The Sigma Factor AlgU Plays a Key Role in Formation of Robust Biofilms by Nonmucoid *Pseudomonas aeruginosa*\*\†

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*Pseudomonas aeruginosa* is a Gram-negative bacterium found in various environments, especially in soil, and is an opportunistic pathogen, responsible in particular for chronic lung infections of cystic fibrosis (CF) patients (21). *P. aeruginosa* frequently adopts a biofilm lifestyle, both in the environment and in the course of pathogenesis (15, 23). Biofilms are microbial communities of cells that are attached to a substrate, an interface, or each other and are embedded in a matrix of extracellular polymeric substances that they have produced (15). Maintenance of long-term infections in the CF lung is associated with the conversion of *P. aeruginosa* to a mucoid phenotype, which results from overproduction of the exopolysaccharide alginate (21, 48). Alginate overproduction leads to highly structured biofilms and is responsible for some biofilm properties, such as increased resistance to the antibiotic tobramycin (24). In contrast, alginate is not a major component of the extracellular matrix when biofilms are formed by nonmucoid *P. aeruginosa* strains, such as PAO1 and PA14 (69).

Consistently, alginate production is not critical for biofilm formation by these strains (17, 57). Biofilm formation by nonmucoid *P. aeruginosa* strains is of primary importance for pathogenesis, since (i) this leads to seeding dispersal of large numbers of cells that might subsequently initiate an infection, (ii) virulent phenotypes might survive and expand within biofilms, and (iii) high cell densities reached in biofilms might activate the quorum-sensing (QS) network which controls the production of virulence factors (23). Furthermore, CF patients are initially colonized by motile, nonmucoid *P. aeruginosa* strains, which attach to mucin-covered epithelial cells and likely develop as a biofilm before switching to a mucoid phenotype (48).

The extracytoplasmic function (ECF) sigma factor AlgU (also known as AlgT, RpoE, σ^E^, and σ^22^) is responsible for transcription of the alginate biosynthetic operon (45). Conversion to mucoidy is due in most cases to spontaneous mutations in *mucA*, leading to hyperactivity of AlgU, which in turn results in alginate operon overexpression (21, 48). AlgU therefore plays a key role in the formation of mucoid *P. aeruginosa* biofilms. AlgU is also involved in other traits, such as resistance to various stresses (oxidative, heat, hyperosmotic, and cell wall stresses) and control of some virulence factor synthesis (2, 16, 45, 67, 68). Surprisingly, few studies have addressed the role of AlgU in nonmucoid *P. aeruginosa*, even though the sigma factor is expressed in these cells (50, 67). Here we examined the potential role of AlgU in the formation of nonmucoid *P. aeruginosa* biofilms. We observed that AlgU is critical for the...
formation of robust biofilms, and we identified genetic and biochemical defects related to biofilm formation and virulence in the algU mutant.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were grown in Luria-Bertani (LB) broth at 37°C. *P. aeruginosa* cells were grown in liquid at 37°C with shaking, using LB or PPGAS medium (20 mM NH₄Cl, 20 mM KCl, 120 mM Luria-Bertani (LB) broth at 37°C. *Escherichia coli* were LB agar (15 g liter⁻¹)/H11002 souse Bois, France).

The system was assembled and prepared as previously described (59). The three-channel flow cell with individual channel dimensions of 1 by 4 by 40 mm.

**Plasmids**
pCR2.1-TOPO E. coli cloning vector for PCR products; Ap⁴ Invitrogen, Paisley, UK
pSMC21 gfp expression plasmid; Kan¹ 6, 11
pEX100Tink Suicide vector; Ap⁴ 47
pUCGMlox Source oflox-aacC1-lox cassette; Ap⁴ Gm¹ 47
pCM157 cre expression vector; Te² 37
pEXalgU pEX100Tink with disrupted algU; Ap⁴ This study
pEXUGL pEXalgU withlox-aacC1-lox cassette; Ap⁴ Gm¹ This study
pBBR1MCS-5 E. coli-P. aeruginosa shuttle vector; Gm¹ This study
pBAUC pBIRR1MCS-5 with a 911-bp fragment includingalgU This study
pMA9 pEX18Gm containing the5' end ofpslA and its upstream region (positions—912 to 591 relative to pslA), with thepsl promoter (positions—267 to—54) replacing byaraC-P_BAG, Gm¹ 36
pMMB207-PA2663 Pisc::PA2663 in pMMB207; Cm² 3

When the strains did not contain pSMC21 [PAOU P_BAG::psl and PAOU(pMMB207-PA2663)], precultures were grown without antibiotic (PAOU P_BAG::psl) or with chloramphenicol [PAOU(pMMB207-PA2663)], bacteria were washed, and biofilms were grown for 24 h under dynamic conditions without antibiotic, as described above. Biofilms were then stained for 10 min with 5 μM Syto 61 Red (Invitrogen, Paisley, United Kingdom) before CLSM observation. Arabinose (0.2%) was included in the PPGAS medium during growth of PAOU P_BAG::psl biofilms.

