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# Synthesis of multiple *Pseudomonas aeruginosa* biofilm matrix exopolysaccharides is post-transcriptionally regulated

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## Summary

Exopolysaccharide is a critical biofilm matrix component, yet little is known about how the synthesis of multiple exopolysaccharides is regulated. *Pseudomonas aeruginosa* can produce several biofilm matrix exopolysaccharides that include alginate, Psl and Pel. Here we demonstrated that AlgC, a key enzyme that provides sugar precursors for the synthesis of alginate and lipopolysaccharides (LPS) is also required for both Psl and Pel production. We showed that forced-synthesis of Psl in alginate-producing mucoid bacteria reduced alginate production but this was not due to transcription of the alginate biosynthesis-operon. Likewise, when either alginate or Psl were overproduced, levels of B-band LPS decreased. Induction of Pel resulted in a reduction of Psl levels. Because the effects of reduced exopolysaccharide synthesis when another is overproduced didn't appear to be regulated at the transcriptional level, this suggests that the biosynthesis pathways of Psl, Pel, alginate, and LPS compete for common sugar precursors. As AlgC is the only enzyme that provides precursors for each of these exopolysaccharides, we propose that AlgC is a key checkpoint enzyme that coordinates the total amount of exopolysaccharide biosynthesis by controlling sugar precursor pool. Our data also provide a plausible strategy that *P. aeruginosa* utilizes to modulate its biofilm matrix exopolysaccharides.

## Introduction

Most bacteria survive in the environment by adopting a surface-associated community (biofilm) life-style by virtue of their extracellular polymeric substances (EPS) that form a matrix to enmesh bacteria in the biofilm (Sutherland, 2001; Whitchurch *et al*., 2002; Allesen-Holm *et al*., 2006; Ma *et al*., 2009). Exopolysaccharide is a key biofilm matrix



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component (Ryder *et al*., 2007) and is also an important carbon source for the bacteria; this may allow biofilm bacteria to survive under nutrient limitation conditions. *Pseudomonas aeruginosa* is a ubiquitous environmental microorganism. This Gram-negative bacterium is also an opportunistic pathogen that can cause life-threatening infections in cystic fibrosis (CF) patients and immunocompromised individuals. *Pseudomonas aeruginosa* can produce several exopolysaccharides that include alginate, Psl, LPS and Pel, which each contribute to biofilm formation (Ma *et al*., 2009; Colvin *et al*., 2011; Ghafoor *et al*., 2011; Yang *et al*., 2011). However, it is not clear how *P. aeruginosa* regulates the production of all these different exopolysaccharides. An understanding of how bacteria control this may lead to the development of anti-biofilm therapies.

*Pseudomonas aeruginosa* cells often become mucoid upon prolonged colonization of the CF lung, despite the fact that they are colonized with nonmucoid strains initially. The mucoid phenotype is due to the overproduction of alginate. Alginate in this bacterial species is a repeating polymer of β-*D*-mannuronate and α-*L*-guluronate residues, and is the most intensely studied exopolysaccaride of *P. aeruginosa* (Ramsey and Wozniak, 2005). The viscous exopolysaccharide alginate coating is believed to play a role in the bacteria's resistance to antibiotics and also to evade host defence mechanisms (Evans and Linker, 1973; Evans *et al*., 1996). While alginate is not essential for the formation of biofilms by nonmucoid strains, it does impact the structure and resistance properties of biofilms formed by mucoid strains (Nivens *et al*., 2001; Wozniak *et al*., 2003; Stapper *et al*., 2004). In nonmucoid strains, Psl and Pel are two key exopolysaccharides known to maintain biofilm structure (Ma *et al*., 2006; 2009; Colvin *et al*., 2011; Yang *et al*., 2011). Pel is a glucose-rich exopolysaccharide, which is required to form pellicles at the air-medium interface or mature solid-surface-associated biofilms (Friedman and Kolter, 2004a,b; Coulon *et al*., 2011; Ghafoor *et al*., 2011). Recently, Colvin *et al*. showed that Pel can serve a structural and protective role in biofilm matrix (Colvin *et al*., 2011). Psl exopolysaccharide is a repeating pentasaccharide containing D-mannose, D-glucose and *L*-rhamnose (Byrd *et al*., 2009). This exopolysaccharide is an essential matrix component that is required for the biofilm formation of nonmucoid as well as mucoid *P. aeruginosa* (Jackson *et al*., 2004; Friedman and Kolter, 2004b; Matsukawa and Greenberg, 2004; Ma *et al*., 2006; 2009; 2011). Yang *et al*. recently showed that Psl had also contributions on antibiotic resistance of *P. aeruginosa* biofilm (Yang *et al*., 2011).

Lipopolysaccharide (LPS) is also a major virulence factor and an integral structural component of the *P. aeruginosa* outer membrane. Two distinct forms of LPS, the common polysaccharide antigen (CPA, also called A-band) and the O-specific antigen (OSA, also called B-band), have been described (Knirel, 1990; Rocchetta *et al*., 1999; Islam *et al*., 2011). A-band LPS consists mainly of a repeating trisaccharide of α-*D*-rhamnose. B-band LPS contains disaccharide to pentasaccharide repeating units that form a heterogeneous length due to varying degrees of polymerization. A-band LPS is anti-genically conserved, while B-band LPS is serologically variable (Knirel, 1990; Islam *et al*., 2011). *Pseudomonas aeruginosa* isolated from CF patients with chronic lung infection often exhibit LPS phenotypes with reduced amounts of long O-antigen side-chains (Hancock *et al*., 1983). At

present, it is not clear whether loss of LPS O-side-chain is coordinately linked to the production of the other exopolysaccharides, such as alginate and Psl.

In *P. aeruginosa*, there are significant overlaps between the biosynthetic pathways of LPS and alginate. The *algC* gene is strategically placed in these pathways, leading to the production of the key intermediate mannose-1-phosphate (Man-1-P) that is necessary for both alginate and LPS (Coyne *et al*., 1994; Deretic *et al*., 1995; Rocchetta *et al*., 1999). The *algC* gene encodes a bifunctional enzyme with phosphomannomutase (PMM) and phosphoglucomutase (PMG) activities that catalyse the conversion of Man-6-P and glucose-6-P (Glc-6-P) into Man-1-P and Glc-1-P respectively (Padgett and Phibbs, 1986). In this report, we show that AlgC is also required for the synthesis of two critical biofilm matrix exopolysaccharides, Psl and Pel. Our data suggest that AlgC is a checkpoint enzyme, which controls the total amount of exopolysaccharides synthesized in *P. aeruginosa* by controlling the amount of the key biosynthetic precursors.

