

Expression of the *psl* Operon in *Pseudomonas aeruginosa* PAO1 Biofilms: PslA Performs an Essential Function in Biofilm Formation

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The *psl* gene cluster, comprising 15 cotranscribed genes from *Pseudomonas aeruginosa*, was recently identified as being involved in exopolysaccharide biosynthesis and biofilm formation. In this study, we investigated the regulation of the *psl* gene cluster and the function of the first gene in this cluster, the *pslA* gene. PslA shows strong similarities to UDP-glucose lipid carriers. An isogenic marker-free *pslA* deletion mutant of *P. aeruginosa* PAO1 deficient in attachment and biofilm formation was used for complementation studies. The expression of only the *pslA* gene, comprising a coding region of 1,437 bp, restored the biofilm-forming phenotype of the wild type, indicating that PslA is required for biofilm formation by nonmucoid *P. aeruginosa*. The promoter region of the *psl* gene cluster, which encodes PslA-PslO, was identified by rapid amplification of cDNA 5' ends. Promoter assays using transcriptional fusions to *lacZ* and *gfp* indicated a constitutive expression of the *psl* cluster in planktonic cells and a highly regulated and localized expression in biofilms, respectively. Expression of the *psl* cluster in biofilms was almost exclusively found in the centers of microcolonies, as revealed by confocal laser scanning microscopy. These data suggest that constitutive expression of the *psl* operon enables efficient attachment to surfaces and that regulated localized *psl* operon expression is required for biofilm differentiation.

The gram-negative bacterium *Pseudomonas aeruginosa* is an important opportunistic human pathogen which causes several life-threatening nosocomial infections in immunocompromised patients and victims with burns (6, 29). Moreover, *P. aeruginosa* is the major and dominant pathogen in cystic fibrosis (4–8, 13, 15, 38, 39).

The ability to form biofilms is the crucial factor in fatal infections by *P. aeruginosa* and has made this bacterium a model organism with respect to biofilm formation. During chronic colonization, *P. aeruginosa* undergoes conversion from a nonmucoid to a mucoid phenotype (16). The most characteristic feature of the mucoid phenotype is the secretion of large amounts of highly viscous exopolysaccharides. The copolymer alginate, which is composed of mannuronic acid and guluronic acid, appears to be the major component of the secreted polysaccharide (9, 31), and besides nucleic acids and proteins, is the key factor in the development of mucoid biofilms which has been intensively studied (5, 16, 26, 30). Biofilms generated by *P. aeruginosa* have been shown to enhance the resistant colonization of cystic fibrosis-diseased lungs and to confer several advantages to the respective microorganism, such as a high-level resistance to antibiotics and to the killing effect of leukocytes (1, 18).

Recently, it was noted that alginate-negative mutants of *P.*

aeruginosa as well as the nonmucoid wild type are also able to form biofilms. These nonmucoid biofilms showed a different architecture from that of biofilms formed by alginate-overproducing mucoid *P. aeruginosa* (25, 40). Moreover, evidence was found only recently of a novel exopolysaccharide involved in the formation of nonmucoid biofilms by *P. aeruginosa* strains (40). An analysis and comparison of the nonmucoid biofilm matrixes of *P. aeruginosa* wild-type strains and alginate-negative mutants clearly indicated that alginate is not required for biofilm formation and is not even a significant component of biofilms. Nonmucoid biofilms produced by *P. aeruginosa* wild-type strains or alginate-negative mutants showed no differences with respect to their architecture or antibiotic resistance (40). A detailed analysis of the *P. aeruginosa* PAO1 genome revealed three putative gene clusters, PA1381 to -1392, PA2231 to -2245, and PA3552 to -3558, harboring genes homologous to exopolysaccharide biosynthesis genes (37). Previously, first investigations showed that the gene cluster PA2231 to -2245 (designated *psl*) is involved in biofilm formation, since corresponding *P. aeruginosa* mutants with disrupted *psl* clusters were severely compromised in biofilm initiation and exhibited thin, unstructured, abnormal biofilms (11, 19, 22).

