

Identification of *psl*, a Locus Encoding a Potential Exopolysaccharide That Is Essential for *Pseudomonas aeruginosa* PAO1 Biofilm Formation

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Bacteria inhabiting biofilms usually produce one or more polysaccharides that provide a hydrated scaffolding to stabilize and reinforce the structure of the biofilm, mediate cell-cell and cell-surface interactions, and provide protection from biocides and antimicrobial agents. Historically, alginate has been considered the major exopolysaccharide of the *Pseudomonas aeruginosa* biofilm matrix, with minimal regard to the different functions polysaccharides execute. Recent chemical and genetic studies have demonstrated that alginate is not involved in the initiation of biofilm formation in *P. aeruginosa* strains PAO1 and PA14. We hypothesized that there is at least one other polysaccharide gene cluster involved in biofilm development. Two separate clusters of genes with homology to exopolysaccharide biosynthetic functions were identified from the annotated PAO1 genome. Reverse genetics was employed to generate mutations in genes from these clusters. We discovered that one group of genes, designated *psl*, are important for biofilm initiation. A PAO1 strain with a disruption of the first two genes of the *psl* cluster (PA2231 and PA2232) was severely compromised in biofilm initiation, as confirmed by static microtiter and continuous culture flow cell and tubing biofilm assays. This impaired biofilm phenotype could be complemented with the wild-type *psl* sequences and was not due to defects in motility or lipopolysaccharide biosynthesis. These results implicate an as yet unknown exopolysaccharide as being required for the formation of the biofilm matrix. Understanding *psl*-encoded exopolysaccharide expression and protection in biofilms will provide insight into the pathogenesis of *P. aeruginosa* in cystic fibrosis and other infections involving biofilms.

Recurrent bacterial infections of the lower respiratory tract are the primary cause of morbidity and mortality in cystic fibrosis (CF) patients (17, 43). In the CF lung, mucociliary clearance is impaired, which results in persistent microbial colonization by a succession of pathogens, usually beginning with *Staphylococcus aureus* and nontypeable *Haemophilus influenzae*, followed by *Burkholderia cepacia* and *Pseudomonas aeruginosa* as the terminal pathogens recovered from the CF lung (17). *P. aeruginosa* is a versatile opportunistic pathogen that is capable of thriving in diverse environments ranging from water and soil to plant and animal tissues. This bacterium has an extensive arsenal of virulence factors that it uses to successfully colonize the lungs of CF patients (30, 43).

Several lines of evidence indicate that colonization of the CF lung by *P. aeruginosa* involves a biofilm mode of growth (7, 27, 51). Bacteria within biofilms are attached to either a substratum or each other and are embedded in a matrix of extracellular polymeric substance (EPS), which may consist of proteins, polysaccharides, nucleic acids, or combinations of these macromolecules (8, 39, 58). One of the most clinically significant characteristics of biofilm communities is that they are more resistant to antibiotics, biocides, and host-mediated clearance strategies than are their planktonic counterparts (33,

53). Since the EPS matrix, in certain cases, may play a critical role in the biofilm resistance phenotype (52, 53), a complete understanding of its organization and composition may assist in the development of therapeutics aimed at disrupting biofilms.

The exopolysaccharide alginate has traditionally been considered the major EPS of *P. aeruginosa* biofilms during CF pathogenesis. Initially, the CF lung is colonized by nonmucoid *P. aeruginosa* strains, but due to poorly understood selective pressures, these strains convert to a characteristic mucoid phenotype. The mucoid phenotype is caused by the overproduction of alginate, a linear copolymer of mannuronic and guluronic acid joined by β 1-4 linkages (12, 17). Despite the extensive work conducted on alginate biosynthesis and regulation (17), to date there is no direct evidence that alginate is required for the development of biofilms by nonmucoid *P. aeruginosa* strains, which are the first to colonize CF patients. In most CF patients, mucoid conversion occurs months or years after the initial colonization (17). There remain significant gaps in our understanding of how *P. aeruginosa* survives the harsh, inflammation-rich environment of the CF lung prior to converting to the alginate-producing phenotype. Recently published data strongly challenged the role of alginate in biofilm development by nonmucoid strains (19, 37, 62). An analysis of the EPS derived from biofilm-grown strain PAO1 revealed that the primary carbohydrate constituents are glucose, rhamnose, and mannose, not mannuronate or guluronate (62). This has led to the hypothesis that polysaccharides other than

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TABLE 1. Oligonucleotides used for this study

Oligonucleotide	Sequence (5' - 3')
<i>psl1</i>	GGAATTCCTCGCAGAAGTCCAGTCCCGCGCT
<i>psl2</i>	CGGGATCCGGCGCTCCGGGGATGCCGAA
<i>algD17</i>	CGCAGAGAAAACATCCTATC
<i>algD20</i>	TGAAGTCGGTGGTCCGACA
PA1389A.....	GGTCTAGAGCCACTACCATTCTCTCG
PA1391A.....	GCCAGAGTTGCTGTCC
PA1390A.....	CGGATGACTGGCTGGCGTCC
PA1390B.....	TGATCAGCTGCCTCGCACC
PA2231A.....	CATGTACCAGGCGCTGGGCC
PA2231B.....	CCGCGGTAGCCGTTGATCTG
PA3059A.....	GTTGATCTCCGGTCTCCCG
PA3059B.....	CGATCAGTTCGCGCAGGAG
PA3559A.....	GCTGTTCGAACGCATCCGG
PA3559B.....	TCAGGCCTGGCGCAACAGGC

alginate may contribute to the formation of the biofilm matrix in nonmucoid strains. In this regard, annotation of the *P. aeruginosa* PAO1 genome revealed at least four novel putative polysaccharide biosynthetic gene clusters (14, 54).

For the present study, we identified a locus, *psl*, which may encode an alternative EPS that is essential for biofilm formation in *P. aeruginosa* PAO1. The disruption of *psl* generated a stable biofilm initiation-deficient phenotype in several assays. Significantly, most of the genes of the *psl* cluster are predicted to encode proteins with sequence homology to enzymes involved in the synthesis of polysaccharides from glucose, mannose, and rhamnose, which are the primary carbohydrate constituents of the EPS purified from biofilm-grown *P. aeruginosa* (62). This suggests that the relationship between the conversion from nonmucoid to mucoid strains and *P. aeruginosa* biofilms is not simply a change in the alginate levels present in the biofilm matrix, but a fundamental change in its carbohydrate constituents. This represents a new paradigm for *P. aeruginosa* biofilm development and pathogenesis that may reveal new avenues for immunological or chemotherapeutic interventions for infections caused by this organism. Two other groups have independently identified the *psl* locus and have shown its requirement for biofilm formation in *P. aeruginosa* in accompanying papers (15, 34).