## Results

### AlgC is required for the synthesis of Psl exopolysaccharide

The report of Byrd *et al*. implied that AlgC was likely the enzyme providing Man-1-P and Glc-1-P for the synthesis of Psl (Byrd *et al*., 2009). In support of this, we did not detect Psl matrix in the biofilm of a Δ*algC* strain (green Psl channel in Fig. 1). In addition, the biofilm of Δ*algC* appeared similar to a ΔPsl strain as previously described, which had much less biomass compared with the biofilm of PAO1 although a few microcolonies can be detected in the entire flow chamber (Figs 1 and 2) (Ma *et al*., 2006). Interestingly, Δ*algC* bacteria bound poorly to the flow cell substratum and other bacteria (Fig. 2). Thus, the biofilm formed by Δ*algC* bacteria had a greater thickness than that of Δ*psl* strain, which usually has a single layer of bacteria on surface (Fig. 2) (Ma *et al*., 2006; 2009). This biofilm phenotype (thicker biofilm, but poor bacterial cell–substratum and cell–cell interactions) is likely due to effects on LPS synthesis of Δ*algC*, because similar biofilm patterns were reported in LPS-deficient strains previously (Palmer and Caldwell, 1995). To determine if loss of Psl in the biofilm of Δ*algC* strain is due to potential feedback effects on transcription of *psl* genes, we constructed a Δ*algC*-derived $P_{BAD}$-*psl* strain WFPA803 (Table 1). WFPA803 formed a few thin bacterial cell aggregates. However, no Psl signal was detected in these cell aggregates of WFPA803 (green Psl channel in Fig. 1). In contrast, the biofilm of WFPA801 had strong Psl staining signal (Fig. 1). Because WFPA803 contains the $P_{BAD}$ promoter driving *psl* expression, it appeared that the block in Psl synthesis in the Δ*algC* mutant was not due to effects on *psl* transcription.

*algC* mutants cannot synthesize the LPS O-antigen and a complete LPS core (Coyne *et al*., 1994; Rocchetta *et al*., 1999). To rule out the possibility that the loss of Psl in the *algC* mutant was a result of effects on LPS, we stained Psl in the biofilm of a PAO1-derived Δ*waaL* strain. This mutant strain lacks the ability to link the LPS O-antigen onto the core LPS. Δ*waaL* formed a thick biofilm that had less bacterial cell density (Fig. 2). Yet Psl was clearly visualized in the biofilm of the *waaL* mutant strain (Fig. 1). This revealed that the lack of Psl in the *algC* mutant was not due to the LPS defect.

To eliminate the possibility that *algC* is involved in the localization of Psl on the bacteria surface and/or export of Psl to extracellular, we utilized the anti-Psl antibody to detect Psl in the whole cell extract (containing bacterium-surface-bound Psl and any Psl inside the bacterial cell), ethanol-precipitated culture supernatant (released Psl from bacteria), and the crude extract of bacterium-surface-bound substances. Immunoblotting results showed the little signal in the three fractions of Δ*algC* mutant and Δ*psl* strain WFPA800, verifying that Psl cannot be synthesized in a Δ*algC* strain (Fig. 3). As a positive control, Psl was detected in the whole cell extract and surface-bound substance of WFPA801. In addition, Psl production in the Δ*algC* mutant was recovered by expression of *algC in trans* (*algC*/pLPS188; Fig. 3). These data indicated that AlgC was involved in the biosynthesis of Psl at a step other than the exportation/localization of Psl.

Our above data showed that *algC* was not involved in the transcription of *psl* and did not appear to affect the exportation/localization of Psl. In addition, the lack of Psl in the *algC* mutant was not due to the LPS defect. Taken together, our data revealed that AlgC was required for the biosynthesis of Psl, likely due to the role of AlgC in generating the precursors as it does in the biosynthesis pathway of alginate and LPS (Coyne *et al*., 1994; Deretic *et al*., 1995; Rocchetta *et al*., 1999).

## AlgC is also involved in the synthesis of Pel polysaccharide

Because AlgC can also convert Glc-6-P into Glc-1-P, which is required for the synthesis of Psl and LPS, we predicted that AlgC might be also the enzyme to make the glucose precursor for the synthesis of Pel. To test this, we constructed a $P_{BAD}$-*pel* strain in Δ*algC*, Δ*pslBCD* and Δ*Psl* (promoter deletion) background respectively (strain named as CIM1, CIM2 and WFPA884 respectively, Table 1). The colony colour and morphology on the Congo red plate can be used to indicate Pel production (Friedman and Kolter, 2004b). Pel production was observed in a $P_{BAD}$-*pel* strain WFPA831 (that had a wild-type *psl* background) as well as the two Δ*psl* $P_{BAD}$-*pel* strains WFPA884 and CIM2 (Δ*Psl* and Δ*pslBCD* respectively). This is indicated by increased red colour of colonies with addition of arabinose (Fig. 4). In contrast, no Pel production was observed in colonies of the Δ*algC* strain as well as the Pel-inducible Δ*algC* strain CIM1 (Fig. 4). These results suggested that Pel synthesis also required AlgC. Interestingly, the $P_{BAD}$-*pel* strain WFPA831 showed a slightly light red colour on Congo red plate than the other two $P_{BAD}$-*pel* strains WFPA884 and CIM2 (Δ*psl* background). This suggested more Pel was produced when cells lack the ability to make Psl. Consistent with this, a recent report also showed that a lack of Psl enhanced the production of Pel (Ghafoor *et al*., 2011).

## Overproduction of individual exopolysaccharides reduces synthesis of the other *P. aeruginosa* exopolysaccharides

Previous reports showed that alginate and LPS shared the sugar precursor Man-1-P, which was converted by AlgC from Man-6-P (Coyne *et al*., 1994; Deretic *et al*., 1995). As Psl is a mannose-rich polysaccharide and AlgC is also the enzyme providing the sugar precursor Man-1-P for the synthesis of Psl, we hypothesized that Psl, alginate, and LPS all share the sugar precursor (Man-1-P). Thus, we reasoned that overproduction of one of these will impact the synthesis of another. In support of this, Psl-producing nonmucoid strain PAO1

synthesized little detectable alginate, yet a PAO1-derived mucoid strain PDO300, produced large amount of alginate and less Psl compared with PAO1 (Fig. 5). To test the hypothesis of a shared sugar precursor pool, we constructed PDO320, a $P_{BAD}$-*psl* strain in the strain PDO300 (Fig. 5, Table 1). Upon induction of Psl synthesis, the amount of alginate was dramatically decreased (alginate reduced 100-fold in 2% arabinose induction sample compared with PDO300) (Fig. 5). Consistent with the reduction of alginate, the colony morphology on plates changed from a large mucoid appearance to a small colony as Psl synthesis increased. This effect appeared not to be due to reduced alginate gene transcription since real-time (RT) PCR (detecting *algD*, the first gene of alginate synthesis operon) results showed similar levels under all Psl-inducing conditions (Fig. 5). These results suggest that Psl and alginate synthesis pathways may be competing for a common precursor, likely Man-1-P.

To further test a shared sugar precursor pool among Psl, alginate and LPS, we also evaluated the levels of A-band LPS (common antigen) and B-band LPS (O antigen) in isogenic wild-type strain PAO1, a PAO1-derived $P_{BAD}$-*psl* strain WFPA801, Δ*psl* strain WFPA800 and a mucoid strain PDO300. Levels of A-band LPS were similar in all strains, but high molecular weight forms of B-band LPS were greatly reduced in the mucoid strain (Fig. 6). This is consistent with the previous report of mucoid *P. aeruginosa* (Deretic *et al*., 1995). The production of LPS increased in Psl deficient strain; however, the overproduction of Psl only decreased the B-band LPS slightly (Fig. 6). Taken together, our data suggested that a common sugar precursor pool was very likely shared within the synthesis pathways of Psl, alginate and LPS.