**Assessment of biofilm stability.** We used the method described by Friedman and Kolter (17) to assess biofilm stability. Briefly, biofilms were grown for 48 h at 37°C in 96-well polystyrene plates (PAC) microtiter plates without shaking. Biofilms were then either gently washed by submerging the microtiter plate in water or harshly washed with running water and were stained with crystal violet, which was quantified by turbidity measurement at 600 nm. Alternatively, bacteria in fresh PPGAS were inoculated into flow cell channels and biofilms were grown for 16 h at 37°C without medium flow to allow biofilm formation under static conditions. PPGAS flows at increasing rates (0.6, 1.2, 3.6, 7.3, 12.0, and 20.0 ml h⁻¹) were then successively applied as follows: the 0.6-ml h⁻¹ flow was applied for 5 min, the flow was turned off to observe biofilms by CLSM, the 1.2-ml h⁻¹ flow was applied for 5 min, and so on.

**Image acquisition and analyses.** CLSM observations were performed with a TCS-SP2 microscope (Leica Microsystems, Heidelberg, Germany), using a 63× immersion objective. Excitation wavelengths for green fluorescent protein (GFP), calciferol white, and Syto 61 Red were 488, 400, and 633 nm, respectively. Emissions wavelengths were 500 to 550, 410 to 490, and 645 nm, respectively. Calciferol white produced a blue light, which we artificially converted to red during image acquisition for better contrast. Images were taken every micrometer at all biofilm depths, at 10 random positions. Three-dimensional (3D) images were obtained using Leica LAS AF software, and biofilm parameters (biovolume and thickness) were quantified with COMSTAT (25).

To observe biofilms by SEM, samples were fixed in 5% glutaraldehyde overnight at 4°C, washed three times for 10 min in 0.1 M phosphate buffer, and then dehydrated by soaking for 10 min in increasing concentrations of alcohol, i.e., 70%, 95%, and absolute ethanol. The samples were dried in ethanol in a CPD 030 critical point dryer (Bal-Tec, France), using CO₂ as a transitional fluid until the critical point was reached. The samples were mounted on aluminum stubs and coated for 120 s at 20 mA with gold-palladium alloy, using a model 501 sputter coater (Edwards Pirani, United Kingdom), and were observed with a JEOL 6460 LV microscope (JEOL Ltd., Tokyo, Japan) at magnifications of

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**TABLE 1. Bacterial strains and plasmids used in this study**

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<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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| **Strains**
| *P. aeruginosa* strains |
| PAO1 | Wild type | 28 |
| PAOU | PAO1 ΔalgU::lox | This study |
| PAOU P_BAD::psl | PAOU araC P_BAD::psl | This study |
| **E. coli** strains |
| Top10 | Electrocompetent cells used as cloning host | Invitrogen, Paisley, United Kingdom |
| S17-1 | recA pro (RP4-Tett::Mu Kan::Tn7), donor and helper strain for conjugation |
| **Plasmids** |
| pCR2.1-TOPO | E. coli cloning vector for PCR products; Ap⁴ | Invitrogen, Paisley, UK |
| pSMC21 | gfp expression plasmid; Kan¹ | 6, 11 |
| pEX100Tink | Suicide vector; Ap⁴ | 47 |
| pUCGMlox | Source oflox-aacC1-lox cassette; Ap⁴ Gm¹ | 47 |
| pCM157 | cre expression vector; Te² | 37 |
| pEXalgU | pEX100Tink with disruptedalgU; Ap⁴ | This study |
| pEXUGL | pEXalgU withlox-aacC1-lox cassette; Ap⁴ Gm¹ | This study |
| pBBR1MCS-5 | E. coli-P. aeruginosa shuttle vector; Gm¹ | This study |
| pBAUC | pBIRR1MCS-5 with a 911-bp fragment includingalgU | This study |
| pMA9 | pEX18Gm containing the5' end ofpslA and its upstream region (positions—912 to 591 relative to pslA), with thepsl promoter (positions—267 to—54) replacing byaraC-P_BAG, Gm¹ | 36 |
| pMMB207-PA2663 | Pisc::PA2663 in pMMB207; Cm² | 3 |
cloned into the EcoRI-HindIII sites of the suicide vector pEX100Tlink (47), generating a 546-bp deletion within the regions were PCR amplified with primer pairs AlgU1-AlgU2 and AlgU3-AlgU4 described by Queneé et al. (47). To construct pEXUGL, the lox of the vector, followed by a search for Gm-resistant (presence of plates were counterselected on 5% sucrose-LB agar plates to force the excision. P. aeruginosa into E. coli. The resulting plasmid, pEXUGL (Table 1), was introduced into the algU in pEX construction by PCR. The mutant strain selected for further studies was named from (47) was then subcloned as a PstI fragment between the 5/H11032 carrying the Gm resistance gene PAO1 was obtained by allelic exchange with a truncated version of plasmid pEXUGL. The full plasmid pEXUGL. The full

