Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms

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The bacterium *Pseudomonas aeruginosa* causes chronic respiratory infections in cystic fibrosis (CF) patients. Such infections are extremely difficult to control because the bacteria exhibit a biofilm-mode of growth, rendering *P. aeruginosa* resistant to antibiotics and phagocytic cells. During the course of infection, *P. aeruginosa* usually undergoes a phenotypic switch to a mucoid colony, which is characterized by the overproduction of the exopolysaccharide alginate. Alginate overproduction has been implicated in protecting *P. aeruginosa* from the harsh environment present in the CF lung, as well as facilitating its persistence as a biofilm by providing an extracellular matrix that promotes adherence. Because of its association with biofilms in CF patients, it has been assumed that alginate is also the primary exopolysaccharide expressed in biofilms of environmental nonmucoid *P. aeruginosa*. In this study, we examined the chemical nature of the biofilm matrix produced by wild-type and isogenic alginate biosynthetic mutants of *P. aeruginosa*. The results clearly indicate that alginate biosynthetic genes are not expressed and that alginate is not required during the formation of nonmucoid biofilms in two *P. aeruginosa* strains, PAO1 and PA14, that have traditionally been used to study biofilms. Because nonmucoid *P. aeruginosa* strains are the predominant environmental phenotype and are also involved in the initial colonization in CF patients, these studies have implications in understanding the early events of the infectious process in the CF airway.

The development of bacterial biofilms involves the regulated and coordinated transition from free-swimming planktonic bacteria to highly differentiated communities of surface-attached organisms. In general, this developmental pathway proceeds from the initial attachment of bacteria to a surface, followed by the accumulation of cell clusters or microcolonies, and ultimately, a "mature" biofilm characterized by large microcolonies of bacteria separated by fluid-filled channels (1–3). Hallmarks of a mature biofilm include the production of an extracellular matrix or EPS (for extracellular polymeric substance) and an increased resistance to antibiotics and antimicrobial stress (2). It has been proposed that one component of EPS, secreted polysaccharides, may contribute to the resistance of biofilm-grown bacteria to certain antimicrobial agents (4–6). Besides polysaccharides, EPS is also thought to be composed of nucleic acids and proteins (7). EPS composition presumably varies from organism to organism; however, its composition is ill defined.

The paradigm organism for studying bacterial biofilms, *Pseudomonas aeruginosa*, has been long thought to produce alginate, a polymer of the uronic acids mannuronic and guluronic acid, as the primary secreted polysaccharide in biofilms (8). *P. aeruginosa* isolated from the lungs of cystic fibrosis (CF) patients, often undergoes a switch to a mucoid phenotype, which is characterized by the formation of shiny colonies on an agar plate (9). Mucoidy results from an overproduction of alginate, which is thought to play a protective role in the relatively harsh environment of the CF lung, perhaps by enhancing the formation of biofilms (9). There are several other lines of evidence, which are consistent with *P. aeruginosa* existing in the CF lung as biofilms. This evidence includes microscopy of *P. aeruginosa* from the sputum of CF patients, the high level antibiotic resistance of *P. aeruginosa* in the CF lung, and the production of quorum-sensing molecules in sputum in ratios typical of biofilm-grown bacteria (10–12). The studies of CF-derived mucoid *P. aeruginosa* isolates, and their link to the biofilm lifestyle, has led to the assumption that alginate is the key secreted polysaccharide in biofilms of both mucoid and nonmucoid strains.

Early studies examining the role of alginate in the initiation or maturation of biofilms often involved the comparison of mucoid strains isolated from the CF lung with nonisogenic, nonmucoid strains (13). These studies generally compared the ability of these strains to form biofilms and the antibiotic resistance of established biofilms. Other approaches included examining the effect of antibodies to alginate on adhesion by mucoid and nonmucoid strains, which demonstrated that the antibodies did not inhibit adhesion of nonmucoid *P. aeruginosa* strains to eukaryotic cells grown *in vitro* (13, 14). Unfortunately, data interpretation is difficult, because compared strains were nonisogenic. Recently, studies comparing isogenic mucoid and nonmucoid strains of *P. aeruginosa*, demonstrated that alginate overproduction and modification had a dramatic effect on biofilm structure and resistance to the antibiotic tobramycin (15, 16). However, because most clinical and environmental isolates of *P. aeruginosa* are nonmucoid, and biofilm formation is important in these niches, a careful examination of the role alginate plays in nonmucoid *P. aeruginosa* biofilms is warranted.