### Induction of Pel also affects the production of Psl polysaccharide

Previous reports showed that Pel was a glucose-rich exopolysaccharide (Friedman and Kolter, 2004b; Coulon *et al*., 2011). As Psl also contains D-glucose (Byrd *et al*., 2009), we hypothesized that the synthesis of Psl and Pel may also affect each other. To test this, we constructed a Pel-negative strain WFPA830, by deletion of the *pel* promoter region (Δ*Ppel*), and a $P_{BAD}$-*pel* (*pel*-inducible) strain WFPA831 (Table 1), both in a PAO1 background. In WFPA830 and WFPA831 without inducer, Psl production was elevated approximately fivefold compared with the wild-type levels (Fig. 7). This indicates endogenous synthesis of Pel impacts Psl levels. However, if Pel synthesis was induced, Psl levels were reduced in an arabinose concentration-dependent manner (Fig. 7). Similar to the results described above, this effect was not due to decreased *psl* transcription (Fig. 7). In addition, the results of *psl-lacZ* translational fusion showed that Pel induction did not affect Psl expression at the translational level (data not shown). Our data suggest that synthesis of Psl and Pel may also compete for common sugar precursors.

## Discussion

*Pseudomonas aeruginosa* can produce many kinds of extracellular polysaccharides that include LPS, alginate, Psl and Pel. Previous reports have shown that the synthesis of rhamnolipid, LPS and alginate require AlgC (Coyne *et al*., 1994; Deretic *et al*., 1995; Pamp and Tolker-Nielsen, 2007). We reported here that AlgC was also involved in the synthesis of

Psl and Pel, two critical biofilm matrix exopolysaccharides. Taken together, the production of all these extracellular polysaccharides are dependent on one key enzyme, AlgC, a bifunctional enzyme with both PMM and PMG activities. So far, AlgC is the only enzyme that is used in the biosynthesis pathways of four biofilm matrix exopolysaccharides in *P. aeruginosa*, Psl, Pel, alginate and LPS. We have also shown that overproduction of individual exopolysaccharides reduces synthesis of the other *P. aeruginosa* exopolysaccharides. This suggests that AlgC can be the checkpoint enzyme to limit the production of *P. aeruginosa* exopolysaccharide.

The control of the surface polysaccharide synthesis is a critical bacterial survival in the environment and human host (Rocchetta *et al*., 1999; Hall-Stoodley *et al*., 2004; Ramsey and Wozniak, 2005; Danhorn and Fuqua, 2007; Hoiby *et al*., 2010). *Pseudomonas aeruginosa* can produce several exopolysaccharides; each has distinct roles in biofilm development (Ghafoor *et al*., 2011; Yang *et al*., 2011). Psl is a primary biofilm matrix component, which promotes bacterial adherence and biofilm formation. Alginate gives *P. aeruginosa* an advantage to persist in the CF airway. Pel gives structural support and protection in biofilm. LPS is a key bacterial outer membrane component and virulence factor. Recently, Coulon *et al*. reported that LPS-like material may also be a biofilm matrix component of *P. aeruginosa* (Coulon *et al*., 2011). Here we show that *P. aeruginosa* post-transcriptionally coordinates synthesis of its periphery biofilm matrix exopolysaccharides by AlgC, which provides the sugar precursors for Psl, alginate, Pel and LPS.

There are three bifunctional enzymes encoded by *algA*, *wbpW* and *pslB*, respectively, which can synthesize Man-6-P and convert Man-1-P to GDP-mannose for synthesis of either alginate, Psl or LPS (Fig. 8). However, AlgC is the only known *P. aeruginosa* enzyme that can convert Man-6-P into Man-1-P (Fig. 8).We propose that AlgC may be a checkpoint enzyme, whose expression level/activity can be coordinated for the synthesis of *P. aeruginosa* surface polysaccharides. This is supported by the standalone location of *algC* in the *P. aeruginosa* chromosome, apart from the alginate-, *psl*-, *pel*- and LPS-synthesis operons. By controlling the expression or activity of AlgC, *P. aeruginosa* is able to control the total amount of polysaccharides on its surface. In mucoid strains, the expression of AlgC is greatly increased (Zielinski *et al*., 1992; Tavares *et al*., 1999). This allows *P. aeruginosa* to make copious amounts of alginate but limit Psl production. Such a modulation strategy would balance the total amount of bacterial extracellular polysaccharides and is also an efficient way for bacteria to use energy. Further studies are needed to discover how *P. aeruginosa* controls the key enzyme, AlgC.

In support of this AlgC-checkpoint model, we have shown in this report that if the production of one polysaccharide is increased, *P. aeruginosa* reduces production of the other polysaccharides in order to maintain the balance of the total pool of exopolysaccharides. Overproduction of Psl largely decreased the amount of alginate synthesis; inductions of Pel reduced the production of Psl polysaccharide; overproduction of alginate also had a profound effect on synthesis of high molecular weight B-band LPS (mannose is the main components of the O-antigen repeating unit). However, overproduction of Psl had little effect on the B-band LPS synthesis. The transcription of *psl* genes was only sevenfold higher in the Psl-overproducing strain WFPA801 than in wild type (Ma *et al*., 2006), but *alg*

genes were hundreds-fold up in mucoid strain than wild type (data not shown). This may be one of the reasons that overproduction of alginate had greater effects on LPS production than Psl. In addition, Byrd *et al*. showed that PslB can replace WbpW for the synthesis of LPS (Byrd *et al*., 2009). Therefore, elevation of *psl* gene expression may also benefit the synthesis of LPS in Psl-inducible strain WFPA801. WbpW, PslB or AlgA can catalyse the conversion of Man-1-P into GDP-*D*-mannose. GDP-*D*-mannose can be further converted to GDP-*D*-rhamnose, which is used for the synthesis of A-band LPS. However, the overproduction of either alginate or Psl does not affect the production of A-band LPS (Fig. 6), suggesting that *P. aeruginosa* may have other pathway to make GDP-*D*-rhamnose for the synthesis of A-band LPS, which is not required GDP-*D*-mannose.

PMG activity of AlgC can convert Glc-6-P to Glc-1-P. The Glc-1-P can be modified to dTDP-*D*-glucose, which directly participates in the synthesis of LPS core. As Glc-1-P is also a precursor for the synthesis of Psl, LPS may share both mannose and glucose precursors with Psl. The dTDP-*D*-glucose is further converted to dTDP-*L*-rhamnose used for either synthesis of rhamnolipid or LPS core (Olvera *et al*., 1999). Rhamnolipid is an important biosurfactant that also has contributions on the *P. aeruginosa* biofilm formation (Pamp and Tolker-Nielsen, 2007). It will be important to examine whether the synthesis of rhamnolipid competes with Psl for glucose precursor.

In this study, we also showed that the synthesis of Pel required AlgC. Because the chemical structure of Pel in *P. aeruginosa* has not been solved, it is not clear whether AlgC is the only enzyme shared by the biosynthesis pathway of Pel and Psl. However, we have shown in this study that forced-synthesis of Pel reduces Psl production and a ΔPsl condition allows more Pel expression (Figs 4 and 7). In addition, Ghafoor *et al*. also reported that lacking of Psl enhanced the production of Pel (Ghafoor *et al*., 2011). These data suggest that the Pel and Psl biosynthesis pathways compete for sugar precursors, implying that AlgC may also be a checkpoint enzyme for the biosynthesis pathway of Pel.