Quantification of mRNA levels by qRT-PCR

16SF CAGGATTAGATACCCCTGGTAGTCACC This study
16SR GACTTAAACCACATCTCAAGCAC This study
PelA1 TGTCGGGTATCTGAAAGAC This study
PelA2 GACCGACAGATAAGCGCAAG This study
PslA1 GTTCTGGCATCTGATGCCTATG This study
PslA2 AGGTAGGGAAACAGGCCCC This study
LccA1 TGGAAGGTGAGTTCTGGG This study
LccA2 AATCGAGTTATCCTGGCCTG This study
LccB3 ACCAAATACGCCTCACTCG This study
LccB4 CTGACTCTTGCACCTGTGC This study
Fhp1 AATTACCTGCATGACCGGGTC This study
Fhp2 CGGGAAACAGATCCAGCGG This study
PpyR1 TTCTCTGACGCCGCTACCTGA This study
PpyR2 AGCGCATGGGGAAAAACACC This study
Promoter mapping by 5′-RACE PCR

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Quantification of mRNA levels by qRT-PCR

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Quantification of mRNA levels by qRT-PCR

×8,500 to ×10,000. The voltage was kept at 10 kV or 15 kV and at an average distance from the electron gun of about 10 mm.

Nucleic acid procedures. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Invitrogen (Paisley, United Kingdom). PCRs were performed using FastStart PC reagent with 2X premix D (Tebu-Bio, Epicentre Biotechnologies, Madison, WI). Plasmids and RNAs were purified using a QIAprep Spin miniprep kit and an RNAeasy Midi kit (Qiagen, Hilden, Germany). E. coli (commercial electrocompetent Top10 cells [Invitrogen, Paisley, United Kingdom] or S17-1 cells) and P. aeruginosa cells were transformed by electroporation as previously described (55) or by conjugation as follows. Exponential-phase cultures of E. coli S17-1 carrying the plasmid to transfer and the recipient strain P. aeruginosa PAO1 were mixed on LB agar medium without NaCl and incubated overnight at 37°C. Bacteria were then resuspended in liquid LB and plated onto solid PIA medium (which inhibits the growth of E. coli but not of P. aeruginosa) containing the appropriate antibiotic.

algU inactivation and complementation of the mutation. The algU mutant of P. aeruginosa PA01 was obtained by allelic exchange with a truncated version of algU carrying the Gm resistance gene aacC1 framed by lor sequences, using plasmid pEXUGL. The full cre-lox gene deletion procedure used here was described by Queneé et al. (47). To construct pEXUGL, the algU-flanking regions were PCR amplified with primer pairs AlgU1-AlgU2 and AlgU3-AlgU4 (Table 2). After PstI digestion, the two fragments were ligated with each other, generating a 546-bp deletion within the algU coding sequence. This fragment was cloned into the EcoRI-HindIII sites of the suicide vector pEX100TlinK (47), resulting in plasmid pEXalgU (Table 1). The lor-aacC1-lor cassette of pUCmCS2 (47) was then subcloned as a PstI fragment between the 5′ and 3′ parts of algU in pEXalgU. The resulting plasmid, pEXalgU (Table 1), was introduced into the E. coli donor/helper strain S17-1, from which it was transferred by conjugation into P. aeruginosa PA01. After mating, Gm-resistant colonies grown on PIA plates were counterselected on 5% sucrose-LB agar plates to force the excision of the vector, followed by a search for Gm-resistant (presence of aacC1) and Ch-sensitive (loss of vector) colonies. The excision of the lox-flanked aacC1 gene was then catalyzed by the Cre recombinase encoded by pCM157 (37) to yield an antibiotic-resistant mutant and to avoid the putative polar effects resulting from aacC1 insertion (47). Primers AlgU1 and AlgU2 were used to verify the construction by PCR. The mutant strain selected for further studies was named PAOU.

To complement the mutation, a 911-bp DNA fragment including the full-length algU gene and its P1, P2, and P3 promoters (52) was amplified by PCR with primers AUCP1 and AUCP2 (Table 2). The amplicon was inserted into pBBR1MCS-5 (33) after EcoRI/BamHI digestion, generating pBAUC (Table 1), which was introduced into P. aeruginosa PAOU. To construct a PAOU strain containing an inducible psl operon, the pMMB207-P2A663 plasmid containing ppyR under the control of the Pscu promoter (3) was introduced into PAOU by conjugation.

mRNA assay by quantitative reverse transcription-PCR (qRT-PCR). RNA quantification was performed as previously described (22), using bacteria grown for 8 h in liquid PPGAS medium. The primers used are given in Table 2. 16S rRNA was used as an endogenous control (10). PCRs were performed in triplicate, and the standard deviations were <0.15 threshold cycle (Ct). The relative quantifications were obtained as previously described (5), using the comparative Ct (2^(-ΔΔCt)) method (34).

