Putative Exopolysaccharide Synthesis Genes Influence *Pseudomonas aeruginosa* Biofilm Development

Masanori Matsukawa and E. P. Greenberg*

Department of Microbiology and the W. M. Keck Microbial Communities and Cell Signaling Program,
Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

Received 16 December 2003/Accepted 20 February 2004

An analysis of the *Pseudomonas aeruginosa* genomic sequence revealed three gene clusters, PA1381-1393, PA2231-2240, and PA3552-3558, in addition to the alginate biosynthesis gene cluster, which appeared to encode functions for exopolysaccharide (EPS) biosynthesis. Recent evidence indicates that alginate is not a significant component of the extracellular matrix in biofilms of the sequenced *P. aeruginosa* strain PAO1. We hypothesized that at least one of the three potential EPS gene clusters revealed by genomic sequencing is an important component of *P. aeruginosa* PAO1 biofilms. Thus, we constructed mutants with chromosomal insertions in PA1383, PA2231, and PA3552. The mutant with a PA2231 defect formed thin unstructured abnormal biofilms. The PA3552 mutant formed structured biofilms that appeared different from those formed by the parent, and the PA1383 mutant formed structured biofilms that were indistinguishable from those formed by the parent. Consistent with a previous report, we found that polysaccharides were one component of the extracellular matrix, which also contained DNA. We suggest that the genes that were inactivated in our PA2231 mutant are required for the production of an EPS, which, although it may be a minor constituent of the matrix, is critical for the formation of *P. aeruginosa* PAO1 biofilms.

Pseudomonas aeruginosa is an important opportunistic pathogen that is capable of causing several different types of infections, including chronic biofilm infections. For example, *P. aeruginosa* infects the lungs of people with the genetic disease cystic fibrosis (CF), and chronic CF lung infections are considered biofilm infections (5, 12, 30). Mature *P. aeruginosa* biofilms consist of groups of cells growing together in clusters embedded in an extracellular polymeric matrix (4, 5). Quite often, *P. aeruginosa* isolated from the lungs of CF patients exhibits a mucoid phenotype resulting from the overproduction of the exopolysaccharide (EPS) alginate (12, 34). However, strains that do not produce much alginate are capable of forming mature biofilms in vitro (18). In fact, the commonly studied laboratory strain PAO1 makes little or no alginate (17, 36).

If alginate is not the extracellular matrix for biofilms of strain PAO1, what is? A recent report showed that a major polymer in the extracellular matrix of *P. aeruginosa* PAO1 biofilms is DNA (35). However, this does not rule out the possibility that *P. aeruginosa* PAO1 makes an EPS other than alginate and that this polysaccharide is an important constituent of the extracellular polymeric matrix.

A clue that polysaccharides other than alginate might be involved in *P. aeruginosa* biofilms came from the *P. aeruginosa* genome sequencing project. The annotation of the genome suggested that there are gene clusters in addition to the alginate biosynthesis gene cluster that may be involved in the synthesis of EPSs. One of these clusters is PA1381-1393, a second is PA2231-2245, and a third is PA3552-3558 (31). The PA1381-1393 cluster has an anomalously low G+C content, and the encoded polypeptides show sequence similarity to

polypeptides encoded by a gene cluster which is required for polymyxin resistance and for the aminoarabinose modification of lipid A in *Salmonella enterica* serovar Typhimurium (9). The PA3552-3558 cluster is adjacent to a cluster of alginate biosynthetic genes. Many of the polypeptides encoded by genes in the PA2231-2245 cluster show similarity to glycosyltransferases, export proteins, or polysaccharide polymerases. The first three genes in this cluster have been studied by Rocchetta et al. (23), who suggested that PA2232 is involved in the biosynthesis of the lipopolysaccharide (LPS) A-band O antigen.

We generated mutations in one gene selected from each cluster (PA1383, PA2231, and PA3552) and studied the influence of the mutations on biofilm development. Our PA2231 mutant showed a severe defect in biofilm formation. Thus, we studied this mutant in more detail. Our results, taken together with those reported in accompanying papers (8, 13), indicate that the PA2231 gene cluster is an important determinant of *P. aeruginosa* biofilm development. We have adopted the nomenclature of Jackson et al. (13), and we will refer to the genes of the PA2231-2245 cluster as *psl* (polysaccharide synthesis locus) genes throughout the remainder of this article.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The strains and plasmids used for this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar. For growth curves and microtiter dish biofilm assays, *P. aeruginosa* was grown in tryptic soy broth (TSB) (Difco). The medium for flow cell biofilms was 1% TSB. Agar plates of Vogel-Bonner minimal medium (33) were used to select exconjugants when pEX18Tc was used as a suicide vector. For EPS purification and for experiments in which double-stranded DNA was visualized by staining the biofilms with PicoGreen (Molecular Probes Inc.), we used M63 minimal medium supplemented with 3% glycerol, 0.4% glutamate, and trace minerals (0.0013% EDTA, 0.0055% ZnSO₄·7H₂O, 0.0025% FeSO₄, 0.00078% MnSO₄·H₂O, 0.0002% CuSO₄·5H₂O, 0.00012% Co(NO₃)₂·5H₂O, and 0.000089% Na₂B₄O₇·10 H₂O). Media were supplemented with antibiotics for marker selection or plasmid maintenance as follows. For *E. coli*, we used 100-μg/ml ampicillin, 15-μg/ml