Despite the great interest in alginate, its biosynthesis, and the environmental signals that regulate its expression, there is little direct evidence to date that alginate is a significant component of wild-type *P. aeruginosa* biofilms. Perhaps the strongest supporting evidence is the detection of low levels of uronic acid-positive carbohydrates in *P. aeruginosa* biofilm EPS, using a general carbazole assay (17). In this article, we describe a series of experiments that demonstrate that alginate is not required for the initiation, maturation, or drug resistance of biofilms formed by two *P. aeruginosa* strains, PAO1 and PA14. In addition, gene fusion analyses, as well as physical and chemical studies of biofilm polysaccharides, reveal no detectable alginate or alginate

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Abbreviations: CF, cystic fibrosis; EPS, extracellular polymeric substance; LPS, lipopolysaccharide.

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gene expression in biofilms formed by PAO1 and PA14, despite the fact that these strains are fully capable of synthesizing alginate. These data suggest that the relationship between the conversion of mucoidy and *P. aeruginosa* biofilms is not simply a change in the alginate levels present in the biofilm EPS matrix, but a fundamental change in its carbohydrate constituents.

Materials and Methods

Strains, Plasmids, Media, and Growth Conditions. The *P. aeruginosa* strains used in this study are PAO1 (18) and PA14 (19), as well as *algD* mutant derivatives WFPA1 (Δ*algD*::*tet*) and PA14Δ*algD*, respectively. WFPA2 is a complemented (*algD*$^+$) derivative of WFPA1, generated by gene replacement of the Δ*algD*::*tet* allele with wild-type *algD* sequences present in pDJW482. *P. aeruginosa* strains WFPA220 and WFPA222, which harbored p*tac-gfp* at the neutral *att* site in PAO1 or WFPA1, respectively, were constructed with pTW16 by techniques outlined elsewhere (20). *P. aeruginosa* WFPA225 (*algD-xylE*), WFPA226 (*xylE*), and WFPA227 (*fliC-xylE*) were generated by similar strategies with plasmids pTW5, pTW8, and pTW13, respectively. The *Escherichia coli* strains used for routine cloning or mating experiments were JM109 (Promega) or SM10 (21). Plasmid pDJW487, which was used to create the Δ*algD*::*tet* mutation in strain PAO1, was generated as follows: an additional *Xho*I site was created in pDJW422 [*Hin*dIII–*Xho*I fragment from pCC27 (22)] cloned in pALTER-1 (Promega) 846-bp upstream of the *algD* translation start site by using the Altered Sites technique (Promega) and oligonucleotide *algD*37 (5′-CGGTAGCGCCTCGAGGCGT-CGGC-3′). The resulting plasmid, pDJW480, was cleaved with *Hin*dIII–*Xho*I and ligated with an 8-kb *Xho*I–*Hin*dIII fragment from pCC27 into the *Hin*dIII site of the gene-replacement vector pDJW527 (23). The subsequent plasmid, pDJW485, which harbored a deletion of the 5′ regulatory and most of the *algD* coding sequence, was cleaved with *Xho*I, treated with treated with the Klenow fragment of DNA polymerase, and a 2.0-kb *Sma*I *tet* fragment from pHP45ΩTc (24) ligated in, generating pDJW487. Plasmid pDJW482, which harbored a 10-kb *Hin*dIII fragment with wild-type *algD* sequences in pDJW527, was used for complementation studies. Plasmid pTW16 (p*tac-gfp*) was described (20). Plasmid pTW5, which contains the *algD-xylE* operon fusion in miniCTX (21), was constructed by cloning a 1.2-kb *Hin*dIII–*Xho*I fragment containing the *algD* promoter adjacent to the promoterless *xylE* gene from pX1918G (25). Plasmid pTW8 contains a promoterless *xylE* gene in miniCTX and was generated by deleting the 1.2-kb *Hin*dIII–*Xho*I *algD* fragment from pTW5, treating with treated with the Klenow fragment of DNA polymerase, and religation. Plasmid pTW13 harbors a *fliC-xylE* operon fusion in miniCTX (20). Media, growth conditions, and antibiotic concentrations for *E. coli* and *P. aeruginosa* are as outlined (20, 23).

Gene Replacement Strategies. Mutants of *P. aeruginosa* were generated by gene replacement techniques essentially as described (23, 25), using *sacB*-mediated counterselection strategies. Introduction of single-copy operon fusions or p*tac-gfp* fusions at the neutral *attB* site were by procedures outlined earlier (20, 21). All allele replacements and *attB*-integration events were confirmed by PCR analyses or Southern hybridizations.