Promoter mapping by 5′-RACE PCR. Total RNAs were isolated from P. aeruginosa PA01 grown in PPGAS medium by use of a MasterPure RNA purification kit (Epicentre Biotechnologies, Madison, WI). The 5′ end of lecB mRNA was amplified using a system for 5′ rapid amplification of cDNA ends (5′-RACE) (version 2.0; Invitrogen, Paisley, United Kingdom) according to the manufacturer’s instructions. The primers used for cDNA synthesis and for the first and second PCRs are listed in Table 2. The final PCR product of 5′-RACE amplifications was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced (Cogenics, Takeley, United Kingdom).

Quantification of the exopolysaccharide Psl. Psl was extracted from the cell surface and its levels were evaluated by an enzyme-linked immunosorbent assay (ELISA) as described in previous work (7).

Twitching motility tests. Twitching assays were performed by inoculating bacteria by stabbing to the bottom of a petri dish through a layer of PPGAS agar (10 g liter\(^{-1}\)) (49). After 24 h of incubation at 37°C, the agar was removed, the petri
Biofilms formed by PAOU(pBAUC) cells were particularly removed about 60% of cells from PAO1 biofilms (Fig. 1B). Most (about 85%) of the cells from PAOU biofilms, whereas it that of PAO1 (Fig. 1B). Harsh washings led to the removal of cell population was reduced only about 2-fold compared with washing revealed that PAOU was able to form biofilms whose water, as described by Friedman and Kolter (17). A gentle submerging the plate in water or harshly washed with running biofilms, cells were grown in a microtiter plate for 48 h only to its reduced attachment ability but also to impaired medium flow was severe, we hypothesized that this was due not to its reduced attachment ability but also to impaired stability of the PAOU biofilm community. To examine biofilm formation under static conditions and to assess the stability of these biofilms, cells were grown in a microtiter plate for 48 h without shaking. Biofilms were then either gently washed by submerging the plate in water or harshly washed with running water, as described by Friedman and Kolter (17). A gentle washing revealed that PAOU was able to form biofilms whose cell population was reduced only about 2-fold compared with that of PAO1 (Fig. 1B). Harsh washings led to the removal of of the surface with PAOU compared to that with PAO1 (Fig. 1A; Table 3). The algU mutant thus displayed dramatically reduced attachment properties. After 24 h of PPGAS flow, a PAOU biofilm defect was particularly obvious: PAOU showed that all three strains had developed biofilms of similar thicknesses (Fig. 1C). However, PAO1 and PAOU(pBAUC) biofilms resisted flows of 2.5 ml h⁻¹ (Fig. 1B) and even 20 ml h⁻¹ (not shown). Importantly, a 2.5-ml h⁻¹ flow rate was sufficient to dissociate cells from PAO1 biofilms (Fig. 1C). This resistant (Fig. 1B), confirming a relationship between AlgU and biofilm stability.

RESULTS

The algU mutant is impaired in attachment to glass and formation of flow-resistant biofilms. In preliminary experiments, two rich media were compared for biofilm formation, namely, the commonly used LB broth and PPGAS, which is limited in phosphate and thereby promotes rhamnolipid production (65). Biofilms of wild-type P. aeruginosa PAO1 grown on glass slides for 16 h at 37°C under static conditions were about 1.5-fold thicker in PPGAS than in LB broth (about 9 versus 6 μm), with similar cell densities (data not shown). Since growth rates did not differ significantly between LB and PPGAS liquid cultures, PPGAS seemed more favorable for biofilm formation and was therefore selected for the subsequent experiments. To test the role of AlgU in biofilm formation by nonmucoid P. aeruginosa, we constructed a P. aeruginosa PAOU mutant in which 546 bp of the 582-bp sequence of algU were deleted (Table 1). This mutant is devoid of antibiotic resistance, allowing its transformation by the GFP-encoding plasmid pSMC21 (11) (Table 1) in order to visualize biofilm-grown cells by CLSM. The algU mutation did not affect the growth rate in liquid PPGAS at 37°C (data not shown). To grow biofilms on a microscope glass coverslip in flow cells, bacteria in 0.9% NaCl were allowed to attach to the coverslip for 2 h, and a flow (2.5 ml h⁻¹) of PPGAS was applied. Attached bacteria were observed by CLSM after 15 min of medium flow, revealing that there was 34-fold reduced coverage of the surface with PAOU compared to that with PAO1 (Fig. 1A; Table 3). The algU mutant thus displayed biologically reduced attachment properties. After 24 h of PPGAS flow, a PAOU biofilm defect was particularly obvious: PAOU failed to produce a biofilm covering the glass surface, whereas the PAO1 biofilm covered the surface (Fig. 1A). To complement the PAO1 mutant, we constructed the low-copy-number plasmid pBAUC (Table 1), which carries the PAO1 algU gene under the control of three (P1, P2, and P3) of its five promoters (52). The cloning of a larger fragment, including algU and all five promoters, was unsuccessful. The presence of pBAUC in strain PAOU increased its attachment ability 11-fold and allowed PAOU to form biofilms similar to those of PAO1 (Fig. 1A; Table 3). This verified that the absence of AlgU was responsible for the PAOU biofilm phenotypes.