Since these previous investigations suggested an essential role of the *psl* gene cluster in the initial biofilm formation of *P. aeruginosa*, we focused on the functional assignment of the *pslA* gene (PA2231), which represents the first gene of this cluster, and on the temporally and spatially resolved regulation of the *psl* operon. An isogenic, marker-free *pslA* knockout mutant was generated and characterized. Complementation

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used for this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence (5'-3')	Source or reference
Strains		
<i>P. aeruginosa</i> PAO1	Prototroph, nonmucoid	ATCC 15692
<i>P. aeruginosa</i> PAO1Δ <i>pslA</i>	PAO1 Δ <i>pslA</i> mutant, biofilm negative	This study
<i>E. coli</i> S17-1	<i>recA</i> ; harbors the <i>tra</i> genes of plasmid RP4 in the chromosome; <i>proA thi-1</i>	35
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 λ⁻lac</i> [F' <i>proAB lacI^qZΔM15 Tn10</i> (Tc)]	3
Plasmids		
pBluescript SK(-)	Amp ^r <i>lacPOZ</i> , T ₇ and T ₃ promoter	Stratagene
pEX100T	Amp ^r <i>oriT sacB lacPOZ'</i>	34
pGEM-Teasy	Amp ^r <i>lacPOZ'</i>	Promega
pPS856	Amp ^r Gm ^r <i>FRT</i>	17
pEX <i>pslA</i>	pEX100T containing GM cassette flanked by 5' and 3' ends of the <i>pslA</i> gene	
pTZ110	Broad-host-range <i>lacZ</i> fusion vector	33
pBBR1-MCS5	Gm ^r <i>lacPOZ'</i> <i>mob</i> , broad-host-range vector	20
pBBR1-MCS5:: <i>pslA</i>	pBBR1-MCS5 containing EcoRI/BamHI PCR fragment of <i>pslA</i>	This study
pSKP _{<i>psl</i>}	pBluescript SK(-) containing EcoRI/BamHI PCR fragment of <i>psl</i> promoter	This study
pTZ110::P _{<i>psl</i>}	pTZ110 containing EcoRI/BamHI PCR fragment of <i>psl</i> promoter	This study
pProbeAT'	Broad-host-range <i>gfp</i> (stable form) fusion vector	24
pProbeAT'::P _{<i>psl</i>}	pProbeAT' containing EcoRI/BamHI PCR fragment of <i>psl</i> promoter	This study
pProbe'- <i>gfp</i> [AAV]	Broad-host-range <i>gfp</i> (unstable form) fusion vector	
pProbe'- <i>gfp</i> [AAV]::P _{<i>psl</i>}	pProbe'- <i>gfp</i> [AAV] containing EcoRI/BamHI PCR fragment of <i>psl</i> promoter	24
pProbe'- <i>gfp</i> [AAV]::ΩGm-P _{<i>psl</i>}	pProbe'- <i>gfp</i> [AAV]::P _{<i>psl</i>} containing a gentamicin resistance gene cassette in the opposite direction to that of the <i>gfp</i> gene	This study
pGEM-Teasy::RACE	pGEM-Teasy containing 5'-RACE PCR product	This study
Oligonucleotides		
PA2231-KO-UA	GTAGATAGCCTGTCGCTGACACGGG	This study
PA2231-KO-DA	AAAAAGGATCCGATCACCGTCCGTTGCAGGTACATG	This study
PA2231-KO-UB	AAAAAGGATCCCGGCTGATCTGGGTCTTCAAGTTCC	This study
PA2231-KO-DB	TCAGTAGACTTCTTGGTCAGGAG	This study
PA2231-UE	AAAAAGAATTCTGGACTGCCCGTGATCGGCAGAGC	This study
PA2231-DB	AAAAAGGATCCGCGAGGGGATCAGCGGGGCGACGGC	This study
P _{<i>psl</i>} UE	AAAAAGAATTCCCGAAATGGCAGGAGCGGCGCTG	This study
P _{<i>psl</i>} DB	AAAAAGGATCCGCGGATCACGGGACAGTCCATTGA	This study
RACE-primer1	CAGGCCAGGCCCTGGTACATGAACAACAGCAGGC	This study
RACE-primer2	CGTCCGGAGTAGACGTCCAGTGCCTGGAAC	This study
RACE-primer3	GCGCAGCTCCACGGGAACCAGGCCGGGG	This study

was achieved by heterologous expression of only the *pslA* gene. Rapid amplification of cDNA 5' ends (5'-RACE) was used to identify the 5' end of the mRNA and the promoter region of the *psl* operon. Reporter gene fusions containing *lacZ* and stable/unstable *gfp* as reporters were created in order to investigate the expression of *psl* in planktonic *P. aeruginosa* PAO1 and in biofilms, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth of bacteria. The *P. aeruginosa* PAO1 and *Escherichia coli* strains and the plasmids used for this study are listed in Table 1. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth. *P. aeruginosa* PAO1 cells were grown at 37°C in either LB broth or *Pseudomonas* isolation agar. If required, antibiotics were added at the following concentrations: for *P. aeruginosa*, 150 μg/ml carbenicillin and 150 μg/ml gentamicin; and for *E. coli*, 10 μg/ml gentamicin, 100 μg/ml ampicillin, and 50 μg/ml kanamycin.