Microtiter Dish and Flow Cell Culturing Techniques for Examining Biofilm Formation. These assays were performed as described (16, 31). COMSTAT software was applied to confocal microscopy images derived from flow cell experiments as described (16, 26). The growth medium used for flow cell experiments was Jensens medium supplemented with succinate (25 mM; ref. 27).

Biofilm Antibiotic Resistance Assays. For the microtiter dish assay, overnight bacterial cultures were subcultured into fresh medium, the bacteria was added to the wells of microtiter dishes (100 μl per well), and the plates were incubated for 24 h. The spent medium was removed and replaced with fresh medium containing the antibiotics and the plates were incubated for an additional 24 h. The antibiotic-containing medium was then removed and replaced with fresh antibiotic-free medium, the plates were incubated for an additional 24 h (during which time, any viable cells in the biofilm grew and replenished the planktonic population), and finally the medium was assessed for viable cells. The MBC-biofilm is defined as the minimal concentration of antibiotic required to kill all of the bacteria in a preformed biofilm. Additionally, the rotating disk assay for antibiotic resistance was performed as reported (16). The antibiotic tobramycin was applied at a range of 0.5–50 μg/ml.

XylE Fusion Analysis of algD Expression in Biofilms. *P. aeruginosa* strains WFPA225 (*algD-xylE*), WFPA226 (*xylE*), and WFPA227 (*fliC-xylE*) were grown in 15 × 100-mm Petri dishes containing 15 ml of inoculated LB medium at 37°C. Biofilms that formed at the air-liquid interface were harvested at the time points indicated in Fig. 2, and were assayed for XylE activity by techniques described elsewhere (20). The XylE enzyme activity was calculated by using the extinction coefficient of the reaction product, 2-hydroxymuconic semialdehyde ($\varepsilon_{375} = 4.4 \times 10^4\ M^{-1}$), and corrected for the number of cells by dividing by the A_{540} of the sample. Activities are thus expressed as nanomolar product liberated per minute per 10^9 cells (1 ml with $OD_{540} = 1$). All *xylE* constructs were placed at the neutral *attB* site (20, 21).

Isolation and Analysis of EPS from *P. aeruginosa* Biofilms. Biofilm EPS was isolated as described (28). *P. aeruginosa* strains depicted in Table 2 were cultured on LB plates covered with cellophane for 24 h at 37°C. PAO1 EPS was also prepared from biofilms grown at 37°C on the inside of 12-inch long (1 inch = 2.54 cm) pieces of silicone tubing [inside diameter (i.d.) 2 mm] with LB medium supplied as the growth medium as described (29).

For carbohydrate analysis, the sample was hydrolyzed with 1.0 M methanolic-HCl for 16 h at 80°C. The released methylglycosides were dried down and *N*-acetylated by using methanol and acetic anhydride (1:1; vol:vol) for 15 min at 45°C. The acetylate sample was trimethyl-sialylated with Tril-Sil and resolved on a 30-m DB-1 column (0.25 × 0.25 i.d.; J &W Scientific, Folsom, CA) in a Hewlett–Packard 5985 GC-MS system by using myo-inositol as an internal standard. The following temperature conditions were used: an initial temperature of 160°C, then raised to 200°C at 2°C per min, and increased to 260°C at 10°C per min. A standard of derivatized methylglycosides were also run alongside the samples for identification.

For linkage analysis, the sample was methylated by using the NaOH/Mel method (2). The methylated material was then isolated by extraction into methylene chloride, dried down, and subjected to sequential hydrolysis (2 M of trifluoroacetic acid at 120°C) reduction ($NaBD_4$) and acetylation (acetic anhydride, pyridine). The resulting partially methylated alditol acetates were analyzed by GC-MS using a fused-silica, 30-m Sp2330 column in a Hewlett–Packard 5985 GC-MS system, using myo-inositol as an internal standard. The following temperature conditions were used: starting with 2 min at an initial temperature of 80°C, which was increased to 170°C at 30°C per min, then to 240°C at 40°C per min, and held at 5 min at 240°C.