Because the biofilm formation defect of PAOU after 24 h of medium flow was severe, we hypothesized that this was due not only to its reduced attachment ability but also to impaired stability of the PAOU biofilm community. To examine biofilm formation under static conditions and to assess the stability of these biofilms, cells were grown in a microtiter plate for 48 h without shaking. Biofilms were then either gently washed by submerging the plate in water or harshly washed with running water, as described by Friedman and Kolter (17). A gentle washing revealed that PAOU was able to form biofilms whose cell population was reduced only about 2-fold compared with that of PAO1 (Fig. 1B). Harsh washings led to the removal of most (about 85%) of the cells from PAO1 biofilms, whereas it removed about 60% of cells from PAO1 biofilms (Fig. 1B). Biofilms formed by PAOU(pBAUC) cells were particularly

FIG. 1. Attachment of P. aeruginosa and biofilm growth and robustness. (A) The indicated strains containing pSMC21 were allowed to attach to the glass surface before application of a medium flow for 15 min (left; attachment) and 24 h (right; biofilm). Each maximal projection is representative of five CLSM observations. (B) Stability of biofilms grown under static conditions in 96-well PVC microtitre plates. Biofilms were quantified by crystal violet staining after gentle washing by immersion into water or harsh washing with running water. Each value is the average for three independent experiments. (C) Side views of biofilms of the indicated strains containing pSMC21. Biofilms were grown under static conditions, and medium flows of 0.6 ml h⁻¹ (left) and 2.5 ml h⁻¹ (right) were applied for 5 min before CLSM observations. These data are representative of two distinct experiments.
showed that AlgU is required for the formation of robust, shear-resistant biofilms.

We used SEM to observe the surfaces of biofilms grown on glass slides. An abundant extracellular matrix masked PAO1 cells, whereas cells were visible within the rare PAOU-attached aggregates (Fig. 2A). Matrix production by PAOU increased upon complementation with pBAUC (Fig. 2A). We used the polysaccharide-binding dye calcofluor white, which increased upon complementation with pBAUC (Fig. 2A). We attached aggregates (Fig. 2A). Matrix production by PAOU in cells, whereas cells were visible within the rare PAOU-at-glass slides. An abundant extracellular matrix masked PAO1 shea-resistant biofilms.

We used SEM to observe the surfaces of biofilms grown on glass slides. An abundant extracellular matrix masked PAO1 cells, whereas cells were visible within the rare PAOU-attached aggregates (Fig. 2A). Matrix production by PAOU increased upon complementation with pBAUC (Fig. 2A). We used the polysaccharide-binding dye calcofluor white, which stains P. aeruginosa biofilm matrix (40), to compare biofilms grown for 48 h under dynamic conditions. This allowed PAOU to form a thin biofilm layer with dispersed microcolonies, whereas PAO1 and PAOU(pBAUC) biofilms were more homogeneous and had higher biovolumes than PAO1 biofilms (Fig. 2B and D; Table 3). PAOU microcolonies were thicker than PAO1 and PAOU(pBAUC) biofilms (Fig. 2D), leading to similar average thicknesses for 48-h biofilms of all three strains (Table 3). Consistent with the SEM images, calcofluor white revealed that PAO1 and PAOU(pBAUC) biofilms were covered by polysaccharidic matrixes, whereas PAO1 biofilms were almost devoid of exopolysaccharides (Fig. 2C and D).

AlgU therefore appears to be required for synthesis of major polysaccharidic components of PAO1 biofilm matrix.

Finally, it should be noted that we also observed a biofilm formation defect with strain PAOU for static biofilms grown in LB and minimal M9 glucose media: no biofilm, but only dispersed PAO1 cells, remained attached to glass slides after washing (data not shown). This indicated that AlgU modulation of biofilm formation occurs under several environmental conditions.

The algU mutant is defective in production of the Psl exopolysaccharide, a key matrix component. Prior studies showed that alginate is not a major component of nonmucoid biofilms (69). Since the PAO1 biofilms appeared to have reduced exopolysaccharides, we hypothesized that AlgU controls production of Psl and/or Pel, two other PAO1 polysaccharides linked to biofilm formation (17, 18, 51). We examined pslA, pslB (the first two genes of the psl operon), and pelA (the first gene of the pel operon) mRNA levels by qRT-PCR. The latter was performed on RNAs extracted from bacteria harvested after 8 h of growth in liquid PPGAS cultures, since we used bacteria at this growth stage for static biofilm formation. Whereas the pelA mRNA level was unaltered, pslA and pslB mRNA levels were 1.7- and 1.8-fold lower, respectively, in PAOU than in PAO1 (Fig. 3A). This indicated that the algU mutant had reduced psl transcription compared with that of wild-type cells. Unfortunately, we were not able to analyze gene expression in biofilm-grown cells because PAOU biofilms did not yield a sufficient biomass to extract adequate amounts of RNA.