For the cultivation of biofilms, M9 medium containing 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 9 mM NaCl, 19 mM NH₄Cl, 2 mM MgSO₄, 100 μM CaCl₂, and 5 mM glucose was used.

Isolation, analysis, manipulation, and transfer of DNA. Plasmid DNA and DNA restriction fragments were isolated and analyzed by standard methods (32). Competent *E. coli* cells were prepared and transformed by using the CaCl₂ procedure (14). Conjugations of *E. coli* S17-1 (donor) harboring hybrid plasmids and *P. aeruginosa* PAO1 (recipient) were performed on solidified NB medium as described by Friedrich et al. (12). DNA sequences of new plasmid constructs

were confirmed by DNA sequencing according to the chain termination method using an ABI370 automatic sequencer (Applied Biosystems).

Generation of isogenic *pslA* knockout mutant of *P. aeruginosa* PAO1. All oligonucleotides used for this study are summarized in Table 1, and PCRs were performed with high-fidelity *Pfx* polymerase. The isogenic *pslA* knockout mutant was obtained according to the method of Pham et al. (28). The following primers were used for PCR: the *pslA* 5'-end region was amplified using PA2231-KO-UA and PA2231-KO-DA, and the *pslA* 3'-end region was amplified using PA2231-KO-UB and PA2231-KO-DB. The isogenic mutant was verified by PCR using the primers PA2231UE and PA2231DB, which hybridize to 31 bp upstream and 32 bp downstream of the *pslA* start and stop codons, respectively. The PCR product was analyzed by restriction site mapping and DNA sequencing. The inserted gentamicin cassette was removed by using plasmid pFLP2 as described elsewhere (17), resulting in *P. aeruginosa* PAO1Δ*pslA*.

Plasmid constructions. For complementation of the isogenic knockout mutant *P. aeruginosa* PAO1Δ*pslA*, the *pslA* gene was amplified by a tailed PCR using the primers PA2231UE and PA2231DB together with genomic DNA from *P. aeruginosa* PAO1 as template DNA. The PCR product was isolated, hydrolyzed with EcoRI (for which a site was present in the upstream primer) and BamHI (for which a site was present in the downstream primer), and cloned into the broad-host-range vector pBBR1-MCS5. In the resulting plasmid, pBBR1-MCS5::*pslA*, the *pslA* gene was arranged colinear to and downstream of the *lacZ* promoter. The mobilizing strain *E. coli* S17-1 was transformed with pBBR1-MCS5::*pslA* and was then used as a donor strain in a conjugation experiment, by which the plasmid was transferred to *P. aeruginosa* PAO1Δ*pslA*.

For construction of the broad-host-range promoter-probe vectors pTZ110::P_{*psl*}, pProbe-AT'::P_{*psl*}, and pProbe'-*gfp*[AAV]::ΩGm-P_{*psl*}, the follow-

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ACGGATATTTCCCTGGGAATGCTAAGATAGCTATCACAAAGCCACTATCGACGAATGAACCTATTC
      - 35                               -10

GACGGGAAAAATGACTAAACCGCGTGGCAAATGAAAAATAGTCACATAAATTGACGCTTCACCGCCTT

      +1                               M H S K S
GCTCTTCCCTATCCACTCAATGGACTGCCCGTGATCGGCAGAGCAAACAACATGCATTGGAAGTCG
                                     pslA -->

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FIG. 1. Promoter region of *psl* operon. The transcription start site A (+1) is located 41 bp upstream of the translation start site of *pslA*. A putative σ^{70} -dependent promoter is indicated.

ing procedures were employed. A 1,007-bp upstream region of *pslA* comprising the putative promoter region of the *psl* gene cluster was amplified by a tailed PCR using the primers P_{psl}UE and P_{psl}DB (Table 1) and genomic DNA from *P. aeruginosa* PAO1 as template DNA. The PCR product was digested with EcoRI (for which a site was present in the upstream primer) and BamHI (for which a site was present in the downstream primer) and cloned into correspondingly hydrolyzed pBluescript SK(-), resulting in the plasmid pSKP_{psl}. The PCR product was analyzed by restriction site mapping and DNA sequencing.