* Corresponding author. Mailing address: Department of Microbiology, 540 F Eckstein Medical Research Building, Carver College of Medicine, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7775. Fax: (319) 335-7949. E-mail: everett-greenberg@uiowa.edu.

TABLE 1. Strains and plasmids used for this study

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
<i>P. aeruginosa</i>		
PAO1	Parent	B. Iglewski
Δ 1383Sm	PA1383 mutant of PAO1, Sm ^r Sp ^r	This study
Δ pslAGm	PA2231 mutant of PAO1, Gm ^r	This study
Δ pslAnull	PA2231 mutant of PAO1, unmarked	This study
Δ 3552Gm	PA3552 mutant of PAO1, Gm ^r	This study
<i>E. coli</i>		
DH5- α		24
S17- λ pir	<i>thi pro hsdR recA RP4-2</i> (Tet::Mu)(Km::Tn7) λ	21
SM10	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i> Km ^r	28
Plasmids		
pKV69	Suicide vector, Cm ^r Tc ^r	3
pGm Ω 1	Source of Ω (<i>aacC1</i> with transcriptional termination signals in both orientations), Amp ^r	27
pHRP315	Source of Ω (<i>aadA</i> with transcriptional termination signals in both orientations), Sm ^r Sp ^r	20
pEX18Tc	Suicide vector, Tc ^r	11
pMRP9-1	GFP expression vector, Cb ^r	6
Mini-CTX- <i>lacZ</i>	Suicide vector, Tc ^r	2
pMM11	pKV69 carrying 4.7-kb (from bp 1500183 to 1505052) fragment containing PA1383, in BamHI-SphI site	This study
pMM12	pMM1 with <i>aadA</i> Sm ^r Sp ^r (SmaI-EcoRV fragment from pHRP315) cassette between the two BstEII sites of ORF PA1383	This study
pMM21	pKV69 carrying 4.4-kb (from bp 24521491 to 2456529) fragment containing PA2231, in BamHI-PstI site	This study
pMM22	pMM2 with <i>aacC1</i> Gm ^r cassette (SmaI fragment from pGm Ω 1) between the two MluI sites of ORF PA2231	This study
pMM31	pEX18Tc carrying 1.2-kb (from bp 3978642 to 3979870) fragment of PAO1 chromosomal DNA in HindIII-BamHI site	This study
pMM32	pMM3 with 1.2-kb (from bp 3981000 to 3982279) fragment of PAO1 chromosomal DNA in BamHI-EcoRI site	This study
pMM33	pMM31 with <i>aacC1</i> Gm ^r cassette (BamHI fragment from pGm Ω 1) in BamHI site	This study
pMMC2231	Mini-CTX- <i>lacZ</i> carrying 1.9-kb (from bp 2453177 to 2455101) fragment of PAO1 chromosomal DNA in BamHI-SphI site	This study

gentamicin (GEN), 25- μ g/ml chloramphenicol, 100- μ g/ml streptomycin (STR), 12- μ g/ml tetracycline (TET), and 20- μ g/ml nalidixic acid. For *P. aeruginosa*, we used 150- μ g/ml carbenicillin, 100- μ g/ml GEN, 500- μ g/ml STR, and 50- or 100- μ g/ml TET.

Construction of *P. aeruginosa* mutants. We used standard methods for manipulating plasmids and DNA fragments (1). PA1383 was interrupted by the insertion of an *aadA* gene cassette (Sm^r Sp^r). A 4.7-kb *P. aeruginosa* PAO1 chromosomal DNA fragment (from bp 1500183 to 1505052) containing PA1383 was amplified by use of an Expand long-template PCR kit (Roche Applied Science). The forward primer contained a BamHI site (5'-CGGGATCCGGAG GACGAGATCGAAACGCCATC-3') and the reverse primer contained an SphI site (5'-ACATGCATCGCAAGGACCAACCTGATCGAACAGG-3'). The PCR fragment was cloned into BamHI- and SphI-digested pKV69 to give pMM11. A 2.0-kb SmaI-EcoRV fragment of pHRP315 containing the *aadA* gene was ligated with BstEII-digested pMM11, which had been polished with T4 DNA polymerase, to create pMM12. There is an insertion in pMM12 575 bp downstream of the translational start site of PA1383 and 736 bp upstream of the stop codon. For construction of the mutant, pMM12 was mobilized from *E. coli* S17- λ pir into *P. aeruginosa* PAO1 by conjugation as follows. Ten microliters of a late-logarithmic-phase culture of *E. coli* S17- λ pir(pMM12) grown at 30°C was mixed with 100 μ l of a late-logarithmic-phase culture of *P. aeruginosa* PAO1 grown at 42°C without shaking. The mixture was spread onto LB agar plates. After 16 to 24 h at 30°C, transconjugants were selected by plating on LB agar containing GEN or STR. We then screened for strains showing sensitivity to TET. Southern blotting was used to confirm that the TET-sensitive strain contained an *aadA* insertion in PA1383.