To directly test Psl levels in the P. aeruginosa strains, PAO1, PAOU, and PAOU(pBAUC) cells were cultured, cell surface polysaccharides were extracted, and amounts of cell-associated Psl were evaluated by ELISA as previously described (7). Consistent with the above qRT-PCR data, Psl synthesis was reduced 2.8-fold in PAO1 compared with that in PAO1, and complementation resulted in enhanced Psl levels (Fig. 3B). In prior studies, 2- to 3-fold changes in psl transcription or Psl synthesis resulted in profound changes in biofilm integrity and architecture (36, 58). For strain PAO1, psl mutants were severely compromised in biofilm formation (8, 29, 38, 42). Psl was recently shown to be anchored on the cell surface in a helical pattern, promoting cell-cell interaction and assembly of a matrix which holds bacteria in the biofilm and on the surface (35). The instability displayed by PAO1 biofilms exposed to a medium flow is therefore consistent with the observed Psl

![FIG. 2. Polysaccharide matrix in P. aeruginosa biofilms. (A) SEM observations of static biofilms of the indicated strains. Magnification, ×8,500 to ×10,000. (B) 3D images resulting from CLSM observations of 48-h biofilms grown as described in the legend to Fig. 1A. The three strains contained pSMC21, and bacteria are shown in green. (C) Polysaccharides of the same biofilms as in panel B, stained with calcofluor white. This dye produced a blue light, which we artificially converted to red in our images for better contrast. Yellow results from the green (bacteria) and red (polysaccharides) overlay. (D) Side views of biofilms shown in panel C. All images are representative of five observations.](http://jb.asm.org/)
production defect. Finally, the \textit{psl} operon was placed under the control of the arabinose-inducible promoter \textit{P\textsubscript{BAD}} in the PAOU background. Inducing \textit{psl} expression restored the ability of PAOU to form flow-resistant biofilms in 24 h, as illustrated by the biovolume and average thickness values, which were both about 2-fold lower than those of PAOU(pBAUC) biofilms (Table 3). This suggested that \textit{psl} downregulation might not be the only cause of the PAOU phenotype.

**Lectin-encoding genes are underexpressed in the absence of AlgU.** We examined if the expression of other genes known to be involved in biofilm formation is compromised in the absence of AlgU. We observed that \textit{lecA} and \textit{lecB} mRNA levels were reduced (2.2- and 3.0-fold, respectively) in the \textit{algU} mutant compared to those in PAO1 (Fig. 3A), which was not reported in published microarray studies (16, 60, 67, 68). The \textit{lecA} and \textit{lecB} genes each encode a lectin, namely, galactophilic LecA (PA-IL) and \(\alpha\)-fucose-specific LecB (PA-III), respectively. Since single \textit{lecA} and \textit{lecB} mutants of PAO1 showed impaired abilities to form biofilms on abiotic surfaces (14, 62), the downregulation of \textit{lecA} and \textit{lecB} genes in the \textit{algU} mutant likely contributes to its biofilm formation defect. Both lectins were proposed to mediate the adhesion of \textit{P. aeruginosa} to other bacterial cells of the same or different species (14, 62), although this role remains debated (56). LecB was shown to be required for posttranscriptional type IV pilus biogenesis, without impairing transcription of \textit{psl} genes encoding pilus components (56). LecB was therefore necessary for twitching motility, which occurs on solid surfaces or interfaces by extension and retraction of type IV pili (54). A \textit{lecB} mutant displayed the same biofilm formation defect as a nonpiliated mutant, leading Sonawane et al. (56) to conclude that pilus biogenesis explains the role of LecB in biofilm formation.