The putative promoter region P_{psl} was isolated from EcoRI/BamHI-hydrolyzed pSKP_{pslA} and cloned into the corresponding sites of pTZ110, pProbe-AT', and pProbe'-gfp[AAV]. In the resulting plasmids, pTZ110::P_{psl}, pProbe-AT'::P_{psl} and pProbe'-gfp[AAV]::P_{psl}, respectively, the putative promoter region was arranged colinear to and upstream of the *lacZ* or *gfp* reporter gene. To enable stable propagation, a gentamicin resistance cassette was inserted into the EcoRI site of pProbe'-gfp[AAV]::P_{psl}. In the resulting hybrid plasmid, pProbe'-gfp[AAV]:: Ω Gm-P_{psl}, the gentamicin resistance gene was cloned upstream of and in the opposite direction to the *gfp* gene. Cells of *E. coli* S17-1 were transformed with pTZ110::P_{psl}, pProbe-AT'::P_{psl} and pProbe'-gfp[AAV]:: Ω Gm-P_{psl}, and the resulting transformants were then used as donor strains in a conjugation experiment, by which the plasmids were transferred to *P. aeruginosa* PAO1.

Plasmid stability was confirmed by plasmid isolation over many subcultures.

β -Galactosidase assay. β -Galactosidase activity was measured as described by Miller (23) and is expressed in Miller units (MU). The data presented below are the results obtained from three independent experiments. The variance is indicated by error bars in the figures.

5'-RACE. 5'-RACE was used to determine the 5' end of the *pslA* mRNA transcript. It was carried out using a commercial 5'/3'-RACE kit (Roche) ac-

ording to the manufacturer's instructions. Total RNA was isolated from a *P. aeruginosa* PAO1 culture grown in LB medium with an optical density at 600 nm of 1.0 by using an SV total RNA isolation kit (Promega). The primer used for the synthesis of cDNA (RACE-primer1) and the first and second nested primers (RACE-primer2 and RACE-primer3) are listed in Table 1. The cDNA was purified using a High Pure PCR product purification kit (Roche). The final PCR product of the 5'-RACE assay was cloned into the pGEM-Teasy vector (Promega) and analyzed by DNA sequencing.

Biofilm experiments. Abiotic solid surface assay (SSA) biofilm formation was analyzed in polyethylene 96-well microtiter plates after 20 h of incubation at 37°C as described previously (10, 27). After crystal violet staining, the absorbance was measured at 595 nm using a microtiter plate reader (Anthos Labtec, Austria).

For the temporal and spatial resolution of *psl* operon expression in biofilms, biofilms were grown in continuous-culture flow cells as described previously (28). *P. aeruginosa* strains were grown in continuous-culture flow cells (channel dimensions, 1 × 4 × 40 mm) at room temperature as described previously (28). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 1 × 10⁹ cells ml⁻¹ and incubated without flow for 1 h at room temperature. Flow was then started with a mean flow velocity in the flow cells of 0.2 mm s⁻¹, corresponding to laminar flow with a Reynolds number of 0.02. Cells expressing the *psl-gfp* transcriptional fusion in biofilms were visualized using a confocal laser scanning microscope (FluoView500; Olympus) with fluorescein isothiocyanate optical filters. The magnification used was ×1,000 for all images.

The attachment assay was carried out in wells of tissue culture plates. Overnight cultures (16 h at 37°C) were adjusted to an optical density at 600 nm of 2.0 and inoculated into wells. Cells were allowed to adhere for 1 h before being washed three times using a multichannel pipette to remove unattached cells.