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, media, and antibiotics. *P. aeruginosa* PAO, its isogenic *pslAB* mutant, WFFA60 (see below), and MS2, an insertion mutant inactivating PA1389-1391 (see below) were used for this study. Other *P. aeruginosa* strains used included PA103 (29), PAK (50), PA388 (4), and FRD1 (38), as well as a collection of CF-derived mucoid and nonmucoid strains (3, 61). WFFA50 is PAO1 *flhC::xylE-aacC1* (63) and PAO AWO is PAO1 *pilA::tet* (J. Mattick); J. Goldberg supplied the PAO1 *algC* mutant. *Escherichia coli* strain JM109 (Promega) was used for all cloning experiments, while strains SM10 and HB101/pRK2013 (13) were used to transfer plasmids to *P. aeruginosa*. PAO1 Sm^r was a spontaneous mutant generated by plating an overnight culture of PAO1 onto a selective medium (100 µg of streptomycin/ml) and screening for spontaneous streptomycin-resistant mutants. PAO1, WFFA60, and MS2 were tagged with the green fluorescent protein (GFP)-expressing plasmid pMRP9 via a linear transformation protocol (11). PCR was used to amplify a 2.5-kb fragment, which included *pslA* (PA2231) and a portion of *pslB* (PA2232) (54), with the primers *psl1* and *psl2* (Table 1). The amplicon was cloned into pALTER1 (Promega) to generate pKDJ1. Huang and colleagues used the PAO1 cosmid library and the PAO1 genome sequence to map a minimal tiling path (22). This library was used to identify two overlapping cosmids, pMO011305 and pMO013305, that span the entire *psl* cluster. Luria broth (LB; 10 g of tryptone/liter, 5 g of yeast extract/liter, 5 g of sodium chloride/liter), LB lacking sodium chloride (LBNS), and Jensen's, a chemically defined medium (23), were used throughout the study. Unless

otherwise indicated, the carbon sources were used at 0.4%. The plasmids were maintained in *E. coli* by antibiotic selection with 15 µg of tetracycline and gentamicin/ml, 100 µg of ampicillin/ml, and 30 µg of kanamycin or streptomycin/ml. In *P. aeruginosa*, antibiotics were used at 100 µg/ml for tetracycline, streptomycin, and gentamicin and 300 µg/ml for carbenicillin. Sucrose (5%) and irgasan (25 µg/ml) were utilized for counterselection. All oligonucleotides used for this study are depicted in Table 1.

Genomic DNA isolation and PCR assays. Genomic DNAs were purified from *P. aeruginosa* strains with Wizard genomic DNA isolation reagents (Promega). PCR assays were performed with 100 ng of genomic DNA, the primer pairs indicated in Table 1, and *Taq* polymerase according to the manufacturer's recommendations (Promega).

Creation of WFFA60 and MS2. A restriction digest of pKDJ1 with BamHI and EcoRI generated a 2.5-kb fragment which was cloned into the gene replacement vector pEX18Ap (21) and designated pDJW630. Plasmid pDJW630 was subsequently digested with the restriction enzyme XmaI, and a 2.2-kb *xylE-aacC1* cassette from pX1918G (21) was cloned in the plasmid to create pDJW634, which carries *pslAB::xylE-aacC1*. The digestion of pDJW630 with XmaI removes the 3' end of *pslA* and the 5' end of *pslB* (54). The *aacC1* marker of pX1918G is reported to create nonpolar insertions during gene disruptions (21). We utilized standard reverse genetics strategies with strain SM10/pDJW634 to disrupt *pslAB* (PA2231-2232) (21, 31, 60). The successful disruption of *pslAB* was confirmed by PCR assays. MS2 construction proceeded as follows. A 7.0-kb region of the PAO1 genome containing open reading frames (ORFs) PA1389 to PA1391 was amplified by using primers PA1389A and PA1391A (Table 1). The PCR product was digested with EcoRI and XbaI and ligated into the corresponding restriction sites of pUC18 (57). A 1.6-kb SmaI fragment from pGM01 (48), containing a marker for gentamicin resistance, was ligated into the EcoRV sites of the construct. The PvuII fragment of the resulting plasmid was ligated into pEX18Ap (21). The MS2 mutant was generated in PAO1 via standard allelic exchange and was verified by PCR (21). The growth kinetics of WFFA60 and MS2 were determined in both LBNS and Jensen's defined medium.

Microtiter dish assay. For biofilm initiation assays, LBNS was inoculated (1:100) with an overnight culture of each strain, and the diluted bacterial suspensions were added to a 96-well microtiter dish (40). The microtiter dish was incubated at 30°C for the indicated times. At each time point, the nonattached and loosely adherent bacteria were removed by discarding the medium and the cells. The wells were rinsed thoroughly with water. Next, 100 µl of a 0.1% solution of crystal violet was added to each well and the plates were incubated at 25°C for 30 min. The washing process was repeated, the crystal violet staining the attached cells was solubilized with 200 µl of 95% ethanol, and 125 µl was removed and placed in a fresh polystyrene microtiter dish to determine the A_{540} .

Motility and LPS assays. For twitching motility assays (50), the strains were stab inoculated into a thin layer of 1% LANS (LBNS plus 1% agar), incubated overnight at 37°C, and incubated an additional 24 h at 25°C to allow motility zones to become more prominent. Flagellum-mediated motility assays were performed by inoculating a single colony onto 0.3% LANS. After overnight growth at 37°C, the motility was assessed by examining the distance the colonies spread beyond the point of inoculation (1). Lipopolysaccharide (LPS) was extracted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and silver stained according to previously published methods (20).

Confocal laser scanning microscopy. Bacteria were cultured in Jensen's medium in a flowthrough biofilm culturing system at 30°C (5, 23). For visualization, each strain was tagged with the GFP-containing plasmid pMRP9 (11). Biofilm growth was assessed at 12-h time points up to 72 h with a Zeiss LSM 510 confocal laser scanning microscope.

Quantitative analysis of biofilm formation by silicone tubing continuous-flow model. To quantitatively analyze biofilm populations of the wild type and the *pslAB* mutants, we used a biofilm tubing assay as previously described (47). The tubing was aseptically inoculated with 200 µl of a cell suspension from a mid-logarithmic-phase culture ($A_{600} = 0.5$, or $\sim 10^7$ cells) of strain PAO1 or WFFA60 by use of a 25 5/8-gauge needle. The bacteria were allowed to adhere to the inner surface of the tubing for 30 min before the flow of the culture medium was resumed (flow rate, ~ 0.7 ml/min). At designated time points, a 15.0-cm-long section of tubing was excised 7.5 cm from the inoculation point. This length of tubing was longitudinally sectioned, and biofilm-grown cells were harvested into 1 ml of medium. The bacteria were serially diluted and enumerated by viable plate counts on LANS. Data are expressed as the log CFU recovered at each time point examined.

