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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2006, p. 3066–3068 Vol. 72, No. 4
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.4.3066–3068.2006

PslD Is a Secreted Protein Required for Biofilm Formation by *Pseudomonas aeruginosa*

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Received 15 December 2005/Accepted 3 February 2006

The function of *pslD*, which is part of the *psl* operon from *Pseudomonas aeruginosa*, was investigated in this study. The *psl* operon is involved in exopolysaccharide biosynthesis and biofilm formation. An isogenic marker-free *pslD* deletion mutant of *P. aeruginosa* PAO1 which was deficient in the formation of differentiated biofilms was generated. Expression of only the *pslD* gene coding region restored the wild-type phenotype. A C-terminal, hexahistidine tag fusion enabled the identification of PslD. LacZ and PhoA translational fusions with PslD indicated that PslD is a secreted protein required for biofilm formation, presumably via its role in exopolysaccharide export.

Pseudomonas aeruginosa is an opportunistic human pathogen which causes severe infections in immunologically compromised patients and is the major pathogen in cystic fibrosis patients. An important virulence mechanism is the formation of a mucoid biofilm. Secreted alginate is a crucial constituent of the mucoid biofilm matrix (16, 17). However, alginate-negative mutants of *P. aeruginosa* are also able to form nonmucoid biofilms, showing an architecture different from that of biofilms formed by alginate-overproducing mucoid *P. aeruginosa* (13, 20). Previous investigations have shown that the gene cluster PA2231-2245 (designated *psl*) is cotranscribed and required for nonmucoid biofilm formation (5, 7, 12). The putative *pslD* gene (PA2234) is part of the *psl* operon and represents an open reading frame comprising 771 bp.

PslD sequence analysis. PslD shows about 72% similarity to hypothetical proteins that are derived from genomes of various pseudomonads and that have a predicted function in exopolysaccharide export. However, the most similar proteins with experimental and functional assignment were Wza_{K30} from *Escherichia coli* strain E69 and Wza_{Vv} from *Vibrio vulnificus*, exerting similarities of 33% and 25% to PslD, respectively (4, 21). Only Wza_{K30} has been experimentally demonstrated as a multimeric lipoprotein in the outer membrane of *E. coli* strain E69, which is presumably involved in the export of the group 1 capsular polysaccharides (4). A nonpolar mutation in the gene putatively encoding Wza_{Vv} in *Vibrio vulnificus* did not abolish capsular polysaccharide biosynthesis, but the polysaccharide was residing in the periplasm and was not exported (21). The PslD sequence analysis by LipoP 1.0 (3) showed a strong indication for a lipoprotein signal peptide recognized by signal peptidase II, with a cleavage site between amino acid residues 15 and 16 (19). Amino acid 16 is a cysteine residue which provides a potential target for lipid modification, enabling anchoring in the outer membrane. No transmembrane domains were predicted both by the dense alignment surface method (2) and by TMHMM 2.0 (9). Signal peptides for lipoproteins are described as containing a positively charged region at the N terminus, a hydrophobic region, and a lipobox with a four-amino-acid consensus region (1). In PslD, the first two amino acid residues (MK) are considered a positively charged region at the N terminus, the hydrophobic TLLMLAMLA sequence is the predicted hydrophobic region, and the lipobox sequence is LAAC. A Conserved Domain Database (11) search shows that PslD contains a conserved pfam02563 domain, which is conserved in a family of outer membrane auxiliary proteins involved in polysaccharide export.

Generation of the isogenic *pslD* knockout mutant of *P. aeruginosa* PAO1. The isogenic *pslD* knockout mutant was obtained and verified as described by Pham et al. (15). The inserted gentamicin cassette was removed as previously described (18), resulting in the marker-free *P. aeruginosa* PAO1Δ*pslD* mutant containing a deletion of 170 bp in the chromosomal *pslD* gene. *P. aeruginosa* PAO1Δ*pslD* showed a biofilm-negative phenotype in the abiotic solid surface assay (SSA) for biofilm formation (Table 1) and in flow chamber analysis (Fig. 1).

Biofilm analysis. For biofilm analysis, *P. aeruginosa* strains were grown in mineral medium continuous-culture flow cells (channel dimensions, 1 by 1 by 120 mm) at room temperature as previously described (14). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 2×10^9 cells ml^{-1} and incubated without flow for 4 h at room temperature. Flow was then started with a mean flow of 0.3 ml min^{-1}, corresponding to a laminar flow with a Reynolds number of 5. Biofilms were stained and visualized using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes Inc., Eugene, Oreg.). The wild-type biofilm was homogenous and showed large microcolonies. A strong prevalence of living cells was observed. The PAO1Δ*pslD* mutant was unable to form such complex biofilms and was characterized by small clusters of cells not developing into the typical microcolonies (Fig. 1). This finding is consistent with the previously identified strong induction of the transcription of the *psl* operon inside microcolonies, which suggested an important function of the *psl* operon in microcolony formation (14). For quantitative analysis of biofilm formation, the abiotic SSA was applied as described previously (14).

Complementation of the *P. aeruginosa* PAO1Δ*pslD* mutant.