**FIG. 3. Alterations in gene expression, Psl exopolysaccharide production, and twitching motility in the absence of AlgU.** (A) Expression levels of \textit{pslA}, \textit{pslA}, \textit{pslB}, \textit{lecA}, \textit{lecB}, \textit{fhp}, and \textit{ppyR} genes in the \textit{algU} mutant PAOU relative to those in PAO1. \textit{pslA} and \textit{pslB} are the first two genes of the \textit{psl} operon, and \textit{fhp} and \textit{ppyR} are the first two genes of the putative \textit{fhp-ppyR-PA2662} operon. RNAs were extracted after 8 h of growth in liquid PPGAS medium and were assayed by qRT-PCR. A relative value of <1 indicates a reduction of gene expression in PAO1. 16S rRNA was used as an endogenous control to normalize the RNA input and reverse transcription efficiency. PCRs were performed in triplicate, and the standard deviations were <0.15 Ct. The values are the means for at least two independent experiments. (B) Quantification of Psl production by ELISA. PAO1, PAOU, and PAOU(pBAUC) cells were grown, and surface exopolysaccharide was extracted and quantified by ELISA using a Psl-specific antibody, as outlined previously (7). Shown below the graph are rapid-attachment biofilm values (7) obtained for strains used for Psl enumeration (means for three replicates). (C) Twitching motilities of the indicated strains were examined at the petri dish-PPGAS agar interface. The pictures are representative of at least three tests.
leaves uncertain the functionality of this putative promoter. The TAGATT sequence lying around position −10 rather suggests that the lecB promoter depends on σ70 (consensus −10 sequence, TATAAT [45]) or on RpoS, which recognizes a similar −10 element and is required for LecB synthesis (66). Interestingly, we observed a lux box-like sequence, which could allow binding of LasR and RhlR QS regulators (64), between positions −52 and −33 (Fig. 4). This is consistent with the report that LecB production requires RhlR and its cognate signal molecule, N-butyroyl-L-homoserine lactone (C4-HSL) (66). The presence of a lux box was highlighted at a similar position (−53 to −34 relative to the +1 base) in the promoter region of lecA, whose expression also depends on RhlR/C4-HSL (66).

These data suggest that AlgU is not directly responsible for transcription of the psl operon or of the lecA and lecB genes. A dramatic reduction in psl gene expression was reported for the ppyR mutant of P. aeruginosa PAO1 compared to the parental strain (3). The fhp and PA2662 genes flank ppyR, and the short sequences separating these genes (54 bp between fhp and ppyR and 12 bp between ppyR and PA2662) suggest that they are cotranscribed (www.pseudomonas.com). Since fhp was the most strongly overexpressed gene (60-fold) in mucoid strains (16), we hypothesized that expression of the fhp-ppyR-PA2662 operon would be reduced in the algU mutant. This was confirmed by qRT-PCR: the mRNA levels of fhp and ppyR genes were reduced 1.6- and 2.2-fold, respectively, in the PAOU mutant strain compared to PAO1 (Fig. 3A). Consequently, our data suggest that AlgU stimulates the expression of the fhp-ppyR-PA2662 operon in PAO1, leading to positive control of the psl operon via PpyR, which is a small protein (85 amino acids) predicted to be located in the cytoplasmic membrane (3; www.pseudomonas.com). Consistent with this hypothesis, expressing ppyR in PAO1 by using the pMMB207-PA2662 plasmid (3) allowed the strain to form flow-resistant biofilms in 24 h, even though the biovolume and average thickness values remained modest compared to those of induced PAOU P_psyR:psl and PAOU(pBAUC) biofilms (Table 3). This prompted us to use another method: we evaluated the stability of PAOU(pMMB207-PA2663) biofilms grown in a microtiter plate under static conditions (17). The crystal violet turbidity values were 1.7 ± 0.1 and 0.74 ± 0.16 after gentle and harsh washing, respectively. These values are similar to those obtained with the wild-type PAO1 strain (Fig. 1B), confirming that expressing ppyR in PAOU increased the biofilm stability.

**DISCUSSION**

The present study revealed that the ECF sigma factor AlgU is required for efficient attachment to abiotic surfaces and for development of robust, shear-resistant biofilms by the nonmucoid P. aeruginosa PAO1 strain. Such a strong biofilm formation defect of the algU mutant strain was unexpected and is likely multifactorial. Likewise, we identified the psl operon and the lecA and lecB genes as being underexpressed in the algU mutant. This led us to propose three mechanisms by which AlgU could contribute to biofilm formation: Psl polysaccharide matrix synthesis, production of LecA and LecB lectins, and type IV pilus biogenesis. It is likely that additional AlgU-dependent genes remain to be identified, but these three mechanisms may be sufficient to explain the attachment and biofilm stability defects of the algU mutant. The critical roles of Psl, LecA, LecB, and type IV pili in adhesion and biofilm formation are indeed extremely well documented (3, 8, 14, 19, 29, 30, 31, 35, 36, 38, 41, 42, 62).