Coinoculation assays. For coinoculation experiments (see Table 3), $\sim 10^7$ PAO1 Sm^r and WFFA60 bacteria were coinoculated into the same biofilm tubing system. The experiments were conducted as described above, except that the cells were enumerated by plate counts on a selective medium containing streptomycin

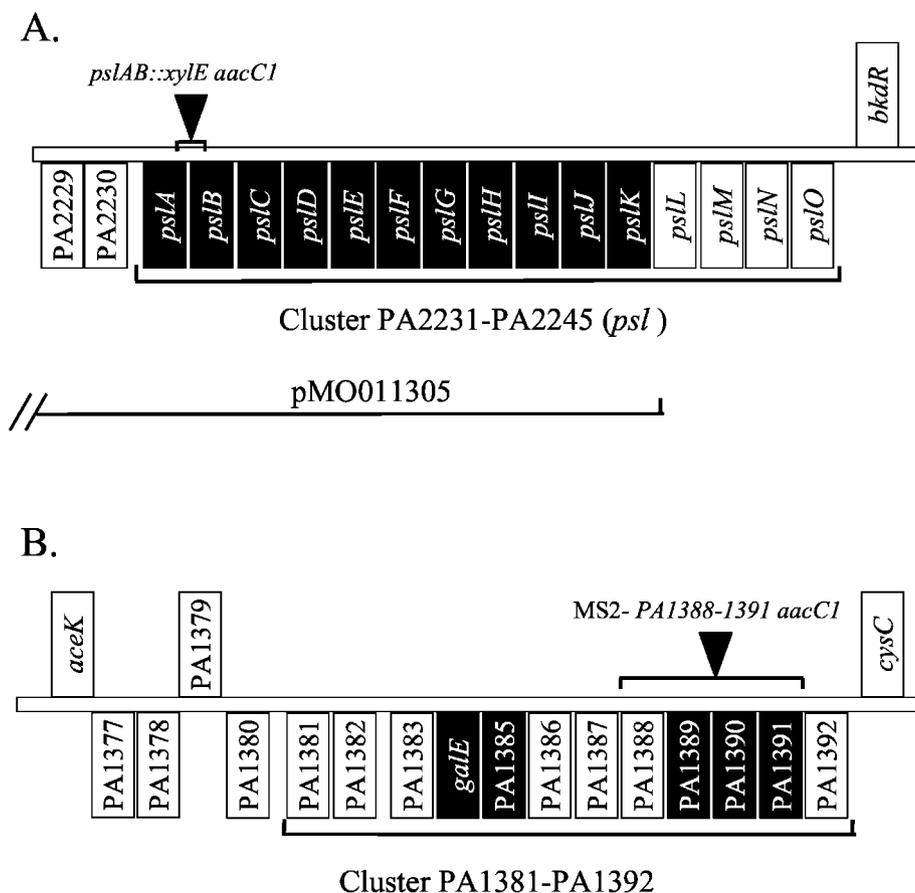


FIG. 1. (A) Schematic of the *psl* gene cluster from the annotated *P. aeruginosa* PAO1 genome. The *psl* gene cluster is located between *opdE* and *bkdR* and contains 15 putative ORFs (~18.7 kb), annotated PA2231 to PA2245 (54). The ORFs of this cluster are tightly linked in the same orientation, which implies an operon structure. The position of the *xylE aacC1* insertion in *pslAB*, as well as a map of cosmid pMO011305, which complements the WFA60 mutation, is indicated. Black boxes indicate ORFs that share homology with proteins involved in polysaccharide synthesis, modification, or transport. (B) Schematic of the PA1381-PA1392 gene cluster from the annotated *P. aeruginosa* PAO1 genome (54). This gene cluster is located between *aceK* and *cysC* and contains 12 putative ORFs (~16.9 kb). The ORFs of this cluster are tightly linked in the same orientation, which implies an operon structure. The position of the *aacC1* insertion of strain MS2, replacing the PA1388-PA1391 cluster, is indicated. Black boxes indicate ORFs that share homology with proteins involved in polysaccharide synthesis, modification, or transport.

for PAO1 Sm^r and gentamicin for WFA60. In sequential coinoculation experiments, ~10⁷ PAO1 Sm^r bacteria were introduced into the tubing system and allowed to establish a biofilm for 24 h, at which time ~10⁷ WFA60 cells were inoculated 0.5 cm upstream of the PAO1 Sm^r inoculation point. WFA60 was allowed to attach for 30 min, and then the medium flow was resumed for an additional 24 h. Biofilm cells were then harvested, resuspended, and enumerated by plating onto selective medium. Data are presented as fractions of the total bacteria recovered.

RESULTS

Analysis of loci potentially involved in EPS production. Recently published data suggest that polysaccharides other than alginate may contribute to the formation of the PAO1 biofilm matrix (19, 37, 62). A prior annotation of the *P. aeruginosa* genome suggested that PAO1 has at least three regions that are potentially involved in the synthesis of novel exopolysaccharides (see the supplemental data for reference 54). A recent report has implicated a fourth locus, designated *pel*, that is required for *P. aeruginosa* pellicle formation on liquid surfaces and that may be involved in the synthesis of an extracellular matrix (14). We determined whether the genes within these

loci are conserved among *P. aeruginosa* strains. Chromosomal DNAs were purified from a variety of commonly used *P. aeruginosa* strains as well as from mucoid and nonmucoid isolates derived from CF patients (see Materials and Methods for a list of the strains analyzed). PCR assays were performed, using primers specific for one gene within each cluster (PA2231 [*algD*], PA1390, PA3059 [*pelF*], and PA3559) (Fig. 1). The *algD* gene was chosen as a control since earlier studies suggested that alginate genes are widely conserved among *Pseudomonas* isolates (16). With the exception of PA1390 and PA3059 (*pelF*), these genes were conserved in all strains examined (data not shown). This suggests that *P. aeruginosa* may utilize common pathways for biofilm matrix formation.