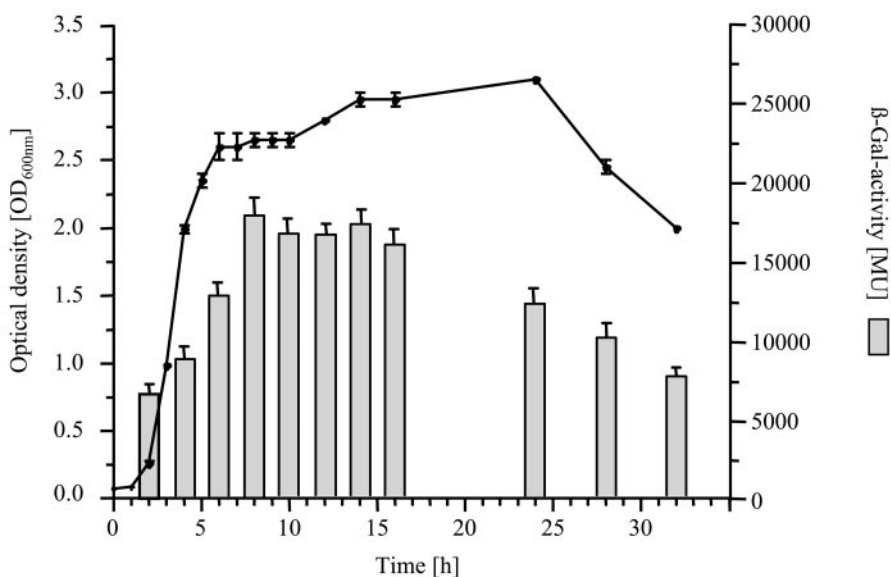


FIG. 2. Analysis of *psl* operon regulation using a transcriptional fusion of the identified promoter region to *lacZ*. The growth and β -galactosidase activity of *P. aeruginosa* PAO1(pTZ110::P_{psl}) under planktonic culture conditions are shown.