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TABLE 1. SSA analysis and reporter enzyme activity of various *P. aeruginosa* strains[a]

Strain	Biofilm formation with SSA	LacZ activity (MU)	PhoA activity (MU)
P. aeruginosa PAO1	1	25.2 ± 1.2	0.42 ± 0.07
P. aeruginosa PAO1Δ*pslD*	0.14 ± 0.02	26.8 ± 6.9	0.12 ± 0.02
P. aeruginosa PAO1Δ*pslD* (pJE608)	0.13 ± 0.02	26.0 ± 3.8	
P. aeruginosa PAO1Δ*pslD* (pJE608::*pslD*)	0.16 ± 0.02	212.9 ± 87.4	
P. aeruginosa PAO1Δ*pslD* (pJE609)	0.17 ± 0.06		0.19 ± 0.03
P. aeruginosa PAO1Δ*pslD* (pJE609::*pslD*)	0.56 ± 0.11		9.46 ± 0.34
P. aeruginosa PAO1Δ*pslD* (pBBR1MCS 5)	0.17 ± 0.02		
P. aeruginosa PAO1Δ*pslD* (pBBR1MCS5::*pslD*)	1.24 ± 0.17		
P. aeruginosa PAO1Δ*pslD* (pBBR1MCS5::HT*pslD*)	0.82 ± 0.10		

[a] Biofilm formation of *P. aeruginosa* PAO1 was assigned a value of 1. All experiments were performed in triplicate. Values are means ± standard deviations. MU, Miller units.

For complementation of the *P. aeruginosa* PAO1Δ*pslD* mutant, the coding region of the *pslD* gene was cloned into the broad-host-range vector pBBR1-MCS5 (8), resulting in plasmid pBBR1-MCS5::*pslD*. The *pslD* gene was inserted downstream of the *lac* promoter and after conjugational transfer, the biofilm formation wild-type phenotype was restored (Table 1 and Fig. 1). Flow cell chamber analysis showed that the Δ*pslD* deletion mutant harboring pBBR1-MCS5::*pslD* showed a fully differentiated and mature biofilm, but LIVE/DEAD staining revealed a larger fraction of dead cells for the mature biofilm than for the wild-type biofilm. Furthermore, the biofilm showed additional smaller cell clusters and appeared to be slightly more heterogenous than the wild-type biofilm. A C-terminal, hexahistidine-tagged PslD protein encoded by plasmid pBBR1-MCS5::HT*pslD*, constructed as described above, also mediated restoration of the biofilm-forming phenotype (Table 1 and Fig. 1).

Detection of PslD. The C-terminal, hexahistidine-tagged PslD protein, with an apparent molecular mass of 29 kDa, was