Two clusters, PA1381-1392 and PA2231-2240 (which we and others have designated *psl*, for polysaccharide synthesis locus), were selected for further study because most of the genes within these clusters are predicted to encode proteins with sequence homology to enzymes involved in the synthesis of polysaccharides (Fig. 1). For the *psl* cluster, these biosynthetic genes were suggested to potentially use the carbohydrates glu-

TABLE 2. *psl* ORFs PA2231 to PA2245 with proposed roles in polysaccharide synthesis

Gene	ORF	No. of amino acids, predicted mass (kDa)	% Similarity or homology to another protein
<i>pslA</i>	PA2231	478, 54.8	69, to colonic acid UDP-glucose lipid carrier, WcaJ (<i>E. coli</i>)
<i>pslB</i>	PA2232	488, 53.5	72, to PMI/GDP-mannose pyrophosphorylase WbpW (<i>P. aeruginosa</i>)
<i>pslC</i>	PA2233	303, 33.6	59, to rhamnosyl transferase RfbN (<i>Salmonella</i>)
<i>pslD</i>	PA2234	256, 27.9	47, to amylovoran export protein AmsH (<i>Erwinia</i>)
<i>pslE</i>	PA2235	662, 74.5	40, to xanthan translocation protein GumC (<i>Xanthomonas</i>)
<i>pslF</i>	PA2236	395, 42.7	38, to beta-xylosidase (<i>Caldicellulosiruptor</i>)
<i>pslG</i>	PA2237	442, 50.0	43, to putative GlcNAc transferase (<i>Bordetella</i>)
<i>pslH</i>	PA2238	402, 44.6	43, to putative galactosyltransferase (<i>Archaeoglobus</i>)
<i>pslI</i>	PA2239	367, 40.6	42, to glycosyltransferase WbpY (<i>P. aeruginosa</i>)
<i>pslJ</i>	PA2240	478, 52.5	43, to putative polysaccharide polymerase (<i>Streptococcus pneumoniae</i>)
<i>pslK</i>	PA2241	469, 49.6	45, to putative membrane-associated virulence factor MviN (<i>E. coli</i>)
<i>pslL</i>	PA2242	355, 39.9	Unknown (hypothetical protein)
<i>pslM</i>	PA2243	577, 61.5	49, to hypothetical protein Rv3537 (<i>Mycobacterium tuberculosis</i>)
<i>pslN</i>	PA2244	333, 38.0	Unknown (hypothetical protein)
<i>pslO</i>	PA2245	101, 10.6	Unknown (hypothetical protein)

cose, mannose, and rhamnose, the primary constituents of the EPS purified from biofilm-grown *P. aeruginosa* (62). An analysis of the *pslA* and *pslC* genes suggested that they encode glucose and rhamnose glycosyl transferases, respectively. The *pslB* gene encodes a probable phosphomannose isomerase/GDP-mannose pyrophosphorylase, and *pslD* encodes an exopolysaccharide transporter (Fig. 1A; Table 2). The *psl* gene cluster, annotated PA2231-PA2245, is located between *opdE* and *bkdR* and contains 15 putative ORFs (~18.7 kb, designated *pslA* to *pslO*). The ORFs of this cluster are tightly linked in the same orientation, which implies an operon structure (Fig. 1A).

An analysis of the PA1381-PA1392 cluster revealed a collection of genes that also encode potential polysaccharide biosynthetic enzymes (Fig. 1B). This cluster harbors four genes, PA1385 and PA1389 to PA1391, that have homology to glycosyl transferases. PA1384, annotated as *galE*, is predicted to encode a UDP-glucose 4-epimerase. Interestingly, the genes of this cluster display a G+C content that is much lower (<60%) than that of the *P. aeruginosa* average (67%), suggesting that these genes may have been acquired through horizontal gene transfer. These genes also cluster fairly tightly, implying an operon structure.

A *pslAB* mutant is deficient in the initiation of biofilm formation. To test whether the *psl* or PA1381-PA1393 gene cluster plays a role in biofilm development, we utilized standard reverse genetics strategies to disrupt both *pslA* and *pslB* or PA1388-1391 in *P. aeruginosa* PAO1 (see Materials and Methods and Fig. 1). The *pslAB* mutant was generated with the *aacC1* (gentamicin resistance) marker of pX1918G, which is reported to create nonpolar insertions during gene disruption (49). We compared the *pslAB* mutant, designated WFPA60, with the parental PAO1 strain for the ability to initiate biofilm formation by utilizing the biofilm microtiter dish assay (Fig. 2). This assay, which measures the early stages of biofilm formation, is based on the quantitation of bacterial cells attached at the medium-air interface of a well of a polyvinyl chloride (PVC) microtiter dish (41). In this assay, WFPA60 was severely deficient in the ability to initiate biofilm formation (Fig. 2A). This defect was not due to differences in the growth rate, as PAO1 and WFPA60 had similar growth rates (data not

shown). The defect in biofilm initiation was clearly mediated by the disruption of *pslAB*, since the introduction of cosmid pMO011305, which harbors a 22.5-kb insert containing the majority of the *psl* locus (22) (Fig. 1A), into WFPA60 restored the biofilm initiation levels (Fig. 2A).

An analysis of strain MS2, which had a mutation in the other potential EPS biosynthetic gene cluster (PA1388-1391), showed no significant defect in biofilm initiation in the microtiter dish assay, even when tested under a variety of growth conditions (Fig. 2B). In flow cell assays, MS2 also produced a robust biofilm that was structurally similar to that of the wild-type strain (data not shown). Because the disruption of PA1388-1391 had no apparent effect on biofilm formation, the remainder of the study focused on the involvement of the *psl* gene cluster in biofilm development.

The *pslAB* mutant has no apparent defect in LPS synthesis or motility. To our knowledge, a function for the *psl* gene cluster in biofilm formation has yet to be reported. Since many of the ORFs may encode enzymes involved in polysaccharide synthesis (Fig. 1; Table 2), *psl* may regulate or constitute a portion of the LPS synthesis pathway. LPS has been implicated in the architecture of *P. aeruginosa* biofilms (44), and a prior study suggested that *pslB* (designated ORF 488) may be involved in A-band LPS synthesis (45). To determine if the *psl*-dependent effects on biofilm formation are LPS related, we compared the LPS profiles of PAO1, WFPA60, a PAO1 *algC* mutant, and the corresponding *algC* complemented strain. *P. aeruginosa* *algC* mutants have a defect in LPS core oligosaccharide synthesis (9). The LPSs from these strains were extracted (20), resolved by SDS-15% PAGE, and silver stained. The silver-stained gel revealed identical LPS profiles for PAO1, WFPA60, and the complemented LPS-deficient mutant (Fig. 3A, lanes 3 to 5), whereas the PAO1 *algC* mutant clearly lacked core oligosaccharides (Fig. 3A, lane 2).