We constructed two PA2231 (*pslA*) mutants. One contained an *aacC1* cassette (Gm^r) with a transcriptional terminator inserted in *pslA*. The other was a deletion mutant. The insertion mutant, *P. aeruginosa* Δ pslAGm, was constructed as follows. A 4.3-kb PAO1 PA2231 chromosomal DNA fragment (bp 2452149 to 2456529) was amplified by Expand long-template PCR (Roche Applied Science). The 5' primer contained a BamHI site (5'-CGGGATCCGCGATATCCTGCA ACTGGTCGAAC-3') and the 3' primer contained a PstI site (5'-AACTGCA GATGTCGTTGAAGCGGACGATGC-3'). The PCR product was cloned into BamHI- and PstI-digested pKV69 to give pMM21. A 1.6-kb SmaI-EcoRV fragment of pGm Ω 1 that contained the *aacC1* gene was ligated with MluI-digested pMM21, which had been polished with T4 DNA polymerase, to create pMM22. This construct has the *aacC1* gene inserted 808 bp downstream of the transla-

tional start site of PA2231 and 208 bp upstream of the stop codon. The *aacC1* insertion mutation was confirmed by Southern blotting. The generation of a PA2231 mutation in *P. aeruginosa* PAO1 with pMM22 involved a procedure similar to that described for the construction of the PA1383 mutant (see above). To generate the *pslA* deletion mutant, we amplified Δ pslAnull, a 1.2-kb PAO1 chromosomal DNA fragment extending from 1,051 bp upstream through 51 bp downstream of the predicted *pslA* start codon, by Expand long-template PCR. The 5' primer contained a HindIII site (5'-CCCAAGCTTGACAACCTCTGC GAGATCT-3') and the 3' primer contained an XbaI site (5'-GCTCTAGAGA AGTACTCGATGAATCCAG-3'). The PCR product was cloned into HindIII- and XbaI-digested pEX18Tc to give pMM201. A 1.2-kb PAO1 chromosomal DNA fragment extending from 23 bp upstream to 1,176 bp downstream of the PA2231 stop codon was amplified with a 5' primer containing an XbaI site (5'-GCTCTAGACTCTGACCAAGGAAGTCTA-3') and a 3' primer containing an EcoRI site (5'-CGGAATTCGGATGCGCTTGATCTTGAAG-3'). The PCR product was cloned into XbaI- and EcoRI-digested pMM201 to generate pMM202. For the generation of *P. aeruginosa* Δ pslAnull, *E. coli* SM10(pMM201) was mated with *P. aeruginosa* PAO1. Equal amounts of late-logarithmic-phase cultures of *E. coli* grown at 30°C with shaking and of strain PAO1 grown at 42°C without shaking were mixed and grown on LB agar. Cells from the agar plates were suspended in phosphate-buffered saline, and a strain with a single crossover was selected on minimal medium plus TET. Exconjugants that were resistant to TET were counterselected by using LB agar plus 5% sucrose. The resulting strain, Δ pslAnull, was shown to have a PA2231 deletion by PCR.

The generation of the PA3552 mutant was done by the insertion of an *aacC1* cassette. A 1.2-kb PAO1 chromosomal DNA fragment upstream of PA3552 (bp 3978642 to 3979870) was amplified by Expand long-template PCR with a forward primer containing a HindIII site (5'-CCCAAGCTTCTGGAAGAACTGAAGA AGCAGCAGGCC-3') and a reverse primer with a BamHI site (5'-CGCGGA TCCGTCAGTGACATGTAATGAAGCCTCGG-3'). The PCR product was cloned into HindIII- and BamHI-digested pEX18Tc to yield pMM31. A 1.3-kb PAO1 chromosomal DNA fragment (bp 3981000 to 3982279) downstream of PA3552 was amplified in a similar fashion by using the forward primer 5'-CGG GATCCGTCGATGAAGCCCTATCCGATCGACCT-3' and the reverse primer 5'-CGGAATTCGGTAGTAGAAGGAGAACAGGAAGTCC-3'. The PCR fragment was cloned into BamHI- and EcoRI-digested pMM31 to create pMM32. A 1.6-kb *aacC1*-containing BamHI fragment of pGm Ω was ligated with BamHI-digested pMM32 to create pMM33. This construct has the insertion 15

bp downstream of the translational start site of open reading frame (ORF) PA3552 and 10 bp upstream of the stop codon. To generate a *P. aeruginosa* PA3552 mutant strain, we used a procedure similar to that described above for the generation of the PA2231 *aacCI* insertion mutant.