In summary, we have shown in this study that AlgC, a bifunctional PMM/PGM enzyme in *P. aeruginosa* is required for the synthesis of two critical biofilm matrix exopolysaccharides, Psl and Pel in addition to its involvement in the biosynthesis of alginate and LPS. Consistent with the exopolysaccharide deficient phenotype of the *algC* mutant, this mutant of *P. aeruginosa* is avirulent in a burned-mouse model of infection (Goldberg *et al*., 1995). Interestingly, we found that the biofilm of Δ*algC* and Δ*waaL* both occupied more space, but less bacterial cell density compared with wild-type strain PAO1, which is consistent with previous reports about the biofilm of LPS deficient strains (Palmer and Caldwell, 1995; Rocchetta *et al*., 1999). We have also provided insights into how *P. aeruginosa* may manage the production of the total exopolysaccharide pool including B-band LPS, alginate, Pel and Psl. This is accomplished by the key enzyme, AlgC. PMM/PGM enzymes are found in many different bacteria, and often play a role in the synthesis of bacterial exoproducts (Regni *et al*., 2004). Using PMM/PGM enzymes as a checkpoint may be a general rule for bacteria to modulate the production of exoproducts. Besides this post-transcriptional regulation, bacteria may have different mechanisms to coordinate the synthesis of exopolysaccharides at transcriptional level.

## Experimental procedures

### Bacterial strains and growth conditions

*Pseudomonas aeruginosa* strains used in this study are listed in Table 1. Strain PDO320 were constructed by the same strategy used for WFPA801 as described previously (Ma *et al*., 2006). In this strain, the *araC*-$P_{BAD}$ has replaced the promoter region of the *psl* operon (−267 to −54 upstream of *pslA*) in strain PDO300. In strain PDO310, the *psl* promoter region (−267 to −54 upstream of *pslA*) was deleted from the chromosome of PDO300. WFPA831, WFPA884, CIM1 and CIM2 were constructed by unmarked in-frame deletion strategy as described previously (Hoang *et al*., 1998). In these strains, the *araC*-$P_{BAD}$ has replaced the promoter region of the *pel* operon (−284 to −65 upstream of *pelA*) in strain PAO1, WFPA800, *algC* mutant and Δ*pslBCD* respectively. The *pslA*-lacZ fusion (transcriptional and translational) strains were constructed as described previously (Irie *et al*., 2010). Unless otherwise indicated, *P. aeruginosa* was grown at 37°C in Luria–Bertani (LB; 10 g $l^{-1}$ tryptone, 5 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ NaCl) lacking sodium chloride (LBNS) or Jensen's, a chemically defined medium (Jensen *et al*., 1980). Biofilms of *P. aeruginosa* were cultured in Jensen's medium at room temperature. To induce the transcription of *psl/pel* operon, arabinose was added to Jensen's medium. *Escherichia coli* strains were grown in LB media. *E. coli* JM109 was used for cloning and SM10 for biparental mating with *P. aeruginosa*. Antibiotics were used for *E. coli/P. aeruginosa* at the following concentrations: gentamycin 15/100 μg $ml^{-1}$; ampicillin 100 μg $ml^{-1}$; carbenicillin 300 μg $ml^{-1}$.

### Psl matrix and biofilm staining

Biofilms were grown at room temperature in three-channel flow chambers with individual channel dimensions of 1 × 4 × 40 mm(Stovall life science, INC) as previously described. The biofilms were stained by membrane stain FM4-64 (1 μM final concentration, Molecular Probes, Invitrogen). Psl matrix was stained by lectin HHA at 100 μg $ml^{-1}$ (EY lab, INC).

### Immunoblotting and ELISA of Psl polysaccharide extracts

Crude bacterial surface-bound polysaccharide extracts (SBPE) were obtained from overnight culture (~ 16 h) that equivalents approximately 10 Optical Density [vol. (ml) = 10/culture $OD_{600}$] as previously described (Byrd *et al*., 2009). Psl in the culture supernatant was extracted as following. The culture supernatants were precipitated by addition of three volume of 100% ethanol, followed by 30 min incubation at −20°C and 15 min spin (14 000 r.p.m.) at 4°C. The pellets were rinsed by 95% ethanol; air dried and resuspended in 100 μl of 3% SDS and boiled for 5 min at 100°C. Samples were directly used for immunoblotting or stored at −20°C. The whole cell extracts were made by resuspending 10 $OD_{600}$ of overnight culture in 100 μl 3% SDS and boiling for 5 min at 100°C. Immunoblotting and ELISA were done as previously described (Byrd *et al*., 2009). Densitometry software Quantity One (Bio-Rad) was used to quantify the immunoblotting data.

### LPS preparation and Western blotting analysis

LPS was prepared by the proteinase K method (Hitchcock and Brown, 1983) from *P. aeruginosa* grown overnight on LB broth or plates at 37°C with addition of 2% arabinose. Bacteria from 1 ml culture with OD of 0.5 were collected and prepared with an overnight proteinase K digestion at 55°C. LPS was separated on a 12.5% PAGE, transferred to a BioTrace172 NT nitrocellulose membrane (Pall) and analysed by immunoblotting as described previously (Rocchetta *et al*., 1998). Mouse-derived monoclonal antibodies N1F10 (Lam *et al*., 1989) and MF15-4 (Lam *et al*., 1987) specific for A-band and B-band LPS, respectively, were used to probe membranes. Blots were developed using goat antimouse alkaline phosphatase-conjugated secondary antibody (Jackson Immuno Research) and BCIP/NBT (Sigma).

### Alginate preparation and carbazole assay

Alginate was prepared as previously described with some modifications. 1 ml overnight grown *P. aeruginosa* culture was mixed with 1 ml 0.85% saline. After 1 min vortex, bacteria were removed by 30 min spin at 25 000 *g*. Alginate was precipitated by addition of 1 ml 2% cetyl pyridinium chloride into 1 ml culture supernatant and collected by 10 min spin at 25 000 *g*. The pellet was dissolved in 1 M NaCl and precipitated again with 1 ml of cold (−20°C) isopropanol. Alginate was collected by centrifuge at 25 000 *g* for 10 min at 4°C, resuspended in 1 ml saline and stored at 4°C. Uronic acid carbazole assay was done in 96-well as previously described (Marina *et al*., 2003).

### Total RNA isolation and quantitative RT-PCR

Total RNA was purified from 10 ml mid-log phase culture grown in Jensen's media using Ambion RNA isolation reagents (Ambion). *Taq*Man one-step RT-PCR Master mix reagents (Roche) were used for PCR reaction with 100 ng of total template RNA. RT-PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystem) using the following primers and probes, *pslB* forward primer: 5′-GCATGCCGAAACCCTTCA-3′; *pslB* reverse primer: 5′-GCGATACGCAGGAAGGTCTT-3′; *pslB* probe: 5′-[DFAM] TGGCCGATG ACCAGAGCCTGT[DTAM]-3′. *algD* forward primer: 5′-GAGCACCGCGATCA AGGA; *algD* probe: 5′-[DFAM]TACGACTTCCCGCCGATGACC[DTAM] −3′; *algD* reverse primer: 5′-TTGTCCAGTTCGCCGATCA. The standard curve method was applied and serial diluted genomic DNA was used as template to obtain a relative standard curve. The constitutively expressed *rpoD* gene was selected as an endogenous control to normalize quantification of the mRNA target. RT-PCR data were analysed validated and calculated as per the instructions of the manufacturer.

