Pseudomonas aeruginosa exopolysaccharide Psl promotes resistance to the biofilm inhibitor polysorbate 80

Michael E. Zegans¹,²#, Daniel Wozniak³, Edward Griffin², Christine M. Toutain-Kidd¹, John H. Hammond², Andrew Garfoot³, Joseph S. Lam⁴

¹Department of Surgery (Ophthalmology), Geisel School of Medicine at Dartmouth, ²Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, ³Departments of Microbial Infection and Immunity, Microbiology, The Ohio State University, ⁴Department of Molecular and Cellular Biology, University of Guelph

#Correspondence to:
Michael E. Zegans, MD
Professor
Department of Surgery (Ophthalmology) and
Department of Microbiology and Immunology
Geisel School of Medicine at Dartmouth
Vail Building, Room 504
Hanover, NH 03755
Tel: (603) 650 - 1007
Fax: (603) 650 - 4434

Running title: P. aeruginosa Psl promotes resistance to PS80

Key Words: polysorbate 80, Psl, biofilm, biofilm inhibitor, Pseudomonas aeruginosa, exopolysaccharide, PS80, Tween 80.
Polysorbate 80 (PS80) is a non-ionic surfactant and detergent that inhibits biofilm formation by *Pseudomonas aeruginosa* at concentrations as low as 0.001% and is well tolerated in human tissues. However, certain clinical and laboratory strains (PAO1) of *P. aeruginosa* are able to form biofilms in the presence of PS80. To better understand this resistance, we performed transposon mutagenesis on a PS80 resistant clinical isolate PA738. This revealed that mutation of *algC* rendered PA738 sensitive to PS80 biofilm inhibition. AlgC contributes to the biosynthesis of exopolysaccharides Psl and alginate as well as lipopolysaccharide and rhamnolipid. Analysis of mutants downstream of AlgC in these biosynthetic pathways established that disruption of the *psl* operon was sufficient to render the PA738 and PAO1 strains sensitive to PS80-mediated biofilm inhibition. Increased levels of Psl production in the presence of arabinose were correlated with increased biofilm formation in PS80. In *P. aeruginosa* strains MJK8 and ZK2870 known to produce both Pel and Psl, disruption of genes in the *psl* but not *pel* operon conferred susceptibility to PS80 mediated biofilm inhibition. The laboratory strain PA14 does not produce Psl and does not form biofilms in PS80. However, when PA14 was transformed with a cosmid containing the *psl* operon, it formed biofilms in the presence of PS80. These findings suggest that Psl can mediate resistance to the biofilm inhibitor PS80. Taken together these data suggest that production of the exopolysaccharide Psl by *P. aeruginosa* promotes resistance to the biofilm inhibitor PS80.
Bacterial biofilm formation on prosthetic medical devices such as contact lenses, indwelling catheters and artificial joints is a common cause of serious infections (8, 9, 12, 22, 32). The formation of biofilms in these clinical settings has prompted interest in developing inhibitors that are compatible with use on human tissues. We have previously reported that polysorbate 80 (PS80), a non-ionic detergent and surfactant, inhibits biofilm formation of the pathogen *Pseudomonas aeruginosa* and many other bacteria at concentrations as low as 0.001% (39). Since PS80 is well tolerated on human tissues at concentrations of 1% or more, this is a clinically relevant finding (17). While the mechanism of action by which PS80 inhibits biofilm formation has not yet been elucidated, PS80’s ability to inhibit bacterial biofilm formation has been noted in other independent publications (1, 13, 26).

Some clinical isolates of *P. aeruginosa* are resistant to PS80 biofilm inhibition, prompting us to investigate the basis of this resistance in order to determine if PS80 formulations can be enhanced or modified to increase their spectrum of activity. As we have reported, one mechanism of PS80 resistance by some strains of *P. aeruginosa* is overexpression of a secreted lipase, LipA, resulting in cleavage of PS80 at its ester bond. Based on these findings we identified a compound related to PS80, polyethoxylated(20) oleyl alcohol, containing a lipase-resistant ether bond rather than an ester bond. This compound was able to inhibit biofilm formation in LipA over-expressing strains (39). However, our data indicate that lipase over-expression was
observed only in approximately 20% of the clinical isolates that exhibited resistance to PS80. Responses to PS80 also vary among different *P. aeruginosa* laboratory strains. Biofilm formation of *P. aeruginosa* PA14 (PA14) is inhibited in the presence of PS80, while *P. aeruginosa* PAO1 (PAO1) forms robust biofilms under these conditions. Similar to most clinical isolates we have investigated, lipase over production (and PS80 cleavage) is not the basis of the resistance to PS80 exhibited by PAO1.