Biofilm experiments. Biofilm formation in a static system was measured as described by O'Toole and Kolter (19), with some modifications. Briefly, TSB (100 μ l/well in 96-well polystyrene microtiter dishes) was inoculated with a mid-exponential-phase culture to give an optical density at 600 nm of 0.005. After 24 h at 37°C in a humidified chamber, the liquid culture was removed by aspiration and the biofilm attached to the microtiter dish wells was stained with 0.1% crystal violet for 15 min. The crystal violet solution was removed by aspiration, and the wells were rinsed three times by submerging the plates in distilled water. The crystal violet remaining in the wells was dissolved in 200 μ l of 95% ethanol–5% acetic acid and measured as the absorbance at 590 nm. We also grew biofilms in flow cell microscopic observation cells. For these experiments, *P. aeruginosa* contained pMRP9-1, which codes for green fluorescent protein (GFP) (6). This plasmid did not influence the planktonic growth of the *P. aeruginosa* strains used for our studies (data not shown). The flow cells and conditions have been described elsewhere (29). Biofilm architecture was monitored by scanning confocal laser microscopy (SCLM). For experiments with the DNA stain PicoGreen, we used a 1:1,000 dilution of PicoGreen gently injected into flow cells in which biofilms of *P. aeruginosa* without the GFP plasmid were grown.

Motility assays. Both swimming and twitching motilities were measured by standard techniques, as described elsewhere (22).

Analysis of extracellular and cell-surface-associated polymers. We obtained extracellular polymeric matrix material by growing *P. aeruginosa* strains in 12 large petri dishes (150-mm diameter), each of which contained 75 ml of M63 minimal broth, at 37°C for 48 h without agitation. The gelatinous material loosely attached to the bottom of the petri dishes was harvested by use of a glass spreader. The harvested material was centrifuged at 15,000 $\times$ g at 42°C for 1 h. The supernatant fluid was removed and the pellet was suspended in 0.4 M NaCl (final volume, 500 ml). For the extraction of extracellular polymeric substances, the suspension was placed in a 2-liter flask and stirred at 42°C for 30 min. The suspension was centrifuged and the pellet was suspended in 250 ml of 0.4 M NaCl. The supernatant fluid was combined with the supernatant fluid resulting from the first extraction. The EPSs and other polymers were extracted with 95% ethanol at 42°C for 12 h. The precipitated material was collected by centrifugation (4,000 $\times$ g at 4°C for 30 min), washed with 95% ethanol, and then washed with absolute ethanol. The washed material was dried and then dissolved in 100 ml of deionized water. Polysaccharides were purified from the resulting viscous solution as follows. One-hundred milliliters of Tris (60 mM, pH 7.5)–MgCl₂ (20 mM) and 10 μ g of DNase I (Sigma) and RNase A (Qiagen)/ml were added and the solution was incubated at 37°C for 6 h. This resulted in a decrease in viscosity. After the nuclease treatment, 100 μ g of proteinase K (Gibco-BRL)/ml was added and the solution was incubated overnight at 55°C. The precipitate formed by this procedure was removed by centrifugation and the supernatant fluid was extracted with hot phenol. Sodium acetate (3 M) was added to bring the acetate concentration to 0.3 M, followed by the addition of ethanol to bring the final ethanol concentration to 80%. After an overnight incubation at 42°C, the precipitate was collected by centrifugation, dissolved in deionized water, and dialyzed against deionized water. The dialysate was lyophilized, and the lyophilized material was analyzed at the University of Georgia Complex Carbohydrate Research Center (37).

To determine the polymer composition of the extracellular matrix, we measured the total carbohydrate, double-stranded DNA, and protein in the crude extracts by a phenol-sulfuric acid assay with glucose as the standard (7), the PicoGreen system with calf thymus DNA as the standard, and a protein assay kit (Sigma) with bovine serum albumin as the standard, respectively. To purify LPS, we used the Hitchcock and Brown method (10). The purified LPS was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15, 32).