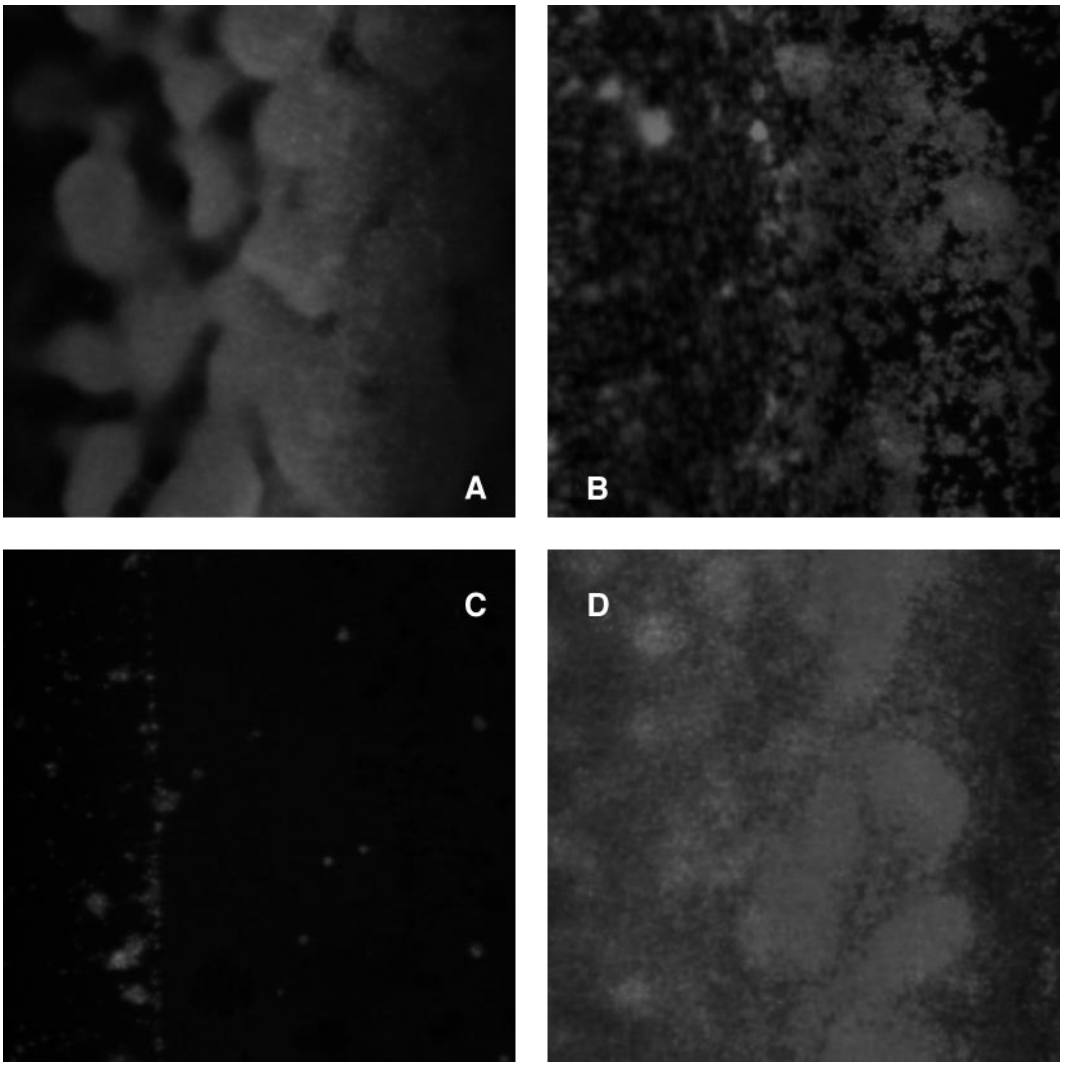

FIG. 1. Fluorescence microscopy analysis of biofilms formed by *P. aeruginosa* after 3 days of growth in flow cell chambers. (A) *P. aeruginosa* PAO1. (B) *P. aeruginosa* PAO1Δ*pslD*. (C) *P. aeruginosa* PAO1Δ*pslD*(pBBR1-MCS5). (D) *P. aeruginosa* PAO1Δ*pslD*(pBBR1-MCS5::*pslD*).

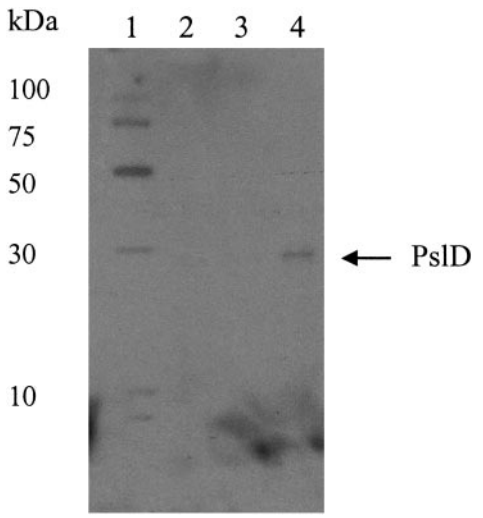

FIG. 2. Immunoblotting of whole-cell lysates of *P. aeruginosa* strains harboring various plasmids. Antihexahistidine antibodies were used to detect C-terminal, hexahistidine-tagged PslD. Lane 1, molecular mass standard of hexahistidine-tagged proteins; lane 2, *P. aeruginosa* PAO1; lane 3, *P. aeruginosa* PAO1Δ*pslD*(pBBR1-MCS5); and lane 4, *P. aeruginosa* PAO1Δ*pslD*(pBBR1-MCS5::HT*pslD*). The arrow indicates the position of C-terminal, hexahistidine-tagged PslD.

detected in whole-cell lysates of *P. aeruginosa* PAO1Δ*pslD* harboring plasmid pBBR1-MCS5::HT*pslD*. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblot analysis, using the SuperSignal West HisProbe kit (Pierce Biotechnology Inc.) according to the manufacturer's protocol (Fig. 2).

Subcellular localization of PslD. The primary structure analysis of PslD suggested a periplasmic and/or outer membrane localization. Translational fusions of the reporter enzymes LacZ and PhoA to the C terminus of PslD were obtained using the respective vectors pJE608 and pJE609 as previously described (6, 18). Only the PslD-PhoA fusion was functional and restored biofilm formation in *P. aeruginosa* PAO1Δ*pslD* (Table 1). Alkaline phosphatase and β-galactosidase enzymatic assays were performed according to the methods of Miller (12a) and Manoil (10), respectively. Reporter enzyme assays showed both high specific alkaline phosphatase activity and β-galactosidase activity in *P. aeruginosa* PAO1Δ*pslD* mutants harboring the respective plasmids (Table 1). The functional PslD-PhoA fusion provides evidence for secretion and localization in the periplasm and/or outer membrane. Immunological detection of PslD-His_6 in whole-cell lysates provided evidence that the protein is not released and remains attached to the cells. Since no hydrophobic domains are present in PslD and no transmembrane helices are predicted, PslD might not be attached to the cytoplasmic membrane. Overall, these data indicate that the *pslD* gene encodes a protein which is localized in the periplasm/outer membrane belonging to the outer membrane auxiliary protein family and which is required for the export of a biofilm-relevant exopolysaccharide.

This study was supported by the Massey University Research Fund and the Massey University Postdoctoral Fellowship.

We thank Zoe Jordens for assistance in biofilm experiments.

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