### β-Galactosidase assays

β-Galactosidase activity was quantitatively assayed as described elsewhere with modification (Hassett *et al*., 1993). *Pseudomonas aeruginosa* strains were grown in Jensen's media (−/+ara) at 37°C with shaking at 250 r.p.m. to $OD_{600}$ of 0.5–0.8. Bacteria from 1.5 ml culture aliquots were resuspended in 200 μl Z-buffer and frozen/thawed three times to lyses bacteria. Cell lysates were assayed for both β-galactosidase activities as well as for total

proteins by BCA assay (Pierce, USA). All β-galactosidase activity units were normalized by total protein per ml aliquots. All assays were done in triplicates.

### Colony morphology

Colonies on Congo red plate were grown as previously described with modifications (Friedman and Kolter, 2004b). Overnight cultures of *P. aeruginosa* strains were diluted in Jensen's media to a final $OD_{600}$ of 0.025 and 5 μl were spotted on Jensen's agar plate (1.0% agar) with 2% (v/v) Congo red solution (Kirisits *et al*., 2005). After 3 days of incubation at room temperature, colonies were imaged by Nikon SMZ1000 (Japan). The single colony was isolated on Jensen's plate (1.5% agar) with arabinose and imaged by Bio-Rad Chemidoc Imagelab system (Bio-Rad, USA).

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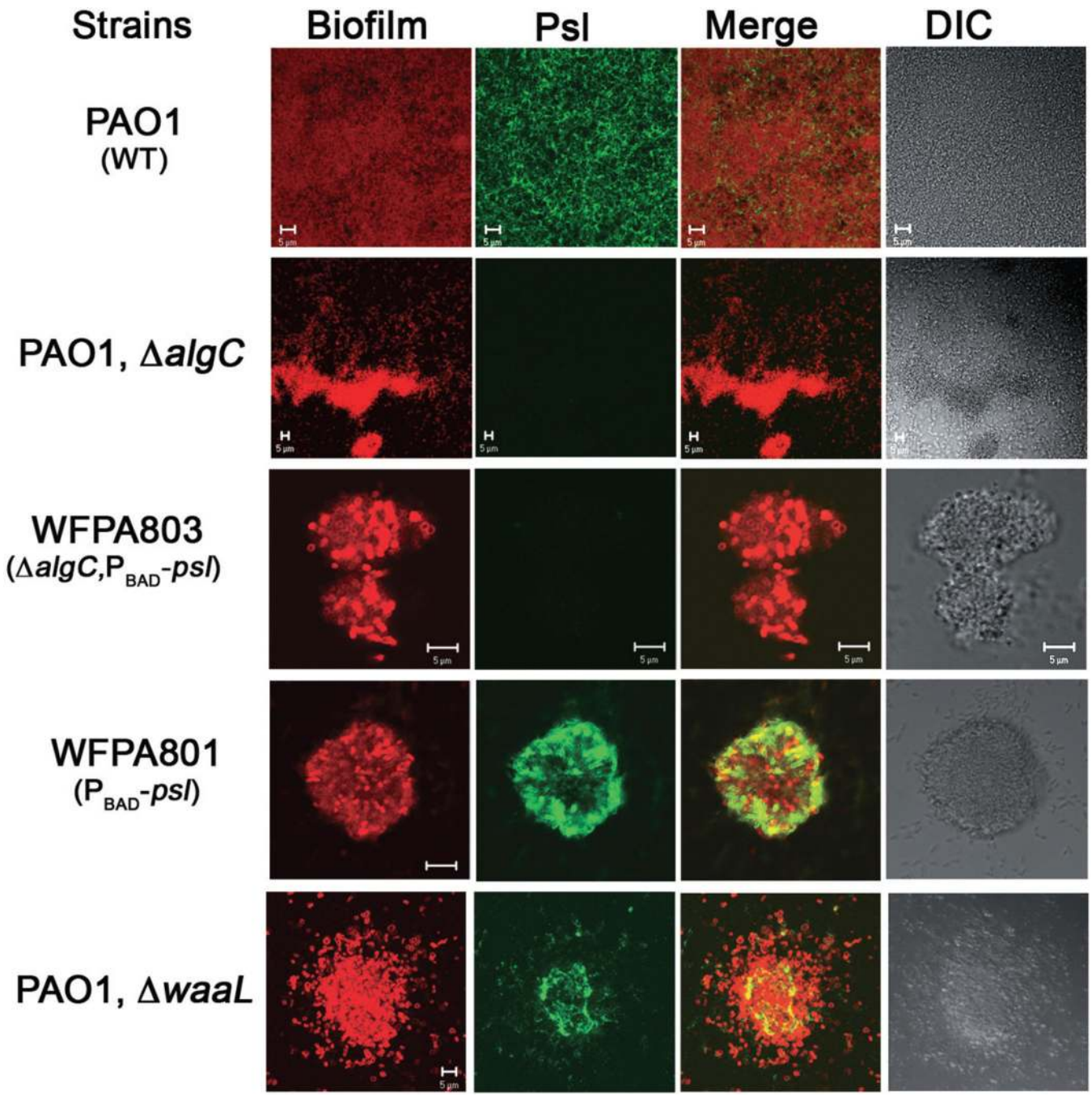


**Fig. 1.**

Biofilms formed by *P. aeruginosa* derivatives and their corresponding Psl staining in biofilms. The biofilms of PAO1, Δ*algC* strain, WFPA803, WFPA801 and Δ*waaL* strain were stained by bacterial membrane stain FM4-64 (red) and Psl stain (green, HHA-FITC) after 44 h growth in the flow cell. 3D reconstructed Psl matrix and biofilm image from each strain was shown. Grey images are corresponding differential interference contrast (DIC) image of biofilms. Scale bar, 5 μm.

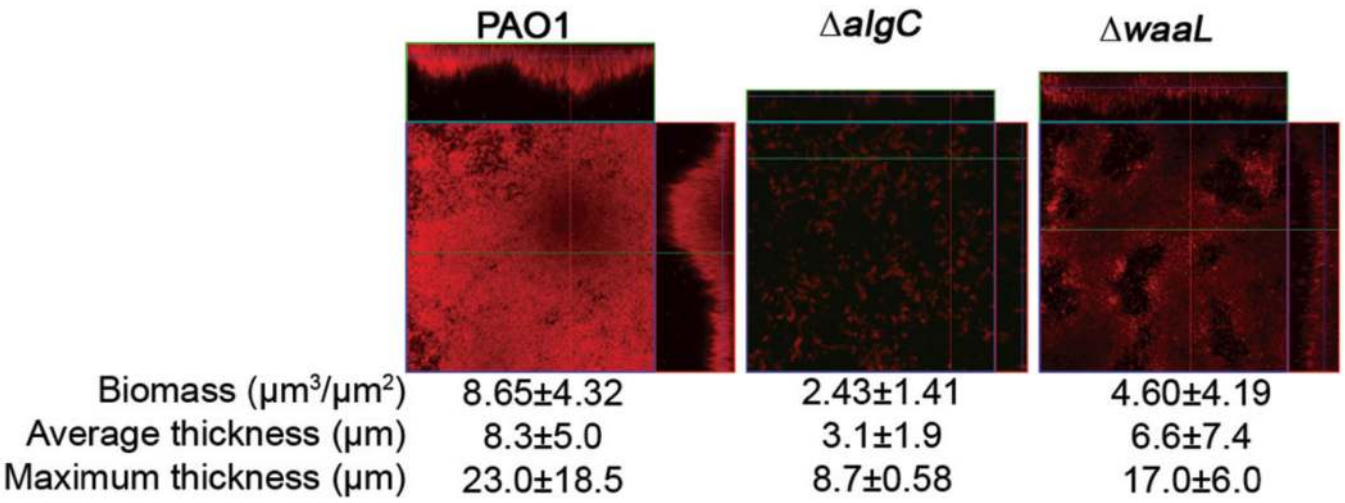


**Fig. 2.**

The biofilms of PAO1, Δ*algC* strain and Δ*waaL* strain. Shown were a selected horizontal and vertical optical-section image from the biofilm (stained by FM4-64) of each strain. Corresponding COMSTAT analysis results were shown under each image.