To better understand non-lipase-dependent mechanisms of PS80 resistance in *P. aeruginosa*, we performed a transposon mutagenesis of a clinical isolate, PA738, which is resistant to PS80 mediated biofilm inhibition, but does not over produce lipase. Based on this screen we found that the genes within the polysaccharide synthesis locus, *psl*, play important roles in resistance to PS80 in PA738, PAO1 and other clinical *P. aeruginosa* strains. Along with Pel and alginate, Psl is one of 3 exopolysaccharides (EPS) produced by *P. aeruginosa* (2, 10, 16). All have been found to contribute to biofilm formation. Some *P. aeruginosa* strains produce only a single EPS while others are capable of producing multiple EPS (20, 34). The chemical structure of Psl is composed of mannose, glucose, and rhamnose and its biosynthesis is directly linked to a 15-gene operon previously reported to be involved in biofilm formation in strains such as PAO1, MJK8 and ZK2870 (2, 10, 20). Our data suggest that Psl can mediate resistance to the biofilm inhibitor PS80.
MATERIALS AND METHODS

Bacterial strains and media

All P. aeruginosa strains used in this study are derived from P. aeruginosa PA14 (31) or PAO1 (14) unless otherwise specified (see list of strains in Table 1). All clinical isolates used unless otherwise noted come from the laboratory strain collection in the Zegans laboratory. Escherichia coli JM109 was used as a host strain for plasmid construction and propagation, whereas S17 was used for conjugations with P. aeruginosa. The Saccharomyces cerevisiae strain InvSc1 (Invitrogen, Carlsbad, California) was used for in vivo homologous recombination for plasmid construction as described (36). Selection of yeast strains was performed using synthetic defined agar medium consisting of 0.67% Yeast Nitrogen Base minus uracil (Research Products International, Mt Prospect, Illinois), 0.076% Complete Supplement Mixture minus Uracil (Sunrise Science Products, San Diego, California), 1.5% dextrose (Sigma, Saint Louis, Missouri), and 2% agar (Difco, Franklin Lakes, New Jersey).

All bacterial planktonic cultures were grown in lysogeny broth (LB medium, also known as Luria Bertani medium) (35) at 37°C, unless otherwise noted and their growth was monitored at the optical density of 600 nm (OD_{600}). Colonies of bacteria were grown on plates of LB agar or LB agar no sodium (LANS) unless otherwise noted. All biofilm cultures for the assays were incubated for 24 h at 37°C in M63 medium with 0.4% arginine and 1 mM MgSO_{4} (M63) unless otherwise specified. Antibiotics were supplemented in the culture media as follows: ampicillin (Am) was used at 150 µg/ml for
E. coli, carbenicillin (Cb) was used at 500 µg/ml for P. aeruginosa, gentamicin (Gm) was used at 10 µg/ml for E. coli and 50 µg/ml for P. aeruginosa, and nalidixic acid (Nx) was used at 20 µg/ml.

Polysorbate 80 (PS80, Spectrum Chemicals, Gardena, California) was prepared as a 10% solution in MilliQ ultrapure water and used at the concentration of 0.1%, unless otherwise noted.

Biofilm assays
Biofilm assays in 96-well PVC or polystyrene microtiter plates with crystal violet staining of biofilms were performed as previously described (25, 27). All biofilm cultures for the assays were incubated for 24 h at 37°C in M63 medium with 0.4% arginine and 1mM MgSO₄ (M63) unless otherwise specified (28). The amount of biofilm present in each well was measured by solubilization of crystal violet with 30% acetic acid and quantified at absorbance at 550 nm (A₅₅₀). All biofilm data presented were collected from one representative experiment, with at least 4 replicates per strain, of a minimum of 3 independent assays. Strains were considered sensitive to PS80 biofilm inhibition if there was a 50% reduction in crystal violet staining when grown as biofilms in PS80-containing medium compared to the control that was grown in a medium lacking PS80.

Psl Dot blot assay
Crude exopolysaccharide extracts and dot blots to demonstrate the presence of Psl production in PAO1 were performed as previously described (2).
Building and screening of the Mariner transposon mutant library in the PA738 strain

A conjugation between *E. coli* SMC1210 (pBT20 in SM10) (37) and *P. aeruginosa* PA738 was performed according to the following protocol. A bacterial lawn of either strain was harvested in order to obtain an OD$_{600}$ of 40 for *E. coli* and 20 for *P. aeruginosa*. The two strains were mixed in a 1:1 ratio (pre-warmed at 37°C) and spotted on a LB agar plate. After an incubation of 3 h at 37°C, the cells were harvested and resuspended in 100 μl of LB broth and plated on LB agar medium supplemented with gentamicin and nalidixic acid. Each culture plate was incubated at 37°C for 2 days. The transconjugants were then isolated in 96-well plates containing LB broth medium supplemented with Gm and Nx. Each library of 96-well plate was inoculated in fresh 96-well microtiter plates containing either LB or LB supplemented with PS80 0.01%. The mutant strains that were found to be sensitive to PS80 were isolated. The localization of the Mariner transposon with respect to the *P. aeruginosa* genome was determined using an established protocol (27).