Control of psl expression in P. aeruginosa appears to be complex. Prior studies showed that the psl genes are transcribed as an operon, which is constitutively expressed in planktonic cells but tightly regulated in biofilm-grown bacteria (38, 42). Several recent studies have reported the regulation of psl by the secondary messenger molecule c-di-GMP (26, 58) as well as by the GacS-GacA-rsmZ-RsmA signal transduction pathway (20). Furthermore, the involvement of PpyR in P. aeruginosa PAO1 biofilm formation (4) was explained by a dramatic reduction in psl gene expression in the ppyR mutant (3). Likewise, the lecA gene is the object of multiple regulation levels: lecA transcription requires the Rhl QS system, the stationary-phase sigma factor RpoS, and the Pseudomonas quinolone signal (PQS), while it is repressed by MvaT (12, 13, 66). As with psl, reports indicate that the posttranscriptional GacS-GacA-rsmZ-RsmA regulatory pathway and other signaling proteins feeding into this pathway control lecA expression, but these controls could occur indirectly through the QS network (20, 44, 51). Little is known regarding lecB regulation, except that similar to that of lecA, lecB transcription requires the Rhl QS system and RpoS (66). Notably, expression of psl, lecA, and lecB was not previously identified as AlgU dependent. This discovery adds another level of complexity to the regulation of these genes. As a sigma factor, AlgU could be directly responsible for their transcription. Analysis of the promoters of these genes, however, indicated that this is probably not the case.
since the transcription initiation sites are not preceded by an AlgU-like promoter sequence with appropriate spacing (Fig. 4) (42, 66). AlgU is therefore likely to act indirectly, via the transcription of genes encoding regulators acting on psl, lecA, and/or lecB. We observed that AlgU contributes to expression of ppyR, whose product positively controls psl expression via an unknown mechanism (3). The control of the fhp-ppyR-PA2662 operon by AlgU is likely indirect, since the fhp promoter is dependent on the alternative sigma factor RpoN (74) and on the activator FhpR (1). The fhp and fhpR genes are adjacent, sharing a bidirectional promoter region. Proteins encoded by PA0779 and PA3697, which both bind the fhp-fhpR intergenic region, also activate transcription of fhp and fhpR (32). We did not observe a clear downregulation of the PA0779 and PA3697 genes in PAOU (not shown). The control of the psl operon by PpyR is also likely indirect, since the latter is a putative membrane protein, suggesting that it plays the role of sensor rather than transcriptional regulator (3, 4). The complete pathway from AlgU to psl thus remains largely unknown. The control exerted by QS on lecA and lecB suggested that the absence of AlgU could somehow affect the QS network, but this hypothesis was invalidated by the following lines of evidence. Production of the Las and Rhl autoinducer molecules and of PQS was not significantly altered by algU inactivation under our conditions (not shown). Consistently, PAOU was able to produce rhamnolipids at a level close to that of PAO1 (not shown), although this process is directly dependent on RhlR and C4-HSL and indirectly dependent on LasR and PQS (39, 43). Therefore, future work will be required to unravel the mechanism(s) by which AlgU contributes to regulation of psl, lecA, and lecB genes.

The AlgU functions and the mechanisms leading to its hyperactivity were studied specifically in the context of mucoid P. aeruginosa strains (16, 21, 46, 48, 61). Relying on the inhibition of AlgU activity by its anti-sigma factor MucA (21, 48), the notion that AlgU activity was negligible in nonmucoid strains likely prevented investigation of AlgU functions in such strains. Although AlgU activity is certainly low in nonmucoid cells, our data showed that it is very far from being negligible. Consistently, whereas 67% of the AlgU pool in PAO1 was held associated with the inner membrane, likely in an inactive state, the remaining 33% was in the cytosol (50), which could lead to a basal AlgU activity. Since the expression of psl, lecA, and lecB genes was partially AlgU dependent in PAO1 (low AlgU activity), we expected that these genes would be identified as strongly induced in transcriptomic studies of mucoid strains (high AlgU activity) and after AlgU activation by stress conditions. This, however, was not the case (16, 60, 67, 68). This could be due to the fact that these genes are only indirectly regulated by AlgU and that other necessary conditions were not met in mucoid cells and/or under the experimental conditions used. Alternatively, it could reflect differential AlgU functions in mucoid and nonmucoid cells. A transcriptome study of the AlgU regulon in nonmucoid cells in the absence of stress would be of great interest to further address this question.

AlgU has been studied primarily for its essential role in mucoidy and therefore in the maintenance of chronic infection in the lungs of CF patients, and it is furthermore known to be involved in stress responses (2, 45, 67, 68). Here we identified new AlgU functions in P. aeruginosa ecology and pathology. Since AlgU is required for attachment of P. aeruginosa to surfaces and for development of robust biofilms, it plays an important role in colonization of abiotic environments by nonmucoid P. aeruginosa strains, providing a source of bacteria for subsequent infections (23). In addition, AlgU contributes to the production of virulence factors by nonmucoid cells, including LecA, LecB, and probably type IV pilus and protease IV as a consequence of a low LecB level (9, 56, 63). Finally, in the process leading to chronic infection of CF patients, it is believed that the lung is initially colonized by nonmucoid strains, which develop biofilms before conversion to mucoidy (48). For this conversion, Wozniak et al. (69) pointed out the importance of a switch from unknown polysaccharides (later identified as Pel and Psl [51]) to alginate in the primary polysaccharide content of the extracellular polymeric substances. Although the involvement of AlgU in the formation of nonmucoid biofilms on biotic surfaces, especially mucin-covered epithelial cells, remains to be investigated, our data suggest that AlgU could play a key role in the initial infection steps (adhesion and development of nonmucoid biofilm) and in the polysaccharide production switch.

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