shows that alginate is important for *P. aeruginosa* biofilm structure and that O acetylation is critical for biofilm formation. To visualize biofilm formation using SCLM, pMF230, encoding constitutive expression of the GFP *mut2* mutation (6), was introduced into each of the FRD strains. Figure 6 shows the three-dimensional reconstruction of 48-h biofilms formed by FRD1, FRD2, and FRD1153. The inoculum, medium, and flow rates were similar to those in the ATR/FT-IR experiments.

The three-dimensional SCLM reconstruction of a biofilm formed by FRD1 is shown in Fig. 6A. The micrograph shows a top and an angled view of the biofilm, examined at ×100 magnification. The micrograph shows nonuniform coverage of the surface by FRD1 microcolonies. An individual FRD1 microcolony is shown in Fig. 6B at ×400 magnification. The top view shows the distribution of bacteria on the surface, with

FIG. 6. SCLM analysis of 48-h biofilms of *P. aeruginosa*, as viewed from the top and at an angle. All strains constitutively expressed the *mut2* GFP. (A) *P. aeruginosa* FRD1 viewed at $\times 100$ magnification. (B) *P. aeruginosa* FRD1 viewed at $\times 400$ magnification. (C) *P. aeruginosa* FRD2 *algT18* viewed at $\times 400$ magnification. (D) *P. aeruginosa* FRD1153 *algJ* viewed at $\times 400$ magnification.

microcolonies covering part of the surface and much of the surface not colonized by bacteria. The angled view shows the depth of an individual microcolony, which was approximately $40\text{ }\mu\text{m}$ in the 48-h biofilms. FRD1 biofilms at 90 h were similar in surface coverage but had depths of approximately $125\text{ }\mu\text{m}$ (data not shown). The micrograph demonstrated that FRD1

biofilms tended to form chains of cells within and between the microcolonies.

The three-dimensional SCLM reconstruction of the FRD2 biofilm at $\times 400$ magnification is shown in Fig. 6C. The top view demonstrates a greater surface coverage by the FRD2 cells compared to the FRD1 biofilms. The cells appear more

densely packed. The angled view shows the depth of the biofilm (z axis equals 12 μm). Much of the surface was covered by a monolayer of cells, and some of the surface was covered by shallow microcolonies that were approximately 6 μm in depth. In contrast to the FRD1 biofilms, the FRD2 cells failed to form chains of cells. SCLM images of the other *algT* mutant, FRD440, and the *algD* mutant, FRD1131, were similar to those of FRD2, showing shallow densely packed biofilms.

SCLM images of 48-h biofilms formed by the *algJ* mutant, FRD1153, demonstrated little attachment and/or surface-associated growth of this mutant strain, which was unable to O acetylate alginate. Figure 6D shows a microcolony formed by FRD1153 at $\times 400$ magnification. The microcolonies formed by FRD1153 were less than 16 μm in depth and sparsely covered the surface. The SCLM results were consistent with the ATR/FT-IR results, which also showed little surface-associated growth of this strain.

DISCUSSION

Two nondestructive techniques, ATR/FT-IR and SCLM, were used for the on-line monitoring of the biofilm development of mucoid and nonmucoid strains of *P. aeruginosa*. The ATR/FT-IR technique was used to obtain chemical information regarding the bacteria directly adjacent to a germanium substratum. SCLM in combination with fluorescent bacteria provided images of the three-dimensional structure of cells within living biofilms. For ATR/FT-IR analysis in an aqueous environment, water was interactively subtracted from each biofilm spectrum, producing IR spectra for living biofilms (20, 39). With attenuated total reflection sampling, the IR radiation is most intense near the interface and rapidly decays (exponentially) with distance from the interface. The analysis range, while difficult to quantify under experimental conditions, can be estimated by calculating the depth of penetration, defined as the distance at which the intensity decreased to 37% of its value at the surface. The depth of penetration depends on the angle of incidence, the refractive indices of the IRE and the outside medium (air or water), and the wavelength of the radiation. For our experiments (i.e., germanium in contact with water), the depth of penetration was calculated to be 0.3 μm for the 1,750- cm^{-1} band, 0.4 μm for the 1,550- cm^{-1} band, and 0.6 μm for the 1,050- cm^{-1} band. The values decrease only slightly when the outside medium is air. For example, the 1550- cm^{-1} band decreases by only 0.02 μm . With SCLM, images of biofilms could be obtained at distances farther from the surface than could be obtained by ATR/FT-IR sampling. As a result, the two techniques provided complementary information on *P. aeruginosa* colonization of surfaces and on the role of extracellular polymer in *P. aeruginosa* surface colonization.