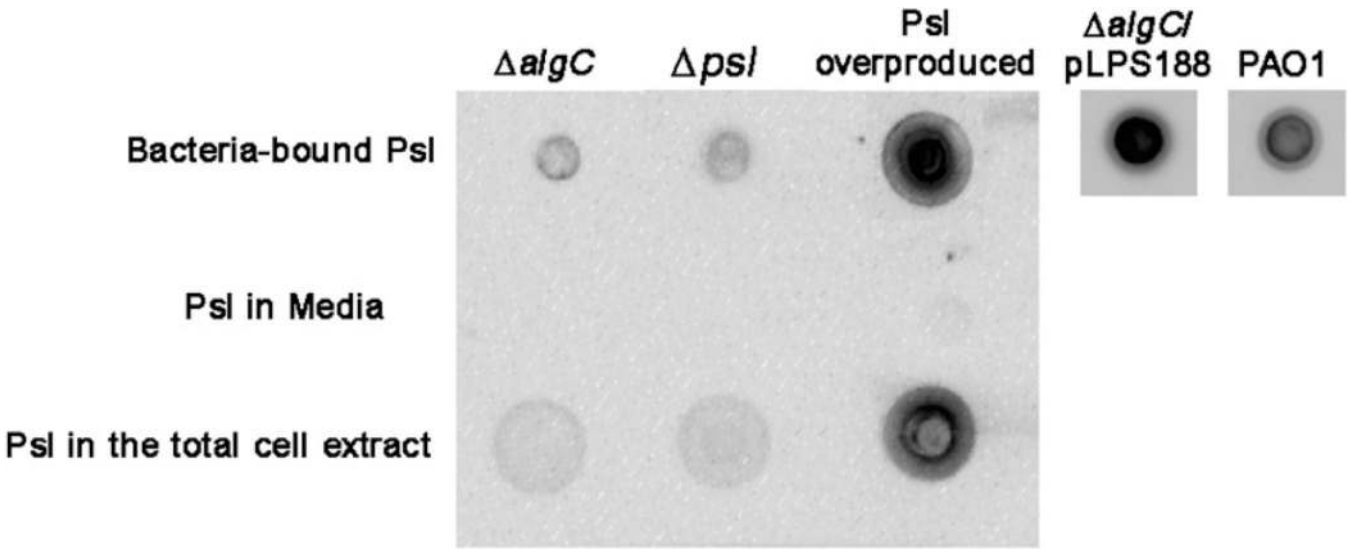


**Fig. 3.**

Psl is not synthesized in *algC* mutant. Immunoblotting with anti-Psl antibody was used to detect Psl in the whole cell extract, crude extract of bacterial surface-bound substance, and precipitation of culture supernatant from PAO1, Δ*algC*, WFPA800, WFPA801 and Δ*algC*/ pLPS188.

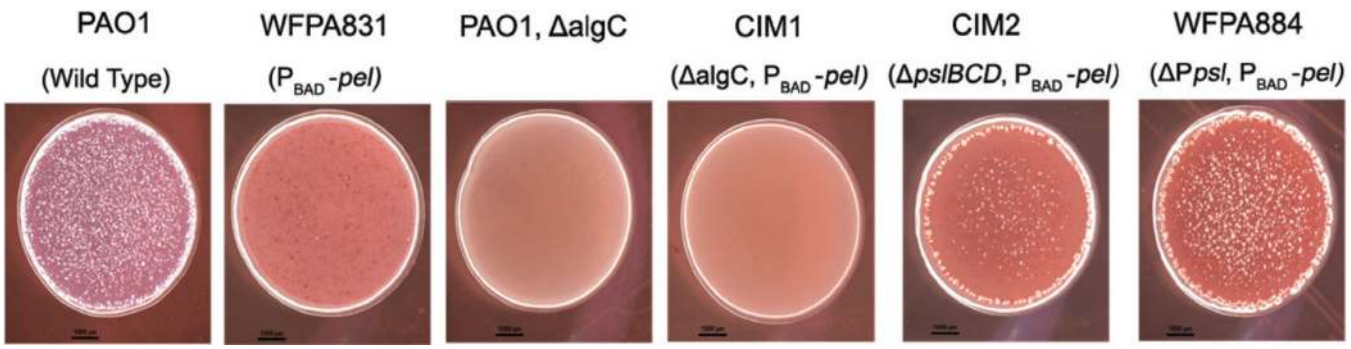


**Fig. 4.**

Pel was not detected in *algC* mutant. Shown were colony morphology of PAO1, *algC* mutant, and a variety of Pel-inducible strains on Congo red plate (1% agar) with 2% arabinose.