RT-PCR. The total RNA was isolated from cells grown to an optical density (600 nm) of 2.0 in LB broth by use of a Qiagen RNeasy kit. The DNA was removed as described elsewhere (26). The RNA was then extracted twice with TRIzol (Invitrogen) and once with chloroform-isoamyl alcohol. DNA contamination of the purified RNA was assessed by Expand long-template PCR using *rplU* primers. Reverse transcription-PCR (RT-PCR) was performed as described elsewhere (16). The cDNA was purified with a Qiagen PCR purification kit and amplified (50 ng) by PCRs (30 cycles) with primers for a series of adjacent genes extending from PA2230 to PA2245 (the sequences of the primers are available upon request). The primers were designed to anneal approximately 250 bp upstream or downstream of the predicted intergenic regions. Genomic DNA was used as a positive control for PCRs.

FIG. 1. Growth of the parent and mutant strains in static biofilm cultures in microtiter dishes (A) and in nonbiofilm broth cultures (B). (A) Bars indicate the standard deviations around the means from five independent experiments, with each sample assayed in triplicate. *, $P < 0.001$ (Mann-Whitney U test). (B) Growth curves for *P. aeruginosa* PAO1 (open circles), Δ pslGm (open squares), Δ 1383Sm (filled squares), and Δ 3552Gm (filled circles).

RESULTS

Influence of mutations in putative EPS synthesis genes on biofilm development. We first examined the biofilm formation of each of the insertion mutants by using a microtiter dish assay (Fig. 1A). In this assay, the parent, the PA1383 mutant, and the PA3552 mutant showed similar levels of biofilm formation. The Δ pslGm mutant produced substantially smaller amounts of biofilm in microtiter dish wells than did the other strains. The biofilm defect was not the result of a growth defect in Δ pslGm. All of the strains showed similar growth in batch cultures (Fig. 1B).

To further assess the influence of the mutations on biofilm development, we transformed the parent and the mutants with a plasmid that codes for the constitutive expression of GFP (6) and monitored the development of biofilms in flow cells over a period of 7 days by using SCLM (Fig. 2). Bacteria attached to the surface within 1 h. There was little difference in the attachment of the parent and that of any of the mutants at this early stage in biofilm formation. After 24 h, it was apparent that microcolonies were forming in the case of all strains but *P. aeruginosa* Δ pslGm. After 2 days, all of the strains except

FIG. 2. Influence of putative EPS gene mutations on *P. aeruginosa* biofilm development. The parent and mutants (indicated on the left) were grown in flow chambers, and SCLM images were acquired at the times shown at the top. Horizontal sections are shown at the top, and vertical reconstructions are shown underneath many of the horizontal images. Bars, 50 μ m (note that the magnification for images acquired on days 3 to 7 was different from that for images acquired during earlier stages of development).

TABLE 2. Influence of mutations in putative EPS synthesis genes on twitching and swimming motilities of *P. aeruginosa*

Strain	Motility (mm of migration from point of inoculation) ^a	
	Swimming	Twitching
PAO1	16 ± 1	14 ± 1
Δ1383Sm	20 ± 0.5	14 ± 1
Δ <i>psl</i> AGm	17 ± 1	13 ± 0.5
Δ3552Gm	20 ± 0.5	16 ± 1

^a The results are means ± standard deviations from six independent experiments for swimming motility and from 12 independent experiments for twitching motility.

Δ*psl*AGm had formed substantial microcolonies that were over 20 μm deep. The PA3552 mutant appeared to be thinner and somewhat less structured than the parent strain. We had the impression that the biofilms of the PA3552 mutant were more fragile than those of the parent, but we do not have a method to measure fragility. The Δ*psl*AGm mutant remained a thin sparse layer of cells at day 2 and even after 3 days. By day 3, mushroom-like structures were apparent in all strains but *P. aeruginosa* Δ*psl*AGm. Upon further incubation in the flow cells, the mushroom-like structures of all strains but the *pslA* mutant were maintained. The *pslA* mutant eventually formed a multilayered growth on the surface, but it was thin and unstructured, with a depth of <10 μm, compared to the depths of the mushroom-like structures formed by the other strains (>25 μm).

PA2231, or *pslA*, is the first gene in a cluster of genes suspected to be involved in the synthesis of an EPS. The insertion of a GEN resistance marker may have had a polar effect on the expression of downstream genes in the cluster. Thus, we constructed a deletion mutant, Δ*pslA*null, which showed a pattern of biofilm development in flow cells that was indistinguishable from that of Δ*psl*AGm (data not shown). However, complementation with a *pslA*-containing plasmid did not restore biofilm development to the parental pattern. The *pslA*-complementing plasmid contained the PA2231-2233 cluster. Note that in an accompanying article, Jackson et al. (13) show that PA2231-2232 mutants form abnormal biofilms with characteristics similar to those of our PA2231 mutants and that the PA2231-2232 mutant biofilm defect can be complemented with a cosmid containing a 22-kb insert including PA2231-2236. Taken together, the data from both investigations suggest that a gene or genes in this cluster are critical for biofilm development.