Since prior studies implicated flagellum (swimming)- and type IV fimbria (twitching)-mediated motility in *P. aeruginosa* biofilm development (25, 42), we conducted standard twitching and swimming motility assays (2, 50) and found no motility defects in WFPA60 (Fig. 3B and C, respectively). These data strongly suggest that the defect in biofilm formation described

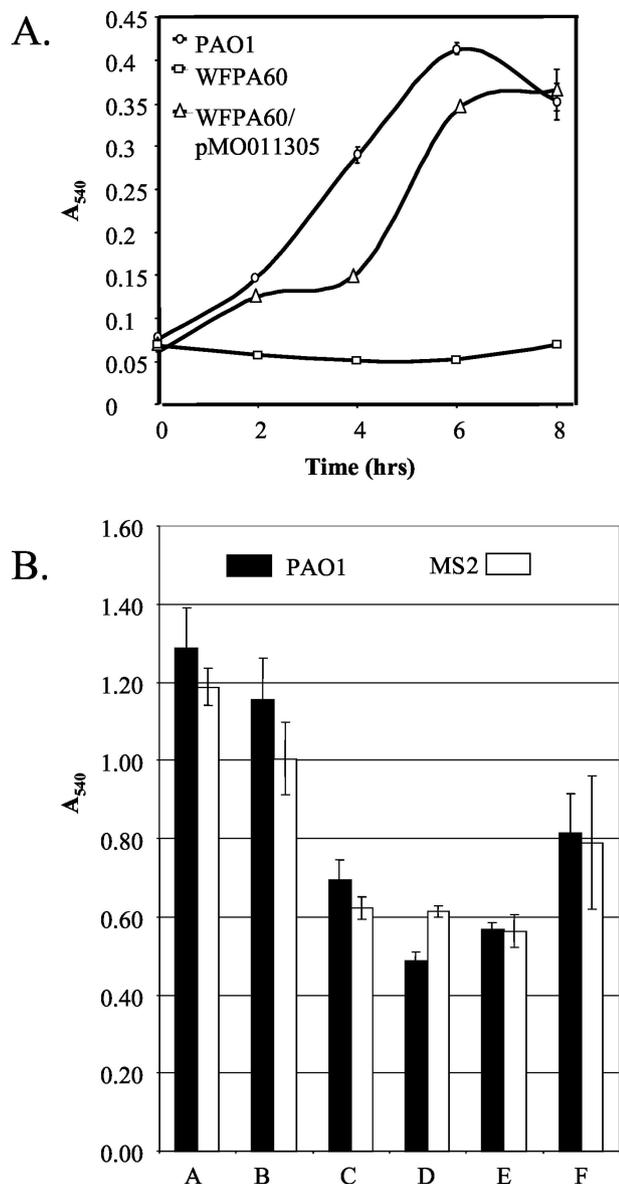


FIG. 2. Biofilm formation by *P. aeruginosa* strains. (A) The biofilm formation of strains PAO1 (circles), WFA60 (squares), and WFA60/pMO011305 (triangles) was assayed at 2-h intervals in a static microtiter plate system (40). Surface-attached cells were stained with crystal violet, the stain was solubilized in ethanol, and the absorbance was analyzed at 540 nm. (B) Biofilm formation of PAO1 (black) and MS2 (white) at 10 h in a static microtiter plate system (40). Bacteria were cultured with the following carbon sources (0.4%): A, LB medium; B, LB medium with glucose; C, 10× glucose; D, succinate; E, glycerol; F, glutamate (F).

above for WFA60 is not due to alterations in LPS biosynthesis or motility.

The *pslAB* mutant is severely compromised in biofilm formation in a flow cell reactor. One limitation of the microtiter dish assay is that nutrients are diminished and waste products accumulate rapidly, making it difficult to examine biofilm development for extended periods of time. In addition, microtiter dish-grown biofilms are not amenable to microscopy. There-

fore, we used flow cell devices to compare the biofilm development of PAO1 and WFA60. In this system, nutrients are continually replenished, allowing for the long-term observation of living hydrated biofilms on a glass surface. We generated GFP-tagged derivatives of PAO1 and WFA60 (11, 64) and combined flow cell culturing technology and confocal scanning laser microscopy to examine the architecture of these strains over time (Fig. 4). The parental strain PAO1 formed robust biofilms throughout the time course of this experiment (Fig. 4, top panels). However, when strain WFA60 was examined, we discovered that it was severely compromised in biofilm development at all time points examined, even though an identical number of PAO1 and WFA60 bacteria were initially inoculated into the flow cells (Fig. 4, bottom panels). At the latest time point examined (72 h), some of the WFA60 bacteria began to develop into microcolonies, suggesting a severe attenuation in biofilm initiation.

Quantitative analysis of PAO1 and *pslAB* biofilm-grown cells. A quantitative assessment of the biofilm formation of WFA60 further strengthened our assertion that the *psl* cluster plays a role in biofilm development. Therefore, we devised a tubing assay to quantify the population of biofilm-grown cells under continuous laminar flow conditions with a constant supply of fresh growth medium. Based on the above results, we hypothesized that there would be a significant difference in biofilm formation between the PAO1 and WFA60 strains. The biofilms were grown in silicone tubes by first priming the substratum with medium and then inoculating the tubing with a culture of PAO1 or WFA60, allowing the cells to attach for 1 h, and initiating a continuous flow of medium. At 6, 12, 24, 36, and 48 h, a defined section of tubing was longitudinally sectioned, and biofilm bacteria were harvested and enumerated by plate counting. WFA60 exhibited a substantial defect in biofilm initiation (up to 10⁵-fold) compared to the isogenic parental PAO1 strain (Fig. 5). Strain WFA60, which was impaired in the initial stages of biofilm formation (Fig. 2), appeared unable to overcome the defect over an extended time period when it was incubated under continuous laminar flow conditions.

Since WFA60 was severely compromised in biofilm development, we reasoned that one or more factors (likely the EPS) that are required for cell-surface or cell-cell interactions are lacking in this strain. Assuming that this was the case, we hypothesized that the factor(s) may be supplied in *trans* by the parental strain in a coculture. To test this, we coinoculated an identical number of PAO1 and WFA60 bacteria into the biofilm tubing system described above. After 48 h, the tubing was sectioned and the cells were harvested and plated onto a selective growth medium. To distinguish between WFA60 and PAO1, we used a streptomycin-resistant PAO1 derivative (PAO1 Sm^r) and WFA60, which is gentamicin resistant. When they were simultaneously cocultured, PAO1 Sm^r was unable to rescue the *pslAB* mutant phenotype (Table 3), suggesting that WFA60 was unable to effectively compete with the wild-type strain for a shared substratum.