Uronic Acids Assay. Levels of uronic acid-positive material, including alginate, were determined directly from the supernatant fraction of planktonic *P. aeruginosa* cultures, using the carbazole assay as described, with modifications (23, 30), and *Macrocystis pyrifera*-derived alginate (Sigma) as a standard. This assay was also performed on biofilm biomass of *P. aeruginosa* strains recovered from microtiter dishes (31). Planktonic and biofilm

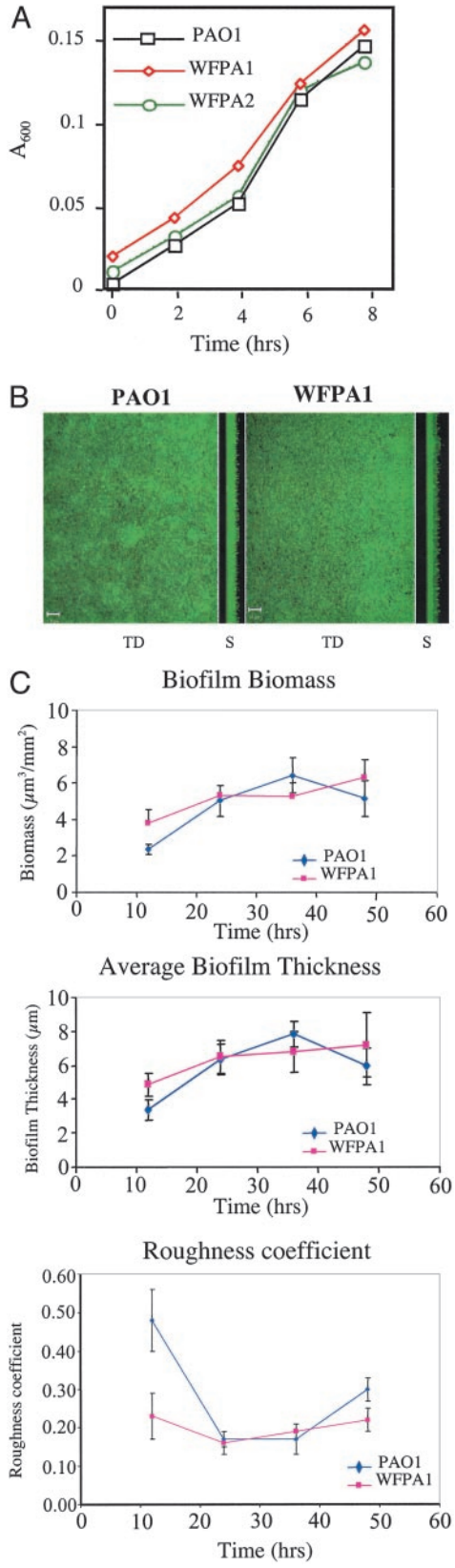

Fig. 1. Biofilm formation by *P. aeruginosa* PAO1 and PA14 strains. (*A*) Biofilm formation of PAO1, WFPA1 (Δ*algD*::*tet*), and WFPA2 (*algD*$^+$ derivative of WFPA1). Biofilm formation was assayed every 2 h during initiation by using the microtiter plate assay (47). Surface-attached cells were stained with crystal violet, the stain was solubilized in ethanol, and the absorbance was analyzed at 600 nm. (*B*) Biofilm formation of *P. aeruginosa* PAO1 and WFPA1 in a flow cell system as described in *Materials and Methods*. The bacteria in the micrograph are labeled with GFP. These micrographs were taken 24 h after inoculation of the system. The micrographs presented are a top-down view of the biofilm (TD) or a side view (S) generated with scanning confocal laser microscopy (SCLM) . The magnification is ×630. (Scale bar, 10 μm.) (*C*) COMSTAT analysis of confocal micrographs generated at different time points in *B*.

cultures were grown for 22 h at 30°C with LB as a growth medium.

Results

Alginate Expression Is Not Required for Initial Stages in Biofilm Development. Secreted polysaccharides have been shown to play a role in both the attachment of bacterial cells to surfaces and dictating the structure of mature biofilms. Therefore, we hypothesized that alginate would play an important role in one stage or possibly multiple stages of *P. aeruginosa* biofilm development. The majority of the genes involved in alginate biosynthesis, modification, and secretion are clustered in a large operon (PA3540-PA3551) (32, 33). The first gene of this operon, *algD*, encodes GDP mannose dehydrogenase, an enzyme essential for alginate synthesis (34). The *algD* gene product catalyzes the first committed step in alginate synthesis and is not known to be required for any other cellular process. We assayed *algD* mutants in two *P. aeruginosa* strains classically used to study biofilms PAO1 and PA14 (35) and compared the kinetics of biofilm formation with isogenic wild-type strains, using the microtiter dish assay. Fig. 1*A* shows the kinetics of biofilm formation of *P. aeruginosa* PAO1, its *algD* derivative, WFPA1, as well as WFPA2, a strain complemented with wild-type *algD* sequences, using gene replacement strategies. To our surprise, all three strains exhibited no observable difference in the rate or extent of biofilm formation over the 8-h testing period. Similarly, the amount of biofilm biomass produced by strain PA14 at 8 h (data not shown) and by PAO1 at 24 h (Fig. 1*B*) was identical to the biofilm formed by the Δ*algD* strain used in this assay. These data suggest that alginate is not required for the early steps in biofilm initiation for either PAO1 or PA14.