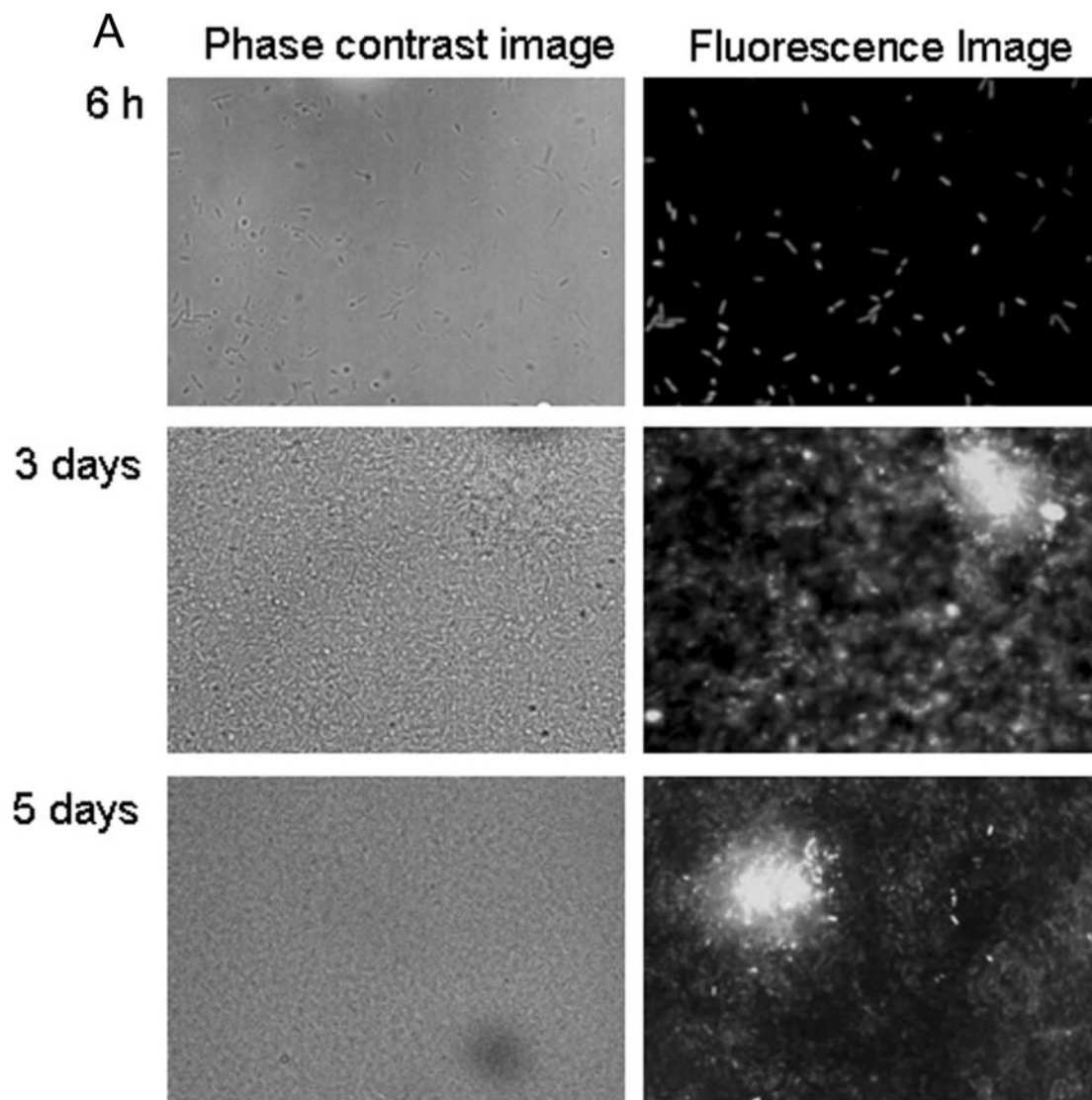


FIG. 3. Expression of stable *gfp* (A) and unstable *gfp* (B) under control of the *psl* promoter in *P. aeruginosa* PAO1 biofilms.

Attached cells were stained using SYTO9 (Molecular Probes Inc., Eugene, Oreg.). The number of attached cells was analyzed by obtaining the relative fluorescence using a plate fluorimeter (Wallac Victor 2; Perkin-Elmer). Mean adhesion values for each strain were determined for 16 wells, and similar results were observed in three replicate attachment experiments.

RESULTS

Molecular characterization of the *pslA* gene. Recently, it has been shown that *pslA* (PA2231) is part of the *psl* operon comprising 15 cotranscribed genes which are involved in the synthesis of an exopolysaccharide that is essential for *P. aeruginosa* PAO1 biofilm formation (11, 19, 22). To evaluate the essential role of *pslA* in biofilm formation, we generated a nonpolar isogenic *pslA* knockout mutant of *P. aeruginosa* PAO1 as described in Materials and Methods. The corresponding isogenic, marker-free *pslA* knockout mutant, *P. aeruginosa* PAO1 Δ *pslA*, contained a deletion of 0.52 kbp in the chromosomal *pslA* gene and was impaired in attachment and biofilm formation. The

mutant showed about 30% less attachment to tissue culture plates than the respective wild type (data not shown). The expected biofilm-negative phenotype of this mutant strain was confirmed by an abiotic SSA biofilm formation assay (10). *P. aeruginosa* PAO1 Δ *pslA* was not able to initiate biofilm formation when grown overnight in the wells of a microtiter dish.

For the complementation of *P. aeruginosa* PAO1 Δ *pslA*, the coding region of the *pslA* gene was cloned into the broad-host-range vector pBBR1-MCS5, resulting in plasmid pBBR1-MCS5::*pslA*. This plasmid was introduced into *P. aeruginosa* PAO1 Δ *pslA*. The ability to produce biofilms was successfully restored in this mutant strain harboring pBBR1-MCS5::*pslA* (data not shown).

An analysis of the PslA sequence using TMHMM (21, 36) revealed five putative transmembrane helices, at amino acid positions 26 to 45, 54 to 72, 88 to 104, 117 to 137, and 289 to 310. A putative cleavage site between amino acid positions 34 and 35 is detectable using SignalP 3.0 (2). A Pfam database