In the above assay, PAO1 and WFA60 were inoculated simultaneously. We also examined whether WFA60 could be recruited into an established biofilm formed by a wild-type strain. To test this, we inoculated PAO1 Sm^r into the continuous flow silicone tubing system and incubated it for 24 h.

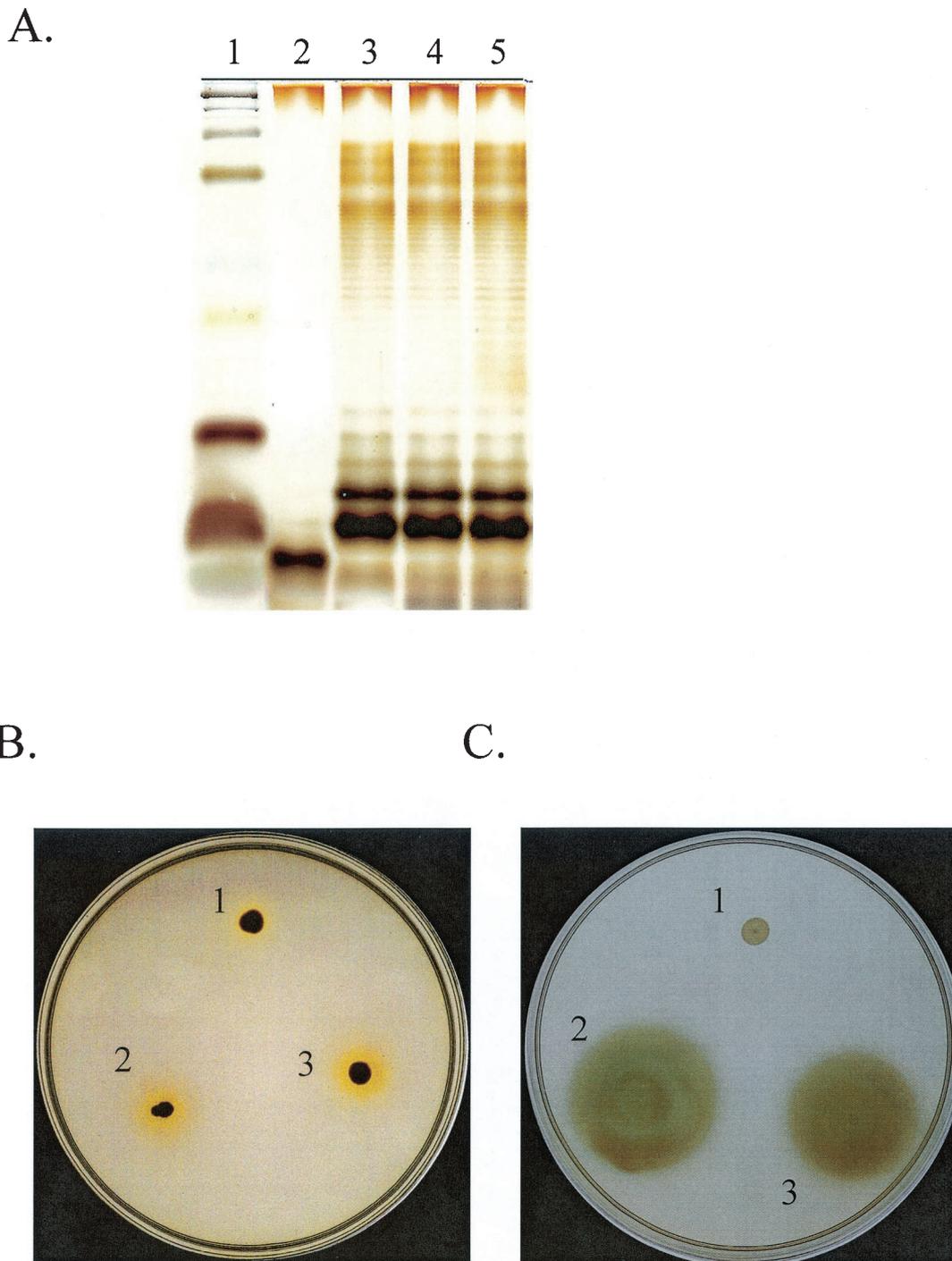


FIG. 3. The *pslAB* mutant has no defects in LPS synthesis or motility. (A) LPS profiles of PAO1 (lane 5) or the following PAO1-derived strains: PAO1 *algC* (lane 2), PAO1 *algC* plus *algC* (lane 3), and WFPA60 (lane 4). LPS was extracted from these strains, separated by SDS-15% PAGE, and visualized by silver staining of the polyacrylamide gel. Lane 1 contains a protein standard. (B) Twitching motility assays of PAO1 AWO (*pilA::tet*) (spot 1), PAO1 (spot 2), and WFPA60 (spot 3). (C) Flagellum motility assays of WFPA50 (*fliC::aacC1*) (spot 1), PAO1 (spot 2), and WFPA60 (spot 3).

PAO1 Sm^r readily attached to the tubing and initiated biofilm development during this period (Table 3). After this initiation, an identical number of WFPA60 cells were introduced into the system and incubated for 30 min without flow, after which the flow was resumed. After 24 h, the numbers of PAO1 Sm^r and

WFPA60 cells were determined. The results were consistent with those of the other biofilm assays; even when cocultured sequentially, WFPA60 was significantly impaired at biofilm initiation (Table 3). These data indicate that even when WFPA60 is deposited onto an established biofilm of wild-type