To further explore the role of alginate, we examined *algD* expression in *P. aeruginosa* biofilms. We constructed two PAO1-derived strains, WFPA225 and WFPA227, which harbored ectopic chromosomal operon fusions of the promoterless *xylE* gene with *algD* or *fliC*, respectively. The *fliC* gene, which encodes flagellin, the major structural subunit of the *P. aeruginosa* flagellum, was chosen as a positive control because earlier studies indicated that flagellar-mediated motility is required for biofilm initiation (31). In addition, strain WFPA226, which harbors a promoterless *xylE* gene was generated. Previous studies (36) have suggested that expression of alginate biosynthetic genes is induced after attachment of *P. aeruginosa* to a surface. Therefore, this study was performed by using microtiter dish-grown biofilms, which facilitates analysis of early events in biofilm development. As depicted in Fig. 2, whereas *fliC-xylE* expression was readily observed, no *algD* expression was detected in biofilm-grown cells. To further verify the functionality of this reporter construct, we introduced the *algT* gene in trans into PAO1 harboring the *algD-xylE* reporter. AlgT is an alternative σ-factor that induces expression of the alginate biosynthetic operon. Expression of the *algD-xylE* fusion was induced at least a hundred-fold when *algT* was supplied in trans (data not shown).

The Architecture and Antibiotic Resistance Profiles of Wild-Type and Alginate-Deficient Biofilms Are Identical. Unlike microtiter-plate-grown biofilms, flow cell biofilm cultures are amenable to microscopy and allow the culturing of biofilms for longer periods of time. Because alginate expression may have an effect on

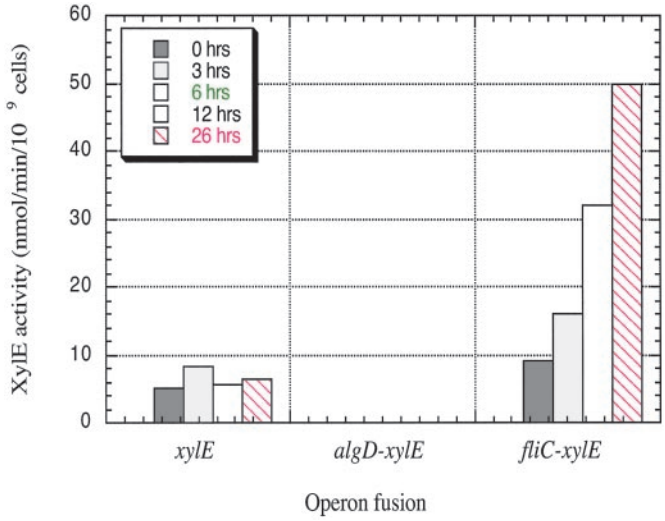

Fig. 2. Alginate gene expression in *P. aeruginosa* biofilms. Expression of alginate (*algD*) and a positive control (*fliC*) at different time points after biofilm formation at an air-liquid interface (see *Materials and Methods*). This graph shows XylE activity of three different reporter strains over time. The negative control (*xylE*) harbors a promoterless fusion gene integrated onto the chromosome. The strains used for this experiment were WFPA225 (*algD-xylE*), WFPA226 (*xylE*), and WFPA227 (*fliC-xylE*).