The IR spectra of most cells—whether living or dead, hydrated or dehydrated—have similar overall characteristics. This is not surprising, since the IR spectrum gives information about the IR-active vibrational modes of chemical bonds found in the molecules of cells and since the cells consist of analogous classes of cellular components (proteins, lipids, carbohydrates, nucleic acids, etc.) with similar functional groups. In previous studies, bacterial cellular components as well as living biofilms were analyzed using FT-IR (37) and ATR/FT-IR (38, 39, 41). Proteins have amide vibrational modes that give rise to IR

bands near 1,650 cm^{-1} (amide I), near 1,550 cm^{-1} (amide II), and in the region of 1,300 to 1,240 cm^{-1} (amide III). Carbohydrates have C—OH and C—O—C stretch modes that absorb IR radiation in the range of 1,200 to 1,000 cm^{-1} . Carboxylate ions have a C=O that absorbs in the range of 1,780 to 1,730 cm^{-1} and contain C—O stretches that absorb near 1,400 cm^{-1} . Phosphates, found in nucleic acids and phospholipids, have a P=O that absorbs at 1,250 cm^{-1} and overlaps the amide III mode. Phosphates also have a P—O stretching mode that absorbs near 1,080 cm^{-1} and overlaps the C—OH stretching modes. The C—H bending of CH_3 groups and C—H scissoring of CH_2 groups absorb near 1,460 cm^{-1} . These IR bands, typically found in all cells, were also detected and monitored in *P. aeruginosa* biofilms described here.

In addition to the IR bands found in all of the *P. aeruginosa* strains, alginate from FRD1 absorbed IR radiation in six different regions, producing spectra with strong absorbance at 1,730, 1,615, 1,410, 1,375, 1,250, and 1,060 cm^{-1} . The band absorbing at 1,615 cm^{-1} was assigned to the carboxylate ion asymmetric stretch, the band absorbing at 1,410 cm^{-1} was assigned to the carboxylate ion symmetrical stretch, and the band absorbing at 1,060 cm^{-1} was assigned to the C—OH stretch. Each of these bands was associated with the purified alginate polymer from FRD1 and FRD1153. The *O*-acetyl groups, linked to the mannuronate residues by ester bonds, had a C=O stretching at 1,730 cm^{-1} and a C—O—C stretching at 1,250 cm^{-1} . These IR bands were observed in purified FRD1 alginate, as well as in FRD1 biofilms, but not in purified alginate from the *algJ* mutant strain, FRD1153.

In the SCLM studies, the cells expressed the GFP. Therefore, it was possible to observe the three-dimensional structure of the biofilms without removing the samples from the flow cells for staining or examination. However, it was possible that the expression of this nonnative protein, GFP, altered the physiology of the bacteria and/or affected the three-dimensional structure of the biofilms. Therefore, in addition to the SCLM studies presented here, we examined the biofilms formed by these strains, but not expressing the GFP, using phase-contrast microscopy. Although it was difficult to obtain focused images of biofilms using phase-contrast microscopy (particularly for the mucoid strains), it was apparent that the non-*gfp*-expressing strains formed biofilms with three-dimensional structures similar to those observed here. The nonmucoid strains formed shallow biofilms, primarily as monolayers, and the mucoid FRD1 formed microcolonies that extended from the surface into the bulk medium. Strain FRD1153 formed sparse microcolonies similar to the bacteria that expressed the GFP. Therefore, expression of the GFP did not appear to have a significant effect on the *P. aeruginosa* biofilm formation observed in these SCLM studies.