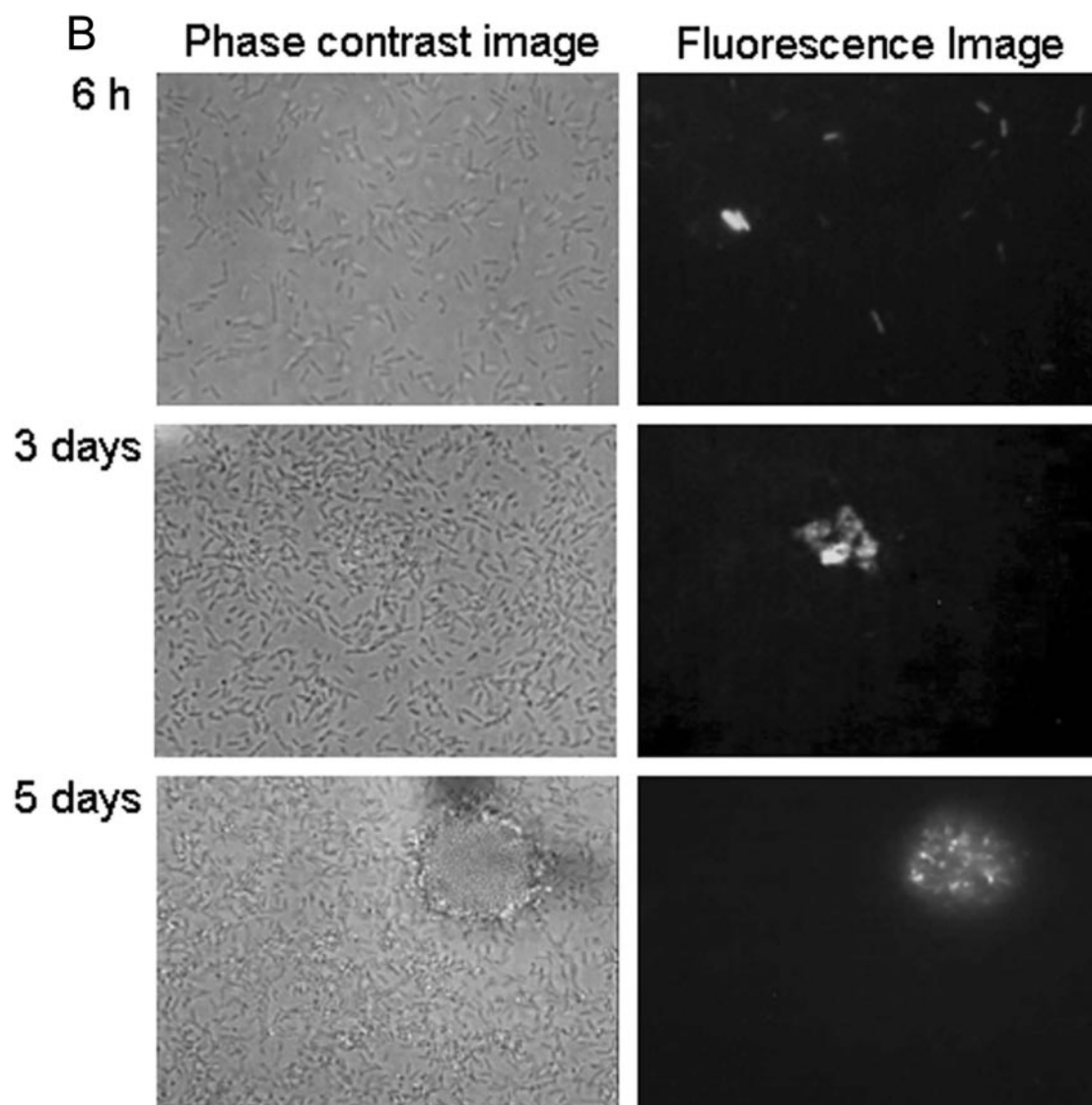


FIG. 3—Continued.

search revealed conserved protein domains, Pfam 02397 and Pfam 02719, which represent conserved regions of different bacterial sugar transferases and diverse bacterial polysaccharide biosynthesis proteins and putative epimerases, respectively.

Identification of the promoter region of the *psl* operon upstream of the *pslA* gene. To determine the transcription start site of the *psl* gene cluster, 5'-RACE was performed as described in Materials and Methods, using RNA isolated from *P. aeruginosa* cells in the late logarithmic growth stage. The final 5'-RACE PCR product was cloned into pGEM-Teasy (Promega), resulting in pGEM-Teasy::RACE, and sequenced. The result of the 5'-RACE experiment is shown in Fig. 1.

Evidence was provided that the transcription start site is located 41 nucleotides (nt) upstream of the *pslA* translational start site. A putative σ^{70} promoter region was found 26 nt upstream of the transcription start site (Fig. 1).

Expression of *psl* operon in planktonic cells. In order to investigate the expression of the *psl* operon in *P. aeruginosa* PAO1, we constructed plasmid pTZ110::P_{*psl*}, which contains the identified promoter region of the *psl* operon upstream of *lacZ* as a transcriptional reporter fusion. *P. aeruginosa* PAO1(pTZ110::P_{*psl*}) was grown under planktonic growth conditions. Growth and the β -galactosidase activity were measured spectrophotometrically over a period of 32 h and are shown in Fig. 2. During the complete experiment, high levels of β -galactosidase activity were measured, ranging from 7,000 MU at the beginning of the exponential growth phase to 18,000 MU at the beginning of the stationary growth phase, suggesting a constitutive expression under planktonic growth conditions. Cells of *P. aeruginosa* PAO1 harboring only the vector pTZ110 showed a similar growth behavior and revealed a β -galactosidase activity of <200 MU (data not shown).