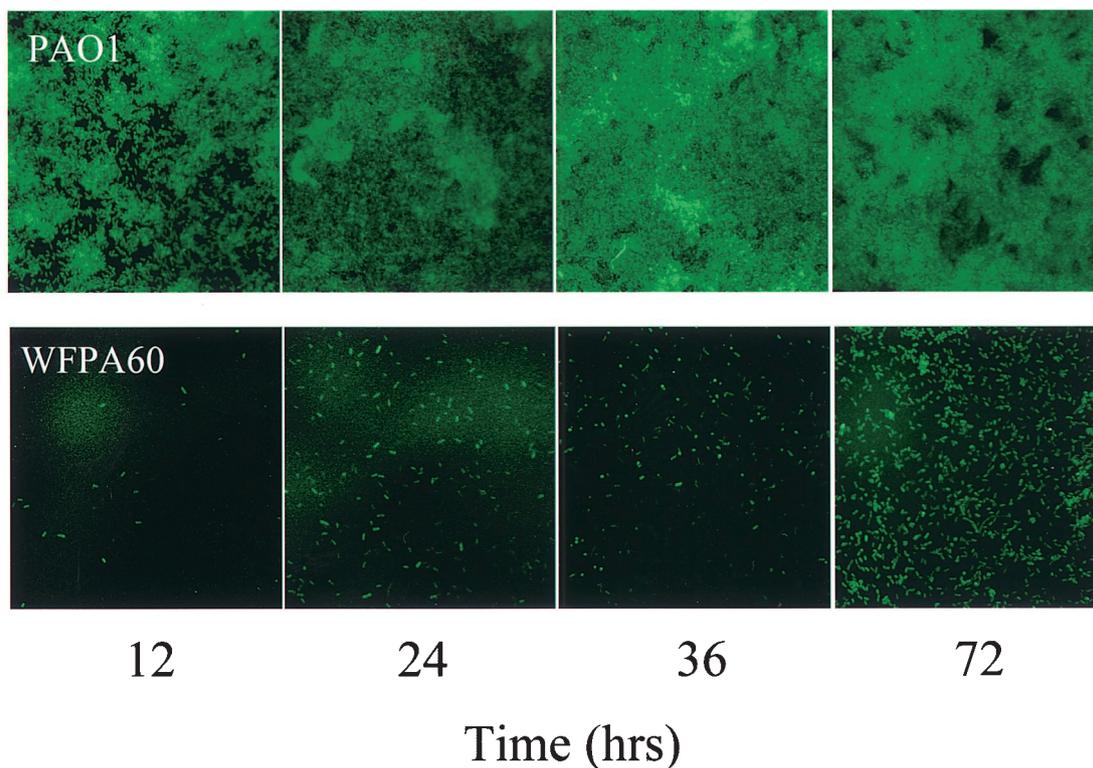


FIG. 4. Qualitative analysis of PAO1 and WFP A60 biofilm populations. Strains harboring pMRP9 were inoculated into respective flow devices and assayed at various time points postinoculation up to 72 h. WFP A60 is deficient in biofilm formation under laminar flow growth conditions compared to PAO1.

cells, it is unable to establish a niche. Taken together, the data in Fig. 2, 4, and 5 and Table 3 suggest that the *psl* gene cluster contributes significantly to cell-surface interactions with abiotic surfaces (glass, PVC, and silicone) as well as to cell-cell interactions with certain biotic surfaces.

DISCUSSION

A critical aspect of *P. aeruginosa* pathogenesis is the ability to form biofilms in the lungs of CF patients and on many other surfaces. For some time, alginate has been considered the major polysaccharide of the biofilm EPS matrix. However, recent studies have indicated that alginate is not involved in the initiation of biofilm formation by nonmucoid *P. aeruginosa* strains, which are the first to colonize CF patients (19, 37, 62). Instead, there appear to be other EPS components that mediate biofilm formation. In this paper, we analyzed two gene clusters that appear to encode enzymes for the synthesis of novel polysaccharides. The inactivation of genes within one cluster (PA1381-PA1393) resulted in no discernible biofilm phenotype. Although we cannot rule out a role for this cluster in biofilm development or polysaccharide synthesis in *P. aeruginosa*, we concentrated our efforts on discerning the function of the *psl* gene cluster. The disruption of the first two genes within the *psl* cluster (strain WFP A60) led to a profound biofilm initiation deficiency. The *pslAB* mutant cells, which were impaired in the initial stages of biofilm formation, were unable to overcome this defect over an extended time period when they

were incubated under continuous flow conditions. This impaired ability of WFP A60 to initiate biofilm formation was observed for several abiotic surfaces (PVC, glass, and silicone tubing) and was not due to defects in motility or LPS biosynthesis. Moreover, by using both qualitative and quantitative assays, we showed that WFP A60 is deficient in biofilm formation for up to 72 h. Finally, we report that either simultaneous or sequential coinoculation of wild-type *P. aeruginosa* cannot rescue the biofilm formation phenotype of WFP A60. Overall, our interpretation of these data is that the *pslAB* mutant is impaired in the initiation phase of biofilm formation. We propose that genes within the *psl* cluster play a critical role in cell-cell and/or cell-surface interactions. This is consistent with the findings in accompanying papers from two other groups (15, 34).

Although our present data do not directly address whether the *psl* locus encodes a novel exopolysaccharide, there are precedents for exopolysaccharides from other bacteria having roles similar to those proposed for *P. aeruginosa psl*. Exopolysaccharides have been shown to play an important role in bacterial plant and animal pathogenesis for a variety of bacterial species (24, 32, 36). Many of the infections caused by these organisms are believed to involve biofilms, and exopolysaccharide-negative strains are typically avirulent.

Exopolysaccharides are often implicated in either mediating attachment or providing protection. The properties of the exopolysaccharide matrix that confer protection depend upon the nature of the antimicrobial agent or stress being applied. In

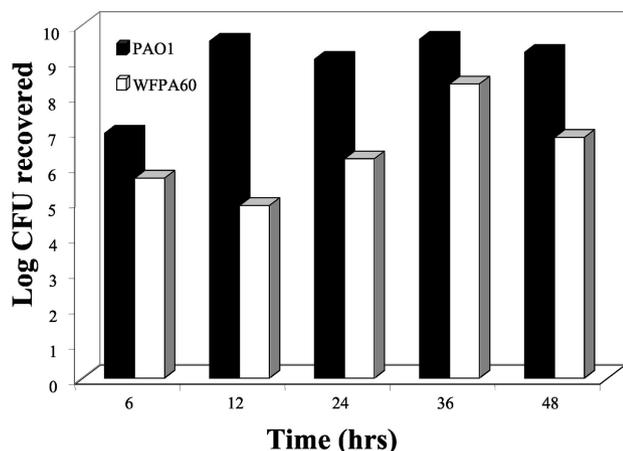


FIG. 5. Quantitative analysis of PAO1 and WFP60 biofilm populations in silicone tubing continuous flow devices. Overnight cultures of each strain ($\sim 10^7$ cells) were inoculated into a section of silicone tubing that had first been primed with medium. At designated end-points (6, 12, 24, 36, and 48 h), the tubing was longitudinally sectioned and the biofilm-grown cells were harvested, resuspended, and enumerated by plate counts (see Materials and Methods). Data are expressed as the log CFU recovered at each time point examined and are the averages of three independent experiments.

general, the exopolysaccharide can act as a physical barrier or function as a chemically reactive barrier. Future studies are aimed at determining if the *psl* locus does indeed encode a novel polysaccharide that plays a protective role in *P. aeruginosa* biofilms. Nonetheless, our observations support the conclusion that the *psl* cluster encodes gene products that are required for mediating cell-surface and cell-cell interactions. Initial attachment requires that a repulsion barrier between a negatively charged bacterial cell and a surface must be overcome (55, 56). Different exopolysaccharides, such as those from *Vibrio* sp., acidic polysaccharides of marine pseudomonads, and *Staphylococcus epidermidis* PIA and PS/A clearly promote adherence (6, 18, 32, 35, 65). The prosthecate marine bacterium *Hyphomonas* produces at least two developmentally regulated exopolysaccharides that serve as adhesins (28). Exopolysaccharides can also influence biofilm structure and function, as has been demonstrated for alginate (19, 37), *Vibrio cholerae* Vps (65), and *E. coli* colanic acid (10).