In this study, the amide II band was used as a marker for interfacial biomass because it was associated with IR absorbance of proteins and, unlike amide I, did not overlap an IR band found in alginate (1,615 cm^{-1}) and water (1,640 cm^{-1}). Furthermore, in a study using *Caulobacter* species, a linear correlation between the amide II band and direct counts of bacteria was observed in the range of 2×10^5 cells/ cm^2 to 2×10^7 cells/ cm^2 (39). However, once the biofilms had more cells per unit area, the correlation was not observed, probably due to the presence of cells outside the analysis range of the ATR/

FT-IR technique. In this study, the amide II band was used to monitor biomass within the evanescent field and to determine a rate of biofilm formation within this field for each of the strains.

Results of both the ATR/FT-IR and SCLM analyses of nonmucoid strains demonstrated that alginate was not required for *P. aeruginosa* biofilm formation, and therefore alginate did not act as a primary adhesin for the *P. aeruginosa* cells to these surfaces. FRD2 and FRD440 have nonmucoid phenotypes on agar medium and did not produce detectable levels of alginate when cultured in liquid medium. However, since these mutations were in the regulatory gene, *algT*, it was possible that FRD2 and FRD440 produced undetectable levels of alginate that may have been required for biofilm formation. For this reason, we assayed biofilm formation by strain FRD1131 that had a Tn501 insertion in the alginate biosynthetic gene *algD*. FRD1131 was unable to produce alginate due to the inability to produce GDP-mannuronate and to the polar effect of the transposon insertion on the downstream alginate biosynthetic operon. Strain FRD1131 showed a delay in biofilm formation but ultimately formed biofilms with spectra similar to those of FRD2 and FRD440. All three nonmucoid strains had amide II absorbances greater than that of FRD1. The reason for the increased lag time of FRD1131 may have resulted from the nonspecific irreversible adsorption of fewer cells during the initial inoculation phase of the experiments. Once biomass was detected, the rate of increase for the amide II band was similar for all three nonmucoid strains. AlgT is an alternative sigma factor and therefore may be responsible for regulation of cellular functions other than alginate production. The ATR/FT-IR results presented here demonstrated that AlgT did not regulate cellular functions that were required for *P. aeruginosa* biofilm formation, since the *algT* mutants, FRD2 and FRD440, formed biofilms with amide II absorbances greater than that of FRD1. The SCLM studies confirmed the ability of the *algT* and *algD* mutant strains to form biofilms. Two conclusions can be drawn from analysis of nonmucoid strains: (i) alginate was not required for interfacial adhesion/growth; and (ii) the regulatory protein AlgT was not required for biofilm development.

Although alginate production did not affect the ability of *P. aeruginosa* to attach to or grow on surfaces, alginate significantly affected the architecture of the *P. aeruginosa* biofilms. The ATR/FT-IR results demonstrated that the nonmucoid strains had amide II absorbances that were as much as eightfold greater than for the mucoid FRD1 strain. However, cell counts revealed approximately the same numbers of cells per unit surface area for FRD1 and for the nonmucoid strains after 88 h. Since the ATR/FT-IR detected only IR bands within approximately 1 μm from the surface, the results suggested that most of the FRD1 cells extended farther from the surface than those of the nonmucoid strains. The SCLM study confirmed those results. The nonmucoid strain FRD2 formed fairly uniform biofilms that completely covered the surface after 48 h of biofilm growth. However, the mucoid FRD1 cells had less surface coverage. The FRD1 biofilms extended farther from the surface and into the medium bulk phase, resulting in approximately the same number of bacteria per unit surface area, but with greater volume and less effective surface coverage. The SCLM studies demonstrated that the nonmucoid *P. aeruginosa* cells were packed more densely than the mucoid

FRD1 cells. This may have been due to the presence of alginate in the space between the individual cells. The ATR/FT-IR subtraction spectra revealed that the alginate produced by the FRD1 cells remained associated with the microcolonies, but primarily outside the analysis range in hydrated biofilms. Therefore, alginate may act as the intercellular matrix that enables the *P. aeruginosa* cells to extend from the surface and form microcolonies.