biofilm structure and may play a role at later stages of biofilm formation, we decided to use flow cells to monitor biofilm development over time. We generated GFP-tagged derivatives of PAO1 (WFPA220) and a Δ*algD*::*tet* derivative (WFPA222) and combined flow cell culturing technology with confocal scanning laser microscopy (CSLM) to examine the architecture of these strains. There were no observable differences in biofilm structure between the two strains throughout the course of biofilm growth (up to 72 h). A representative fluorescent micrograph displaying the architecture of a 24-h-old biofilm is shown in Fig. 1*B*. PA14 and PA14Δ*algD* also produced biofilms that were indistinguishable by microscopy (data not shown). We used the COMSTAT software package to quantify three key parameters of biofilm architecture from the CSLM images generated from flow cell biofilms: average biofilm thickness, biofilm roughness (a measure of biofilm heterogeneity), and total biofilm biomass (Fig. 1*C*). Interestingly, PAO1 produced biofilms with little structural heterogeneity in this growth medium, although this phenomenon has been previously reported for this bacterium. These data support the finding that alginate is not required for the formation and maintenance of biofilm architecture in *P. aeruginosa* PAO1 and PA14. Taken together, the data in Figs. 1 and 2 strongly suggest that alginate plays no significant role in biofilm development.

Alginate and Antimicrobial Resistance. A hallmark characteristic of biofilm populations is their increased antimicrobial resistance to planktonic bacteria of the same species (2, 3). Previous studies have demonstrated that exopolysaccharide production can influence the antimicrobial resistance properties of biofilms. Therefore, we compared the antibiotic resistance profiles of wild-type and *algD* mutant biofilms using two experimental systems. In the first system, biofilms are formed in microtiter dishes for 24 h, treated with antibiotics for 24 h, then viable cells remaining are determined. In this assay, antibiotic resistance increases from 10- to 100-fold over planktonically grown cells, depending on the antibiotic tested. The results of these assays are shown in Table 1. We observed no difference in the antibiotic resistance of the wild-type or Δ*algD* mutant in the *P. aeruginosa*

Table 1. Antibiotic resistance profiles of *P. aeruginosa* biofilms

Drug/strain	PA14	PA14 *algD*	PAO1	PAO1 *algD*
Gm	0.25	0.25	0.25	0.25
Tb	0.1	0.1	0.1	0.1
Cip	0.005	0.25	0.025	0.025

MBCs are all in mg/ml.

PA14 background for the three antibiotics tested. Similarly, the resistance profiles of the PAO1 strains are nearly identical for tobramycin and gentamycin. Interestingly, the *algD* mutant of *P. aeruginosa* PA14 appears to have increased resistance to ciprofloxacin, although the basis for this increased resistance is not clear.

The second experimental system is a spinning disk reactor, previously used to evaluate antibiotic resistance in *P. aeruginosa* biofilms (16). This reactor allows cultivation of biofilms on 18 polycarbonate chips, which are inserted into a lexan disk. This disk is rotated in a bacterial chemostat liquid culture. The chips can be removed and the biofilms subjected to antibiotic treatment. The antibiotic resistance of the biofilm population can then be compared with the chemostat liquid culture. Besides providing complementary data for the microtiter plate analysis, this reactor system also allows a more careful statistical comparison of antibiotic-resistance profiles of biofilm populations. PAO1 and Δ*algD* biofilms grown for 24 h were subjected to a range of tobramycin concentrations (0.5–50 μg/ml) for 5 h. The antibiotic-resistance profiles were similar for the two strains, with the MBC for tobramycin at 25 μg/ml (data not shown). These results indicate little or no role for alginate in the enhanced antibiotic-resistance phenotypes of biofilm-grown PAO1 and PA14 *P. aeruginosa*.

Analysis of Planktonic and Biofilm EPS Using a Uronic Acids Assay. Previous studies (37, 38) implicating alginate in *P. aeruginosa* biofilms relied on a chemical carbazole-based assay for detection of uronic acids (17). We applied this assay to planktonic and biofilm-grown PAO1, the Δ*algD* strain WFPA1, PDO300, a *mucA* derivative of PAO1, which produces alginate (16), and FRD1, an alginate-producing CF isolate (39). We readily detected uronic acids from both PDO300 and FRD1, yet were unable to observe differences between PAO1 and WFPA1, even though they both produced low amounts of material that reacted in the carbazole assay (Table 2). This finding suggests that some component of EPS derived from a source other than alginate is responsible for the weak activity detected in this assay.