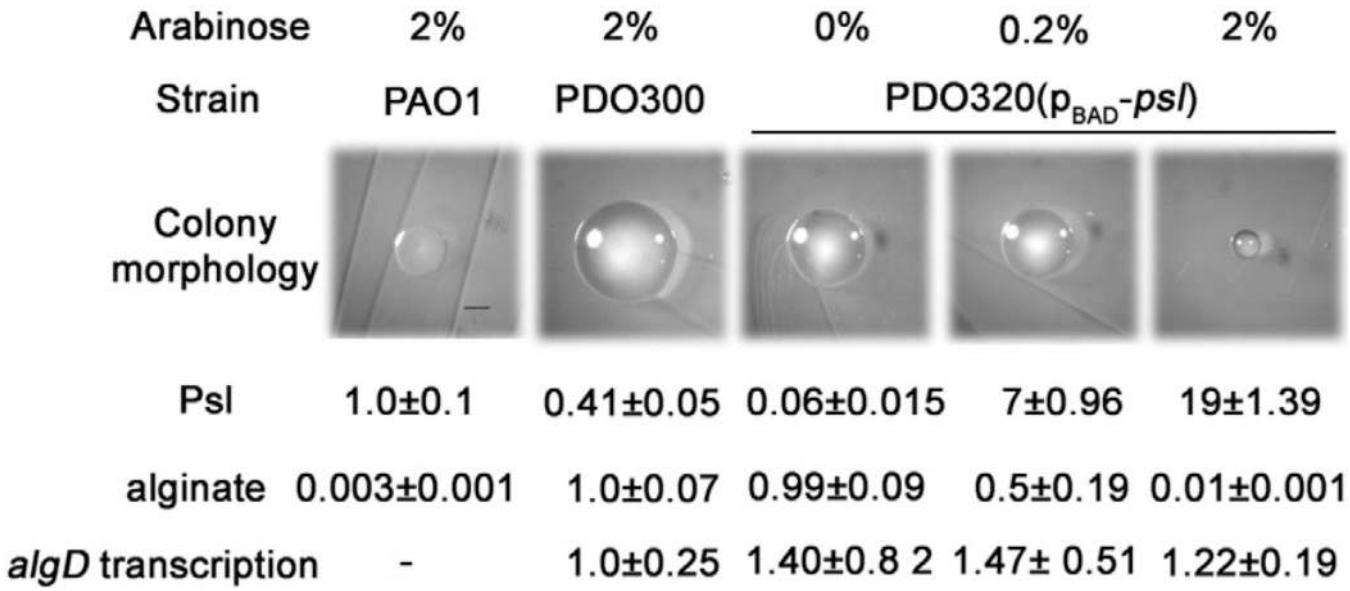


**Fig. 5.**

Overproduction of Psl reduces the amount of alginate synthesis. Shown were colonies morphology of PAO1-derived strains on Jensen's agar (1.5%) plate with different arabinose concentration. The corresponding Psl polysaccharide and alginate production as well as the *algD* transcription were shown under each colony. Psl values have been normalized to the result of wild-type strain PAO1 (32 μg $ml^{-1}$ crude Psl) and alginate values have been normalized to the result of PDO300 (709 μg alginate/$OD_{600}$). The *psl* and *alg* transcriptional level were normalized to the result of PAO1. Scale bar, 1 mm.

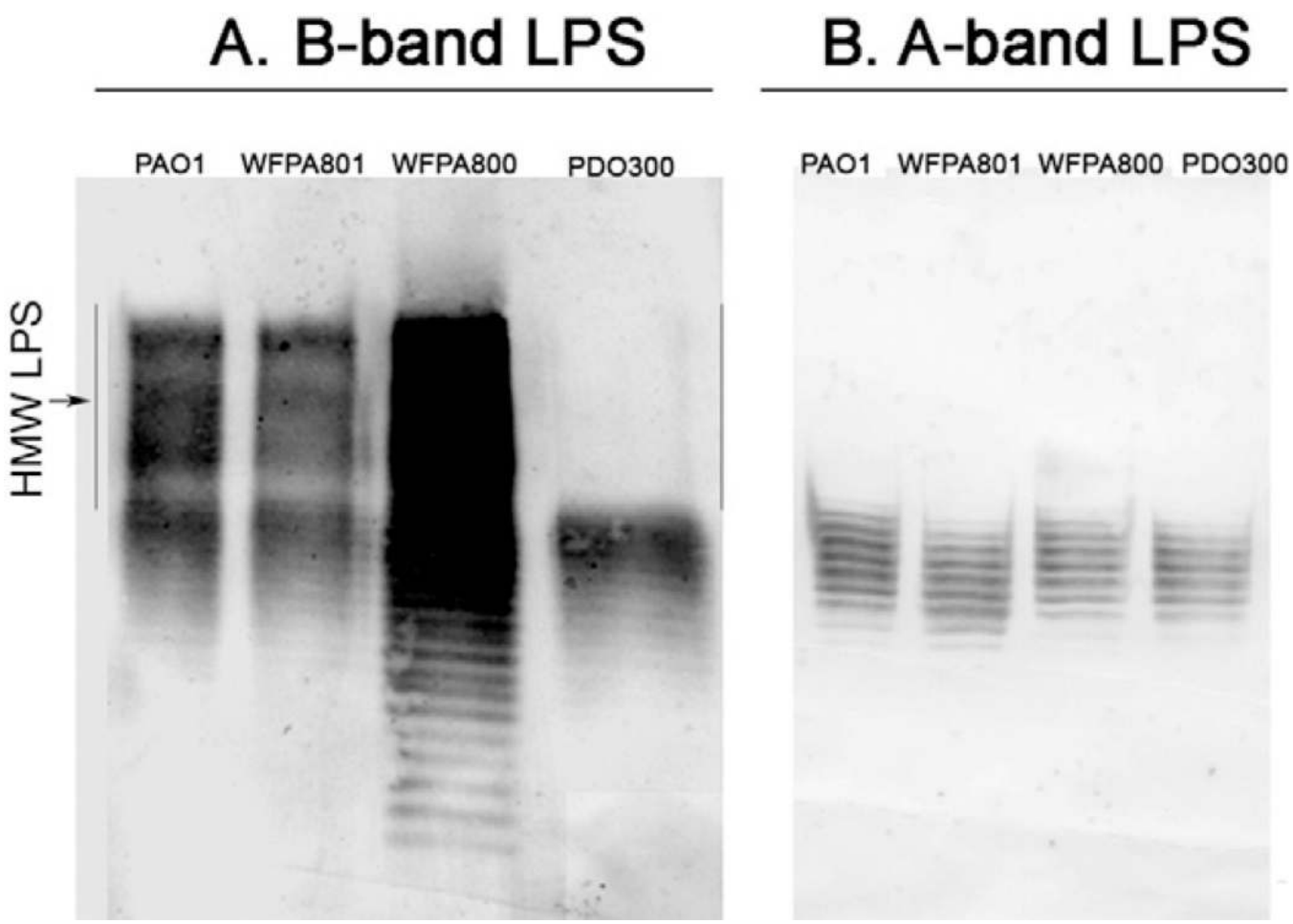


**Fig. 6.**

Lipopolysaccharides production in PAO1-derived strains. Shown are Western blotting results of LPS levels of PAO1, WFPA801, WFPA800, PDO300 using anti-B-band LPS antibody (A) and anti-A-band LPS antibody (B) respectively. All strains were grown with 2% arabinose. The arrow indicates the high molecular weigh (HMW) LPS.

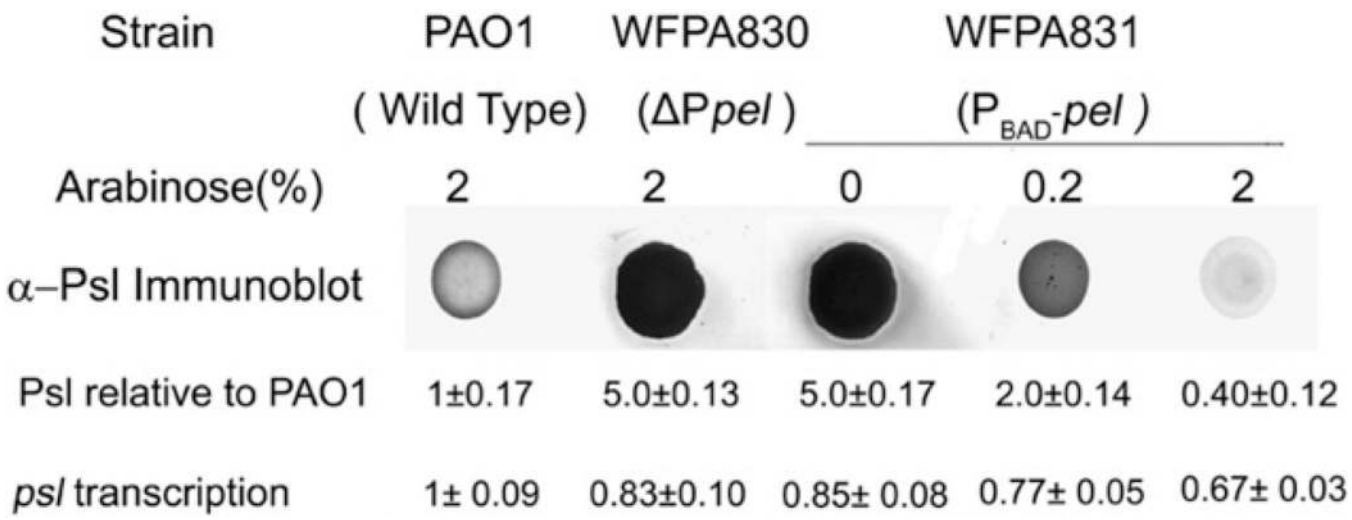


**Fig. 7.**

Overproduction of Pel reduces the amount of Psl. The production of Psl was detected by immunoblotting with anti-Psl antibody in PAO1, *pel* promoter deletion strain, and Pel-inducible strain. The density of dot blotting was quantified and normalized to PAO1 level and showed under each corresponding dot. Shown were also the values of *psl-lacZ* at transcriptional level of each strain with corresponding arabinose induction.