Flagella are involved in the initial stages of *P. aeruginosa* biofilm development, and type IV pilus-mediated twitching motility is involved in biofilm formation (19), particularly in the maturation of microcolonies to mature mushroom-like structures on a glass surface (14). Thus, we determined whether the Δ*psl*AGm strain had a defect in either swimming or twitching motility that could explain the abnormal biofilm development. We discerned no significant differences in swimming or twitching among any of the strains (Table 2).

The extracellular matrix of *P. aeruginosa* biofilms. The defect in biofilm development shown by *pslA* mutants does not appear to result from a general growth defect (Fig. 1B) or from a motility defect (Table 2). This and the fact that PA2231 is the first gene in a cluster of genes annotated as EPS synthesis

genes led us to analyze the extracellular matrix of the parent and the Δ*psl*AGm strain. To obtain enough polysaccharide for our analyses, we grew attached bacteria in relatively large volumes in thin layers under static conditions. We do not know how the biofilms that develop under these conditions compare to those grown in flow cells. The matrix from either strain grown under static conditions consisted primarily of DNA, which was about five-fold more abundant than protein, which was about three or four times more abundant than carbohydrates (Table 3). The finding of abundant DNA in the purified extracellular matrix was consistent with a previous report that DNA is an important polymer in the matrix of *P. aeruginosa* PAO1 biofilms (35). To extend and confirm this observation, we grew biofilms of the parent in a defined DNA-free medium and stained these biofilms with PicoGreen, a specific fluorescent double-stranded DNA stain. The biofilm matrix was brightly fluorescent, and both the cells and the intercellular matrix were stained (Fig. 3). It is not possible to tell from images such as those shown in Fig. 3 whether the matrix of the entire biofilm is fluorescent or whether there is a specific region encased in DNA. As a control, a PicoGreen-stained planktonic culture fluid showed no extracellular fluorescence.

Although carbohydrates constituted only a small part of the matrix by weight, an EPS produced by the parent, but not by the Δ*psl*AGm strain, might yet play an important role in biofilm development (the EPS might also constitute a larger fraction of the matrix in flow cell biofilms). Thus, we removed DNA and protein from the purified matrix material and analyzed the remaining soluble carbohydrate. For the parent, the most abundant sugar monomers were glucose, rhamnose, and mannose (a ratio of about 4 glucose to 3 rhamnose to 1 mannose). For the Δ*psl*AGm mutant, the polysaccharide was enriched in glucose (glucose constituted about 50% of the parent EPS and 75 to 80% of the Δ*psl*AGm EPS); however, in separate analyses the levels of rhamnose and mannose were low but variable. Friedman and Kolter (8) report in an accompanying paper that mutations in the *psl* gene cluster in a strain of *P. aeruginosa* other than PAO1 result in a mannose-depleted polysaccharide.

In *P. aeruginosa*, there can be an overlap between EPS synthesis gene functions and LPS functions (23). Thus, *pslA* could be involved in LPS synthesis and an LPS modification could be responsible for the abnormal biofilm phenotype of *pslA* mutants. To address this question, we purified LPS from the mutant and the wild type and showed that the two preparations were indistinguishable in a silver-stained sodium dodecyl sulfate-polyacrylamide gel (data not shown). Although this does not exclude the possibility of a subtle alteration in the LPS structure, it is consistent with our conclusion that the mutant

TABLE 3. Composition of extracellular matrix of *P. aeruginosa* PAO1 and Δ*psl*AGm

Strain	Total carbohydrate (mg/100 mg or protein) ^a	Total double-stranded DNA (mg/100 mg of protein) ^a
PAO1	27.6 ± 4.6	529.1 ± 103.7
Δ2231Gm	30.0 ± 3.4	608.9 ± 53.9

^a Results are means and standard deviations from three independent experiments.

FIG. 3. Images from SCLM of a 7-day *P. aeruginosa* biofilm stained with the double-stranded DNA stain PicoGreen. (A) Reconstructed three-dimensional image of a mushroom-like structure. Bar, 50 μ m. (B) Horizontal section of the biofilm at the glass surface showing that the material between cells is stained as well as the cells in the biofilm. Bar, 10 μ m.

produces a defective EPS that is important for normal biofilm development.