Chemical Analysis of Biofilm-Grown *P. aeruginosa* EPS. The data described above indicated that alginate was neither expressed nor required for biofilm structure or antibiotic resistance. We wished to directly examine the exopolysaccharide composition of the EPS of biofilm populations. Specifically, we wanted to determine whether mannuronate or guluronate residues, indic-

Table 2. Uronic acids assay on planktonic supernatants and biofilm EPS

		Uronic acid, μg uronic acid/OD_{600} culture	
Strain	Genotype	Planktonic	Biofilm
PAO1	Wild-type	2.2 ± 0.2	7.6 ± 1.0
WFPA1	PAO1 Δ*algD*::*tet*	2.4 ± 0.3	9.3 ± 1.2
PDO300	PAO1 *mucA22*	9.6 ± 0.4	26.4 ± 4.0
FRD1	CF isolate *mucA22*	38.8 ± 1.7	48.7 ± 6.5

Table 3. Biofilm EPS carbohydrate monomer composition profiles

Carbohydrate	PAO1	PDO300	*algD*	PA14
Mannuronic acid	0.0	100.0	0.0	0.0
Glucose	41.0	0.0	56.0	37.9
Rhamnose	14.3	0.0	6.4	20.7
Galactose	0.0	0.0	12.4	1.9
Mannose	13.9	0.0	8.5	4.7
Xylose	9.7	0.0	0.0	0.0
KDO	9.1	0.0	7.0	5.3
N-acetyl galactosamine	1.9	0.0	0.0	0.0
N-acetyl fucosamine	7.5	0.0	6.1	0.0
N-acetyl glucosamine	2.6	0.0	3.6	3.8
N-acetyl quinovosamine	0.0	0.0	0.0	18.1
Unknown amino sugar	0.0	0.0	0.0	7.5

Data are presented as percent molar composition.

Table 4. Biofilm EPS carbohydrate linkages

Linkage	Relative ratio*
Terminal glucose	0.53
Terminal mannose†	
3-linked glucose	1.0
4-linked glucose	0.40
6-linked glucose	0.73
2-linked rhamnose	0.07
3-linked rhamnose†	
2-linked mannose	0.24
4-linked mannose†	
4-linked xylose	0.14
3,6-linked glucose	0.52
2,3-linked rhamnose	0.22
2,6-linked mannose	0.25

*Ratio of the GC peak areas with 3-linked glucose set to 1.0.
†GC peaks not fully resolved.

ative of alginate, could be detected. To accomplish this goal, we used a procedure recently performed to define the *Vibrio cholerae* rugose-associated EPS (28). EPS was partially purified from biofilms of several *P. aeruginosa* strains grown on solid medium and the sugar monomer and linkage composition was determined (see *Materials and Methods*). The carbohydrate composition of EPS prepared from PAO1 and the isogenic Δ*algD* strain were nearly identical (Table 3). Significantly, there was no detectable mannuronic or guluronic acid present in the EPS preparations of either PAO1 or PA14. The primary carbohydrate constituents of the biofilm EPS were glucose, rhamnose, and mannose. An analysis of the *algD* mutant of PAO1 revealed an EPS profile similar to PAO1 (Table 3). Interestingly, an unknown amino sugar was detected in the PA14 EPS. The presence of *N*-acetyl sugars, as well as ketodeoxyoctulosonate (KDO), indicated the presence of a low level of lipopolysaccharide in the EPS sample. As a control, EPS was purified from an isogenic mucoid PAO1-derivative, PDO300. As expected, an analysis of the EPS harvested from PDO300 revealed that the primary sugar detected was mannuronic acid. These results are consistent with previous analyses of mucoid *P. aeruginosa*, which demonstrated that mannuronic acid is by far the primary carbohydrate component (40). To verify that the EPS of biofilms grown on solid medium is comparable to biofilms grown in flow cells and other liquid/solid interface biofilm culturing methods used in this article, we determined the monomer composition of PAO1 EPS isolated from biofilms grown on the interior of silicone tubing fed with a continuous supply of LB. We found that the monomer composition was almost identical to the EPS data presented in Table 3 (data not shown). The biofilm carbohydrate EPS was further characterized by glycosyl linkage analysis (Table 4). The primary linkages observed in the EPS were 3-linked, 4-linked, and 6-linked glucose, 2-linked and 3-linked rhamnose, and 2-linked and 4-linked mannose.

Discussion

In this article, we provide experimental evidence that alginate is not a major constituent of the extracellular matrix of *P. aeruginosa* PAO1 and PA14 biofilms. Wild-type and alginate biosynthetic mutants formed biofilms that had no difference in structural architecture or antibiotic sensitivity. Furthermore, transcriptional reporter fusion data indicate that alginate biosynthetic gene expression is not induced on initiation of biofilm formation in a microtiter plate assay. A uronic acids assay traditionally used to detect alginate in biofilm samples was shown to have similar, low levels of activity on samples prepared from both a wild-type and an *algD* mutant strain. This finding indicates something other than alginate is crossreacting in the assay. Finally, a chemical analysis of carbohydrate monomers of PAO1 biofilm EPS failed to detect either of the primary sugars of alginate.