The *pslAB* mutant attaches poorly to abiotic and biotic surfaces under both static and dynamic culture conditions. This phenotype is most readily apparent for attachment to these surfaces under dynamic culture conditions. However, under extended static culture conditions (>36 h), WFP60 eventually will form biofilms that are comparable to those formed by the wild-type strain (K. D. Jackson and D. J. Wozniak, unpublished data). When the WFP60 cells are left undisturbed for extended periods, they begin the biofilm formation process, but this is substantially delayed in the dynamic flow tubing system. This assay was designed to mimic indwelling device biofilms such as those that form in catheters. Our interpretation of this is as follows. If WFP60 is deficient at forming cell-surface and cell-cell interactions but is left undisturbed, it will eventually overcome these barriers. However, if it is subjected to laminar or turbulent flow, it is incapable of mediating such interactions. It is also possible that the growth conditions

TABLE 3. Quantification of bacteria recovered during simultaneous and sequential coinoculation biofilm tubing assays^a

Strain	Inoculation	Time of harvesting (h)	Fraction of bacteria recovered
PAO1 Sm ^r	Simultaneous	48	0.999
WFP60	Simultaneous	48	0.001
PAO1 Sm ^r	Sequential	48	0.999999
WFP60	Sequential	24	0.000001

^a At 48 h, the biofilm cells were harvested, resuspended, plated onto selective media, enumerated, and expressed as a percentage of the total population of cells recovered.

in these long-term static experiments result in the activation of another set of exopolysaccharide genes, such as *pel* or PA1388-1391, and that this can compensate for the loss of *psl*.

Sauer et al. proposed that during biofilm formation, cells undergo a two-step attachment process consisting of reversible and irreversible phases (see reference 46 and references therein). In the reversible attachment phase, the cells contact the surface via the cell pole and are transiently affixed to the substratum, contrasting with the irreversible attachment stage, in which the cells reorient to the longitudinal cell axis and begin cell cluster (microcolony) formation. In future experiments, it would be worthwhile to investigate more thoroughly at which stage WFP60 is impaired (i.e., reversible versus irreversible attachment).

The biochemical composition of the *P. aeruginosa* biofilm matrix in mature nonmucoid biofilms has remained poorly defined. Until recently, it was assumed that the matrix is composed of alginate, but several reports have challenged this (14, 19, 37, 62). A recent study indicated the importance of extracellular DNA for the initial establishment of *P. aeruginosa* biofilms (58). More recently, Friedman and Kolter identified a seven-gene locus in *P. aeruginosa* strain PA14, designated *pel*, that is required for the formation of pellicles at the air-liquid interface (14). Most of the predicted proteins of the *pel* genes have amino acid sequence similarities with enzymes involved in carbohydrate processing. Carbohydrate and glycosyl linkage analysis of the matrix material derived from PA14 and PA14 *pel* mutants supported the conclusion that the matrix is composed of a glucose-rich component that is distinct from cellulose or LPS. Although we did not investigate the involvement of the *pel* locus in *P. aeruginosa* PAO1 biofilms, it was reported that the laboratory domestication of strain PAO1 may have precluded identification of the *pel* genes in PAO1 biofilm formation (14). Likewise, strain PA14 appears to lack the *pslA* to *-D* genes (15). A recent analysis examined the genomic contents of 18 *P. aeruginosa* isolates derived from diverse sources ranging from the environment to acute and chronic infections (59). The data from that analysis showed that the *psl* and *pel* loci were present in every strain analyzed, indicating that most *P. aeruginosa* genes possess the genetic potential to produce these putative EPS types. Interestingly, the putative EPS locus PA1381-1392 was present in only 11 of the isolates examined.

An earlier analysis of the *P. aeruginosa* PAO1 biofilm matrix revealed that the primary carbohydrate constituents are glucose, rhamnose, and mannose (62). A neutral extracellular polysaccharide containing D-glucose, D-mannose, and L-rham-

nose was previously purified from a clinical *P. aeruginosa* immunotype 4 isolate (26). However, there have been no subsequent reports regarding the genetic basis for the production of this polysaccharide. Although carbohydrate analysis of the PA14 pellicle matrix did not identify mannose as a component (14), this sugar subunit is clearly present in the PAO1 biofilm matrix (62). Since the *pslB* gene product is a probable phosphomannose isomerase/GDP-mannose pyrophosphorylase, it is tempting to speculate that the *psl* locus may be involved in the synthesis of a mannose-containing polysaccharide that contributes to biofilm formation. Data in one of the accompanying papers (15) support this hypothesis. It is also possible that *P. aeruginosa* has the capacity to synthesize several alternative polysaccharides that play a role in the biofilm matrix.

Because of their intrinsic resistance to antimicrobial agents, biofilms are significant in medicine and industry. The chemical structure of the biofilm matrix is believed to be composed of nucleic acids and exopolysaccharides. Our data indicate that a previously uncharacterized locus, *psl*, may encode a novel polysaccharide that is required for the formation of the biofilm matrix. Future experiments will include a thorough comparison of the carbohydrate constituents and the glycosyl linkages of polysaccharides purified from PAO1 and WFP60. In addition, the role of the remaining *psl* genes (*pslC* to *pslO*) in biofilm formation requires further study. In this regard, data in an accompanying paper reveal that all 15 of the genes within the *psl* locus are cotranscribed, suggesting that there is a single operon (34).

Since mucoid conversion typically occurs months or years after initial colonization (17), we hypothesize that *P. aeruginosa* has the capacity to express alternative exopolysaccharides that are essential for biofilm formation and the persistence of *P. aeruginosa* infections. Further investigations of these early events in conjunction with data derived from genetic regulation studies of the *psl* cluster may open up new avenues for immunological or chemotherapeutic interventions for infections caused by this important opportunistic pathogen.

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