Evidence that *pslA* is the first gene in a 15-gene operon. In the *P. aeruginosa* genome, *pslA* (or PA2231) is the first gene in a cluster that appears to encode polysaccharide synthesis functions. This cluster extends through PA2245. The next gene, PA2246, is oriented in the opposite direction. To assess whether the genes in the PA2231-2245 cluster are cotranscribed, we first interrogated the existing *P. aeruginosa* microarray database generated in our laboratory over the past several years (25, 26). The analysis revealed only that the transcripts of these genes are present in a very low abundance in a variety of planktonic cultures. Thus, we used

RT-PCR with the RNA isolated from the parent strain, PAO1, and primer pairs spanning the intergenic regions through the PA2231 gene cluster (Fig. 4). The results indicate that PA2230 is not cotranscribed with PA2231. The analysis suggested that each gene in the cluster from PA2231 to PA2245 is cotranscribed with the adjacent gene. This is consistent with the conclusion that the cluster constitutes a large operon in *P. aeruginosa*. Furthermore, in an independent study, Friedman and Kolter (8) have identified multiple mutations in this gene cluster that result in a specific biofilm phenotype, the lack of pellicle formation. They found that mutations in the PA2231-2245 cluster downstream of *pslA* result in the production of an EPS consisting

FIG. 4. Analysis of the PA2231-2245 gene cluster by RT-PCR of intergenic regions. Lane 1, size markers; lane 2, PCR amplification product of PA2230-2231 cluster with genomic DNA as the template (this serves as a control for RT-PCR of this region); lane 3, RT-PCR of PA2230 and -2231 (the lack of product indicates that PA2230 and -2231 are not cotranscribed); lane 4, size marker; lane 5, RT-PCR of PA2231 and -2232; lane 6, PA2232 and -2233; lane 7, PA2233 and -2234; lane 8, PA2234 and -2235; lane 9, PA2235 and -2236; lane 10, PA2236 and -2237; lane 11, PA2237 and -2238; lane 12, PA2238 and -2239; lane 13, PA2239 and -2240; lane 14, PA2240 and -2241; lane 15, PA2241 and -2242; lane 16, PA2242 and -2243; lane 17, PA2243 and -2244; lane 18, PA2244 and -2245. Control experiments without reverse transcriptase reactions prior to PCR were negative.

primarily of glucose and rhamnose, but not mannose. Thus, we propose that these genes be called *pslA* to *-O*.

DISCUSSION

We have presented evidence that of three gene clusters that may encode functions for nonalginate EPS synthesis in *P. aeruginosa* PAO1, the PA2231-2245 (*pslA* to *-O*) cluster plays an important role in biofilm development. PA2231 (*pslA*) mutants showed a biofilm defect in both static microtiter dish experiments (Fig. 1) and flow cell experiments (Fig. 2). This is consistent with the findings reported in the two accompanying papers (8, 13). We call the genes in this cluster *psl* genes by agreement among the authors of all three papers. We also showed that mutants with Ω insertions in the other two gene clusters (PA1383 and PA3552) developed biofilms similar to those of the parent, although the PA3552 mutant biofilms seemed somewhat thinner (Fig. 2) and we had the impression that they were more fragile than those of the parent. Although we introduced the Ω cassette near the beginning of each cluster, we have not investigated whether any of the genes in these clusters are cotranscribed. For this and other reasons, we caution against concluding that the clusters other than the *psl* cluster are not important for biofilm development. We can only say that a gene or genes in the *psl* cluster are important for biofilm development.

There are many possible reasons why genes in the *psl* gene cluster may be required for normal biofilm development. We know that motility is involved in biofilm formation, but both the twitching and swimming motilities of a *pslA* mutant were comparable to those of the parent (Table 2). There is evidence that LPS influences biofilm architecture (23). One might imagine that the *psl* gene cluster affects LPS synthesis, but at least by comparing LPSs in silver-stained sodium dodecyl sulfate-polyacrylamide gels, the mutant and the parent were indistinguishable.

The most obvious explanation for an involvement of *psl* genes in biofilm formation is that they direct the synthesis of an essential EPS. Our finding that the purified EPS from Δ *pslAGm* is different from that of the parent and the findings of Friedman and Kolter (8) support this explanation. However, in both the parent and the mutant, the EPS may be a minor constituent of the total extracellular material. We judged DNA to be the most abundant polymer in the matrix (Table 3). This was consistent with a previous report that DNA was a major component of the *P. aeruginosa* PAO1 biofilm extracellular matrix (35). The DNA could have resulted from contamination of the matrix preparation with whole *P. aeruginosa* cells or there may have been substantial cell lysis as a result of the specific conditions in which we grew the biofilms for matrix analysis. Although this could account for some of the DNA in these preparations, we visualized DNA in both the cells and the matrix of a biofilm growing in a flow cell by staining with PicoGreen (Fig. 3). This provides evidence consistent with, but not in proof of, the hypothesis that there is abundant DNA in the extracellular matrix of flow-cell-grown biofilms. Regardless of the abundance of polysaccharide in the extracellular matrix, both we and Friedman and Kolter (8) have found that mutations in different genes in this locus alter the monomeric constituents of the polysaccharide from extracellular matrix frac-

tions. Thus, the *psl* (polysaccharide synthesis locus) terminology used to describe the gene cluster seems appropriate. Our evidence indicates that PA2231 is the first of 15 genes in an operon (Fig. 4) and thus we called it *pslA*. For the parent strain, the most abundant sugars in the EPS were glucose, rhamnose, and mannose. The *psl* mutants produced an EPS that was predominantly glucose and rhamnose (8). We noted that the sugars that constitute alginate were not detected. This was consistent with previous findings that strain PAO1 biofilms make little or no alginate, at least under certain conditions (36).