Expression of *psl* operon in biofilms. To monitor the expression of the *psl* operon in biofilms, the identified promoter region upstream of the *pslA* gene was transcriptionally fused to genes encoding stable and unstable green fluorescent protein (GFP). With stable GFP, nearly all cells were fluorescent 6 h after inoculation (Fig. 3A). After 3 and 5 days, GFP fluorescence was considerably and increasingly brighter in the microcolonies. Enhanced GFP expression in microcolonies is visible at the single-cell level, i.e., individual cells in microcolonies have clearly upregulated GFP production compared to those in the bulk biofilm (Fig. 3A). With unstable GFP, these cells exhibited weak fluorescence. The exposure times necessary to capture the images were 2 to 4 seconds, in contrast to 0.2 to 0.4 seconds for stable GFP. There was some variation in GFP fluorescence after 6 h; most cells showed weak fluorescence, but some cells were very bright, and others showed no fluorescence at all (Fig. 3B). After 3 and 5 days of biofilm development, GFP production was detectable only inside the microcolonies (Fig. 3B). Many regions of the biofilm without microcolonies showed no fluorescence at all. The production of unstable GFP occurred almost exclusively in the microcolonies.

DISCUSSION

The *psl* gene cluster, comprising 15 cotranscribed genes (*pslA* to *pslO*) encoding proteins involved in exopolysaccharide biosynthesis, was recently identified by three independent research groups as playing an important role in biofilm formation by nonmucoid *P. aeruginosa* (11, 19, 22). In this study, we focused on the role of *pslA*, encoding a putative UDP-glucose carrier protein, in biofilm formation and on the regulation of the entire *psl* operon in planktonic and biofilm cells. A marker-free *pslA* deletion mutant was constructed to enable complementation studies by expressing only the *pslA* gene. A comparative attachment analysis indicated that *P. aeruginosa* PAO1 Δ *pslA* was impaired in attachment, suggesting a role of the *psl* operon-mediated exopolysaccharide in the attachment process. This mutant was also strongly impaired in biofilm formation, as observed for other mutants with mutations affecting *psl* genes (11, 19, 22). A plasmid mediating the expression of only the 1,437 bp comprising the coding region of the *pslA* gene under the control of the *lac* promoter showed a restoration of biofilm development in *P. aeruginosa* PAO1 Δ *pslA* to the wild-type level. In a previous study, complementation of a *pslA* null mutant of *P. aeruginosa* could not be achieved with a plasmid containing the genes *pslA* to *pslC* (22). Jackson and coworkers (19) isolated a *pslA-pslB* insertion mutant which could be restored to wild-type biofilm development by using a cosmid with a 22-kb insert comprising the genes *pslA* to *pslF*. In this study, we identified the designated *pslA* gene as a functional gene essential for biofilm development in nonmucoid *P. aeruginosa* and capable of complementing an isogenic *pslA* deletion mutant to have wild-type biofilm formation.

Since the *psl* genes are cotranscribed and the *pslA* gene is the first gene in this operon, we identified the promoter region upstream of *pslA*. 5'-RACE was used to locate the transcriptional start point 41 nt upstream of the start codon of *pslA*. Similarities to σ^{70} promoter sequences indicated that the *psl* operon is constitutively expressed (Fig. 1).

In order to understand the regulation of the *psl* operon, which plays a crucial role in biofilm development, we constructed transcriptional fusions of reporter genes with the identified *psl* promoter region comprising 1,007 bp upstream of the translational start codon of *pslA*. With planktonic cells, an analysis of the *lacZ* fusion indicated a deregulated constitutive expression of the *psl* operon (Fig. 2). A similar observation was made when a stable *gfp* fusion was investigated to study the localized regulation of the *psl* operon in biofilms. Almost all cells showed a rather high level of fluorescence after early attachment to the surface (Fig. 3A). However, after 3 and 5 days of biofilm development, the induction of the *psl* operon appears to be increasingly localized to the centers of microcolonies (Fig. 3A). To achieve a higher temporal resolution of *psl* operon expression, we used unstable *gfp* fusions. These data indicated that after attachment to the surface, the *psl* operon is repressed (Fig. 3B). However, with increasing biofilm development and microcolony formation, the *psl* operon is induced and localized to the centers of microcolonies (Fig. 3B). Since we found an important role for *pslA* in attachment, the constitutive expression of the *psl* operon might be required for efficient attachment to surfaces mediated by the synthesized exopolysaccharide. After attachment, *psl* operon expression is repressed and increasingly localizes to the centers of microcolonies, which strongly suggests an important role in biofilm differentiation. Future experiments will comprise the identification of factors involved in the regulation of the *psl* operon.

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