Although alginate was not required for biofilm formation, a mutant form of alginate, lacking *O*-acetyl groups, apparently inhibited *P. aeruginosa* attachment to or growth on the surfaces used here. During planktonic growth, the *algJ* mutant strain, FRD1153, had growth rates similar to those of FRD1. However, FRD1153 showed less surface-associated attachment and/or growth. Evidence for the inhibition of biofilm growth by the mutant form of alginate included (i) the ATR/FT-IR studies, which showed less interfacial absorbance of the amide II band, as well as the other IR bands associated with *P. aeruginosa* cells; (ii) viable cell counts, which demonstrated fewer FRD1153 cells associated with the surface while the cells remained mucoid; and (iii) the SCLM studies, which demonstrated that the FRD1153 cells sparsely populated the surface after 48 h of growth. FRD1153 showed significant surface-associated growth only after a portion of the cells switched to the nonmucoid phenotype. The mechanism for inhibition of biofilm formation on these surfaces by the mutant form of alginate is not known. However, the chemical and physical properties of non-*O*-acetylated alginate are very different from those of the *O*-acetylated form. Differences include the reduced viscosity of the deacetylated alginate (53) and increased susceptibility of the deacetylated alginate to degradation by alginate lyases (2, 3, 45). These properties of the mutant alginate may have provided a less stable intercellular matrix for microcolony formation than the *O*-acetylated alginate.

P. aeruginosa microcolonies have been observed in vivo associated with pulmonary tissue isolated from CF patients (29). The experiments reported here demonstrated that alginate plays a role in the formation of three-dimensional microcolonies in vitro and suggest that alginate may play a similar role in vivo. Since alginate appears to be associated with the biofilm matrix, this polymer may exert its antiphagocytic effect on microcolonies, rather than on individual *P. aeruginosa* cells.

ACKNOWLEDGMENTS

We thank David C. White and Wendy Cochran for their contributions to this work.

This work was supported by Veterans Administration Medical Research Funds (D.E.O.) and in part by Public Health Service grants AI-19146 (D.E.O.) and AI-46588 (M.J.F.) from the National Institute of Allergy and Infectious Diseases. Support for this work was also provided by the Center for Biofilm Engineering at Montana State University, an NSF-supported Engineering Research Center (NSF Cooperative Agreement EEC-8907039).

REFERENCES

1. Baltimore, R. S., and M. Mitchell. 1982. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. *J. Infect. Dis.* **141**:238–247.
2. Boyd, A., and A. M. Chakrabarty. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **60**:2355–2359.
3. Boyd, A., M. Ghosh, T. B. May, D. Shinabarger, R. Keogh, and A. M.