If DNA and protein are abundant polymers in the *P. aeruginosa* PAO1 biofilm, why does an EPS have such a strong influence on the ability of the bacteria to form a biofilm? There are several possible explanations. Perhaps the EPS is synthesized early in biofilm formation and serves as a base upon which the mushroom-like structures are built. Another possibility that we favor is that the EPS functions as a scaffold on which other polymers like DNA become entangled. One might imagine that bacterial cells in a biofilm exist in a cloud of matrix materials, including DNA and protein, and that the EPS serves as a backbone that defines the area in which the cloud exists.

ACKNOWLEDGMENTS

Support for this study was provided by a grant from the National Institute of General Medicine (GM59026) and from the W. M. Keck Foundation.

The contents of this study do not necessarily represent the official views of the National Institute of General Medicine.

REFERENCES

- Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1997. Short protocols in molecular biology, 3rd ed. John Wiley & Sons, Inc., New York, N.Y.
- Becher, A., and H. P. Schweizer. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *BioTechniques* 29:948-950, 952.
- Chugani, S. A., M. Whiteley, K. M. Lee, D. D'Argenio, C. Manoil, and E. P. Greenberg. 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 98:2752-2757.
- 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.
- Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318-1322.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280:295-298.
- Fox, J. D., and J. F. Robyt. 1991. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem.* 195:93-96.
- Friedman, L., and R. Kolter. 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J. Bacteriol.* 186:4457-4465.
- Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 68:6139-6146.
- Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154:269-277.
- Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77-86.
- Hoiby, N. 1993. Antibiotic therapy for chronic infection of *Pseudomonas* in the lung. *Annu. Rev. Med.* 44:1-10.
- Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak. 2004. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J. Bacteriol.* 186:4466-4475.

14. Klausen, M., A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol. Microbiol.* **50**:61–68.
15. Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella. 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Immunol. Methods* **126**:109–117.
16. Martin, P. R., A. A. Watson, T. F. McCaul, and J. S. Mattick. 1995. Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **16**:497–508.
17. Mathee, K., O. Ciofu, C. Sternberg, P. W. Lindum, J. I. Campbell, P. Jensen, A. H. Johnsen, M. Givskov, D. E. Ohman, S. Molin, N. Hoiby, and A. Kharazmi. 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* **145**:1349–1357.
18. Nivens, D. E., D. E. Ohman, J. Williams, and M. J. Franklin. 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J. Bacteriol.* **183**:1047–1057.
19. O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motilities are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**:295–304.
20. Paraless, R. E., and C. S. Harwood. 1993. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for gram-negative bacteria. *Gene* **133**:23–30.
21. Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**:1203–1210.
22. Rashid, M. H., and A. Kornberg. 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **97**:4885–4890.
23. Rocchetta, H. L., J. C. Pacan, and J. S. Lam. 1998. Synthesis of the A-band polysaccharide sugar D-rhamnose requires Rmd and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **29**:1419–1434.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. Schuster, M., A. Hawkins, C. S. Harwood, and E. P. Greenberg. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol. Microbiol.* **51**:973–985.
26. Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* **185**:2066–2079.
27. Schweizer, H. P. 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. *BioTechniques* **15**:831–833.
28. Simon, R., U. Priefer, and A. Puhler. 1983. A broad-host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:37–45.
29. Singh, P. K., M. R. Parsek, E. P. Greenberg, and M. J. Welsh. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature* **417**:552–555.
30. Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**:762–764.
31. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrenner, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K.-S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**:959–964.
32. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
33. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**:97–106.
34. Welsh, M. J., B. W. Ramsey, F. Accurso, and G. R. Cutting. 2001. Cystic fibrosis, p. 5121–5189. In C. L. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, B. Childs, and B. Vogelstein (ed.), *The metabolic and molecular basis of inherited disease*, 8th ed. McGraw-Hill, New York, N.Y.
35. Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* **295**:1487.
36. Wozniak, D. J., T. J. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc. Natl. Acad. Sci. USA* **100**:7907–7912.
37. Yildiz, F. H., and G. K. Schoolnik. 1999. *Vibrio cholerae* O1 E1 Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. USA* **96**:4028–4033.