Many previous studies have focused on the role alginate plays in mucoid strains of *P. aeruginosa*. In these studies, alginate overproduction was shown to influence biofilm architecture and sensitivity to the antibiotic tobramycin (15, 16). *O*-acetylation of alginate has also been shown to affect the architecture of *P. aeruginosa* biofilms (15). Previous studies by Davies *et al.* (36) measured expression of *algC*, an alginate biosynthetic gene, and found that expression was induced upon attachment to a surface. These data were generated in a mucoid derivative of PAO1, 8830. Interpretation of this data is complicated by the fact that the *algC* gene product is also involved in the biosynthesis of rhamnolipid and lipopolysaccharide (LPS). Wingender *et al.* (41) analyzed the sugars present in a mucoid strain of *P. aeruginosa*, SG81, and demonstrated that besides uronic acids, other carbohydrates were present in the sample. Perhaps the strongest evidence that alginate might be an important component of biofilms is the fact that EPS prepared from *P. aeruginosa* biofilms have detectable levels of uronic acids. Combined with the data that PAO1 and *algD* supernatants show identical levels of low activity in the assay suggests that some other component of biofilm EPS is responsible for this activity.

Our findings indicate that alginate is not the primary structural matrix or "scaffolding" of *P. aeruginosa* PAO1 and PA14 biofilms. Structural and antibiotic-resistance profiles of wild-type and *algD* mutant biofilms are indistinguishable, both in PAO1 and PA14. This result raises the question as to what, if any, secreted polysaccharides are important in *P. aeruginosa* biofilm development. A recent study indicates the importance of nucleic acids for the structural integrity of a *P. aeruginosa* biofilm in the early stages of development (42). Interestingly, this nucleic acid reacted in the carbazole assay and may contribute to the low level of reactivity we observe in PAO1 and *DalgD* liquid culture supernatants or biofilms (42). Another candidate molecule that might provide structural integrity to a biofilm is LPS. LPS has been demonstrated to affect attachment of bacteria to a surface (43). Many of the sugars identified in our carbohydrate analysis correspond to sugars found in *P. aeruginosa* LPS (e.g., KDO, *N*-acetyl sugars, glucose, etc). Both rhamnose and glucose are core monosaccharides, whereas rhamnose is also an important component of A-band LPS (44, 45). The presence of 2-linked and 3-linked rhamnose sugars further supports that our EPS samples contain LPS sugars (Table 4). However, there are other sugars present in the EPS samples that are not associated with LPS, such as xylose and mannose. Why xylose is present in PAO1 biofilm EPS and not in the *algD* mutant is unclear. Interestingly,

PA14 biofilm EPS not only lacked detectable uronic acids, but indicated the presence of an unidentified amino sugar. These data suggest that some other, yet unidentified exopolysaccharide, might be the predominant scaffolding in *P. aeruginosa* wild-type biofilms. Previously, *P. aeruginosa* was reported to produce a secreted polysaccharide composed of primarily mannose and rhamnose (46). This polysaccharide could potentially play a role in PAO1 and PA14 biofilms. However the linkage data (Table 4) suggest against the presence of this type of EPS, because the type of mannose and glucose linkages specific to that EPS were not detected in our analysis. We should also emphasize that although alginate was not found to be important for PAO1 or PA14 biofilms for any of the culturing methods or assays used in this article, we cannot rule out the possibility that alginate expression is important for biofilm development or function under yet unidentified environmental conditions.

The transition of *P. aeruginosa* from a nonmucoid to a mucoid phenotype has severe consequences in CF pathogenesis and biofilm formation. The role of alginate in mucoid biofilms is unquestionably important. However, one must consider that the onset of biofilm formation in the CF lung during initial colonization likely precludes the switch to a mucoid phenotype. Although these data were generated from laboratory biofilms grown on abiotic surfaces, they suggest that this transition from a nonmucoid to a mucoid phenotype may involve a switch from an as-yet-unidentified exopolysaccharide as being the primary polysaccharide component of EPS to alginate. Understanding the dynamic nature of *P. aeruginosa* biofilm EPS production and regulation will shed new light on understanding chronic biofilms in CF.

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