- Chakrabarty. 1993. Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. *Gene* **131**:1–8.
4. Chitnis, C. E., and D. E. Ohman. 1990. Cloning of *Pseudomonas aeruginosa* *algG*, which controls alginate structure. *J. Bacteriol.* **172**:2894–2900.
 5. Chitnis, C. E., and D. E. Ohman. 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol. Microbiol.* **8**:583–590.
 6. Cormack, B. P., R. H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**:33–38.
 7. Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49**:711–745.
 8. Davidson, J. W., C. J. Lawson, and I. W. Sutherland. 1977. Localization of O-acetyl groups in bacterial alginate. *J. Gen. Microbiol.* **98**:603–606.
 9. Deretic, V., J. F. Gill, and A. M. Chakrabarty. 1987. Gene *algD* coding for GDP-mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.* **169**:351–358.
 10. DeVries, C. A., and D. E. Ohman. 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternative sigma factor, and shows evidence for autoregulation. *J. Bacteriol.* **176**:6677–6687.
 11. Evans, L. R., and A. Linker. 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J. Bacteriol.* **116**:915–924.
 12. Figurski, D., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. USA* **76**:1648–1652.
 13. Flynn, J. L., and D. E. Ohman. 1988. Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. *J. Bacteriol.* **170**:1452–1460.
 14. Flynn, J. L., and D. E. Ohman. 1988. Use of a gene replacement cosmid vector for cloning alginate conversion genes from mucoid and nonmucoid *Pseudomonas aeruginosa* strains: *algS* controls expression of *algT*. *J. Bacteriol.* **170**:3228–3236.
 15. Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman. 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. *J. Bacteriol.* **176**:1821–1830.
 16. Franklin, M. J., and D. E. Ohman. 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. *J. Bacteriol.* **175**:5057–5065.
 17. Franklin, M. J., and D. E. Ohman. 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. *J. Bacteriol.* **178**:2186–2195.
 18. Gacesa, P. 1988. Alginates. *Carbohydr. Polym.* **8**:161–182.
 19. Gacesa, P. 1998. Bacterial alginate biosynthesis—recent progress and future prospects. *Microbiology* **144**:1133–1143.
 20. Gendreau, R. M. 1986. Biomedical Fourier transform infrared spectroscopy: applications to proteins, p. 22–52. *In* R. M. Gendreau (ed.), *Spectroscopy in the biomedical sciences*. CRC Press, Boca Raton, Fla.
 21. Goldberg, J. B., W. L. Gorman, J. L. Flynn, and D. E. Ohman. 1993. A mutation in *algN* permits *trans* activation of alginate production by *algT* in *Pseudomonas* species. *J. Bacteriol.* **175**:1303–1308.
 22. Gorin, P. A. T., and J. F. T. Spencer. 1966. Extracellular alginic acid from *Azotobacter vinelandii*. *Can. J. Chem.* **44**:993–998.
 23. Govan, J. R. W., J. A. M. Fyfe, and T. R. Jarman. 1981. Isolation of alginate-producing mutants of *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas mendocina*. *J. Gen. Microbiol.* **125**:217–220.
 24. Govan, J. R. W., and G. S. Harris. 1986. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiol. Sci.* **3**:302–308.
 25. Grasdalen, H. 1983. High-field, ¹H-n.m.r. spectroscopy of alginate: sequential structure and linkage conformations. *Carbohydr. Res.* **118**:255–260.
 26. Grasdalen, H., B. Larsen, and O. Smidsrod. 1981. ¹³C-N.M.R. studies of monomeric composition and sequence in alginate. *Carbohydr. Res.* **89**:179–191.
 27. Griffiths, P. R., and J. A. deHaseth. 1986. Fourier transform infrared spectrometry. John Wiley & Sons, Inc., New York, N.Y.
 28. Hershberger, C. D., R. W. Ye, M. R. Parsek, Z. Xie, and A. M. Chakrabarty. 1995. The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^c). *Proc. Natl. Acad. Sci. USA* **92**:7941–7945.
 29. Lam, J., R. Chan, K. Lam, and J. R. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.* **28**:546–556.
 30. Lawrence, J. R., D. R. Korber, B. D. Hoyle, J. W. Costerton, and D. E. Caldwell. 1991. Optical sectioning of microbial biofilms. *J. Bacteriol.* **173**:6558–6567.
 31. Learn, D. B., E. P. Brestel, and S. Seetharama. 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infect. Immun.* **55**:1813–1818.
 32. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 33. Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic. 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **90**:8377–8381.