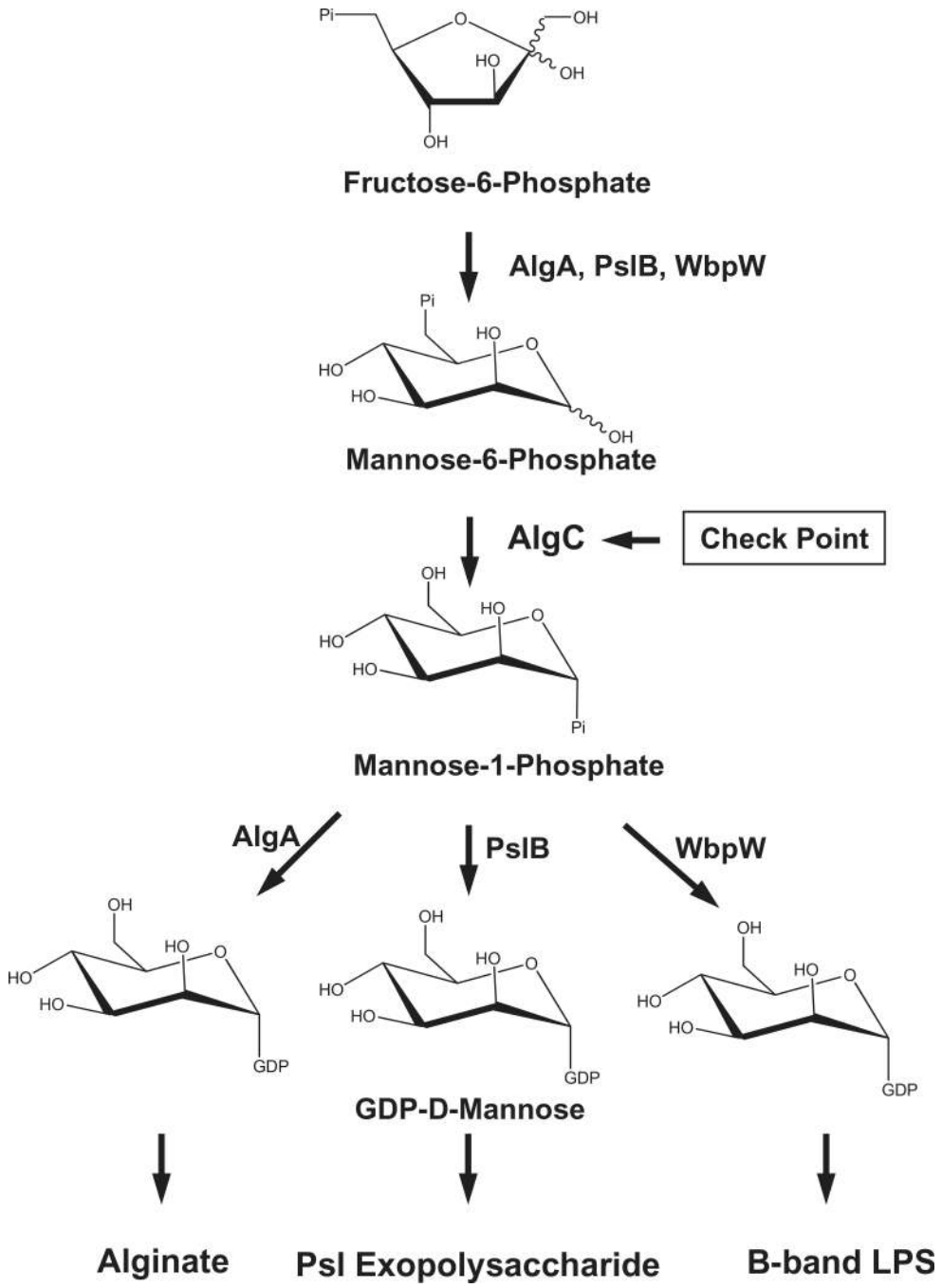


**Fig. 8.**

Schematic representation of the *P. aeruginosa* PAO1 metabolic route for the production of alginate, Psl and LPS. The phosphomannomutase activity of AlgC is required for the biosynthesis pathways of three exopolysaccharides in *P. aeruginosa*, Psl, alginate, and LPS. Our data showed that overproduction of individual exopolysaccharide reduces synthesis of the other exopolysaccharides. This suggested AlgC can be the checkpoint enzyme that limits the production of *P. aeruginosa* exopolysaccharides.

## Table 1

*Pseudomonas aeruginosa* strains used in this study.

| Strains | Relevant characteristics | Source or reference |
|---|---|---|
| *P. aeruginosa* PAO1-derived strains | | |
| PAO1 | Nonmucoid, wild-type strain, | |
| WFPA800 | Nonmucoid, *psl* operon promoter deletion mutant, Δ*Ppsl* | Ma *et al*. (2006) |
| WFPA801 | Nonmucoid, *psl*-inducible strain, $P_{BAD}$-*psl* | Ma *et al*. (2006) |
| Δ*algC* | Nonmucoid, *algC*:Tet, $Tet^r$ | Coyne *et al*. (1994) |
| Δ*algC*/pLPS188 | Nonmucoid, Δ*algC* containing pLPS188, a plasmid has constitutively expressed *algC*. | Coyne *et al*. (1994) |
| WFPA803 | Nonmucoid, Δ*algC*, Δ*Ppsl*/$P_{BAD}$-*psl* | This study |
| Δ*waaL* | Nonmucoid, *waaL*:$Gm^r$ | Abeyrathne *et al*. (2005) |
| WFPA830 | Nonmucoid, *pel* operon promoter deletion mutant, Δ*Ppel* | This study |
| WFPA831 | Nonmucoid, *pel*-inducible strain Δ*Ppel*/$P_{BAD}$-*pel* | This study |
| CIM1 | Nonmucoid, *pel*-inducible strain Δ*algC* background, Δ*Ppel*/$P_{BAD}$-*pel*, $Tet^r$ | This study |
| CIM2 | Nonmucoid, *pel*-inducible strain in Δ*pslBCD* background, Δ*Ppel*/$P_{BAD}$-*pel* | This study |
| Δ*pslBCD* | Δ*pslBCD*, markerless | Kirisits *et al*. (2005) |
| PDO300 | Mucoid PAO1, *mucA22* | Mathee *et al*. (1999) |
| PDO310 | Mucoid, *psl* operon promoter deletion mutant in PDO300 background, Δ*Ppsl, mucA22* | This study |
| PDO320 | Mucoid, *psl*-inducible strain, Δ*Ppsl*/ $P_{BAD}$-*psl, mucA22* | This study |