 34. Martin, D. W., M. J. Schurr, H. Yu, and V. Deretic. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to σ^E and stress response. *J. Bacteriol.* **176**:6688–6696.
 35. Mathee, K., C. J. McPherson, and D. E. Ohman. 1997. Posttranslational control of the *algT* (*algU*)-encoded σ^{22} for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). *J. Bacteriol.* **179**:3711–3720.
 36. Naumann, D. 1984. Some ultrastructural information on intact, living bacterial cells and related cell-wall fragments as given by FT-IR. *Infrared Phys.* **24**:233–238.
 37. Naumann, D., D. Helm, H. Labischinski, and P. Giesbrecht. 1991. The characterizations of microorganisms by Fourier transform infrared spectroscopy, p. 43–96. *In* W. Nelson (ed.), *Modern techniques for rapid microbiological analysis*. VCH Publishers, New York, N.Y.
 38. Nichols, P. D., J. M. Henson, J. B. Guckert, D. E. Nivens, and D. C. White. 1985. Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria-polymer mixtures, and biofilms. *J. Microbiol. Methods* **4**:79–94.
 39. Nivens, D. E., J. Q. Chambers, T. R. Anderson, A. Tunlid, J. Smit, and D. C. White. 1993. Monitoring microbial adhesion and biofilm formation by attenuated total reflection/Fourier transform infrared spectroscopy. *J. Microbiol. Methods* **17**:199–213.
 40. Nivens, D. E., R. J. Palmer, and D. C. White. 1995. Continuous nondestructive monitoring of microbial biofilms: a review of analytical techniques. *J. Ind. Microbiol.* **15**:263–276.
 41. Nivens, D. E., J. Schmit, J. Sniatecki, T. Anderson, J. Q. Chambers, and D. C. White. 1993. Multichannel ATR/FT-IR spectrometer for on-line examination of microbial biofilms. *Appl. Spectrosc.* **47**:668–671.
 42. Ogle, J. W., J. M. Janda, D. E. Woods, and M. L. Vasil. 1987. Characterization and use of a DNA probe as an epidemiological marker for *Pseudomonas aeruginosa*. *J. Infect. Dis.* **155**:119–126.
 43. Ohman, D. E., and A. M. Chakrabarty. 1981. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect. Immun.* **33**:142–148.
 44. Rehm, B. H., H. Ertesvag, and S. Valla. 1996. A new *Azotobacter vinelandii* mannuronan C-5-epimerase gene (*algG*) is part of an *alg* gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**:5884–5889.
 45. Schiller, N. L., S. R. Monday, C. M. Boyd, N. T. Keen, and D. E. Ohman. 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli*. *J. Bacteriol.* **175**:4780–4789.
 46. Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic. 1994. Gene cluster controlling conversion of alginate-overproducing phenotype in *Pseudomonas aeruginosa*: a functional analysis in a heterologous host and role in the instability of mucoidy. *J. Bacteriol.* **176**:3375–3382.
 47. Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic. 1996. Control of AlgU, a member of the σ^E -like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol.* **178**:4997–5004.
 48. Schwarzmann, S., and J. R. Boring III. 1971. Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. *Infect. Immun.* **3**:762–767.
 49. Shinabarger, D., T. B. May, A. Boyd, M. Ghosh, and A. M. Chakrabarty. 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa* *algF* gene controlling acetylation of alginate. *Mol. Microbiol.* **9**:1027–1035.
 50. Simpson, J. A., S. E. Smith, and R. T. Dean. 1988. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. *J. Gen. Microbiol.* **134**:29–36.
 51. Simpson, J. A., S. E. Smith, and R. T. Dean. 1989. Scavenging by alginate of free radicals released by macrophages. *Free Radic. Biol. Med.* **6**:347–353.
 52. Skjak-Braek, G., H. Grasdalen, and B. Larsen. 1986. Monomer sequence and acetylation pattern in some bacterial alginates. *Carbohydr. Res.* **154**:239–250.
 53. Skjak-Braek, G., F. Zanetti, and S. Paoletti. 1989. Effect of acetylation on some solution and gelling properties of alginates. *Carbohydr. Res.* **185**:131–138.
 54. Stretton, S., S. Techarnjanaruk, A. M. McLennan, and A. E. Goodman. 1998. Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Appl. Environ. Microbiol.* **64**:2554–2559.
 55. Wozniak, D. J., and D. E. Ohman. 1994. Transcriptional analysis of *algR*, *algB*, and *algD* in *Pseudomonas aeruginosa* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *J. Bacteriol.* **176**:6007–6014.
 56. Xie, Z. D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty. 1996. Sigma factor-anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. *J. Bacteriol.* **178**:4990–4996.