Assembly of constructs and generation of mutants

Deletion and complementation constructs were assembled as follows, using the respective primers as listed in Table 2. The vectors pMQ30 and pDPM73 (24, 36) were used for deletion and complementation, respectively. The 5’ end of each primer used for cloning has homology to the vector into which it was cloned. PCR was used to amplify fragments from the PAO1 genome, which were cloned into their respective vectors by *in vivo* homologous recombination in yeast. Recombinant plasmids were recovered from yeast as previously described (36) transformed into *E. coli* S17 by electroporation, and
candidates were screened by antibiotic selection and PCR using specific primers to check for the insertion of the fragments into the plasmids. The $\Delta psl$-promoter construct was created by amplifying the regions flanking the $psl$ promoter to have overlapping sequences that omit the promoter sequence. Flanking regions of the $psl$ promoter were amplified by PCR using the primers pMQ30_upper-psl-pro_Fwd with $\Delta psl$-promoter_OLR, and pMQ30_lower-psl-pro_Rev with $\Delta psl$-promoter_OLF. The fragments were cloned into pMQ30 by in vivo-homologous recombination in yeast. Constructs were harvested and screened as previously described and transformed into $E. coli$ S17 by electroporation. Transformants of deletion constructs were then conjugated into $P. aeruginosa$ strains to generate mutants. Complementation assays were performed by transforming plasmid constructs containing specific wild-type genes into the mutants by electroporation. Conjugates of deletion constructs were counter-selected on LB agar medium containing 5% sucrose, but without NaCl. Candidates were re-streaked on LB agar medium and tested for deletions by PCR (36).

**Genetic complementation of $psl$ operon in $P. aeruginosa$ PA14**

A cosmid pMO011305 (15) containing the 22.5-Kb $psl$A-K locus, as confirmed by PCR was introduced into PA14 by biparental mating using SM10 $E. coli$ (16). Exconjugates were selected on LANS plates containing tetracycline and irgasan, pLAFR3 without the $psl$ insert was used as a vector control. Randomly picked colonies were analyzed for Psl expression by dot blot.

**Semiquantitative RT-PCR**
Using the High Pure RNA Isolation Kit (Roche, Indianapolis, IN) total RNA was isolated from 1 ml of each overnight culture that was used to test for biofilm formation and treated with DNase per protocol of this kit. Total isolated RNA was then used as the template for cDNA synthesis using the DyNAmo™ cDNA Synthesis Kit (Thermo Fisher Scientific, Lafayette, CO). The synthesized cDNA was then used as the template for PCR reactions to amplify a 198-bp fragment of \textit{pslA} template cDNA and a 127-bp fragment of \textit{rplU} template cDNA. Amplification and visualization of \textit{rplU} transcripts was used as a positive control for reaction and template competency. No reverse transcriptase controls were also performed to prove there was not genomic DNA contamination of the samples used for PCR. Forward and reverse primers for the PCR amplification of \textit{pslA} template cDNA were 5’-TCCCTACCTCAGCAGCAAGCTGGT-3’ and 5’- CGGATGTGCTGTTGCGTACCAGGTAT-3’, respectively. Forward and reverse primers for the PCR amplification of \textit{rplU} template cDNA were 5’-TTACCGGTGGCAAGCAGCACAAAG-3’ and 5’-TTCACGTCTTCGCCATTGGCAACC-3’, respectively. Amplicons were visualized using gel electrophoresis in 1% agarose.
RESULTS

Transposon mutagenesis of PS80 resistant strain PA738 yields a PS80-sensitive mutant with disruption of \textit{algC}

\textit{P. aeruginosa} 738 (PA738) is a clinical isolate which forms biofilms despite the presence of PS80 in the culture medium and like strain \textit{P. aeruginosa} PAO1, it does not appear to overproduce lipase, which had been shown to be one of the mechanisms used by \textit{P. aeruginosa} to inactivate PS80 (39). Since the basis of PA738's resistance did not appear to be inactivation of PS80, we postulated that a genetic approach to isolate mutants, that conferred sensitivity to biofilm inhibition by PS80, would help to identify the mechanism of PS80 resistance.

We used the Mariner transposon to perform a mutagenesis of PA738 in order to isolate mutants which were unable to form biofilms when grown in the presence of PS80. We screened 13,118 mutants for isolates that could form biofilms in the absence of PS80 but exhibited a 50\% or more reduction in biofilm formation in the presence of 0.01\% PS80. The location of the transposon insertions in 4 candidate mutants was mapped (see Materials and Methods). This screen led to the identification a particular mutant with a Mariner transposon inserted in a gene that shares high sequence identity to
PA5322, the \textit{algC} gene in PAO1. Therefore we concluded that our transposon had disrupted the \textit{algC} homolog of strain PA738 and will be referred to as \textit{algC} hereafter. To ensure that mutation of \textit{algC} rather than an unknown mutation elsewhere in the chromosome was responsible for the observed PS80 susceptible phenotype, we tested strains with independent \textit{algC} mutations in PAO1 (listed in Table 1). These previously isolated \textit{algC} mutants were also resistant to PS80 and could form biofilm despite the presence of this inhibitory compound. Like in PA738, disruption of the \textit{algC} gene in PAO1 rendered this isogenic PAO1\textit{algC} mutant to be sensitive to PS80 mediated biofilm inhibition (Figure 1). Transforming strain PA738 \textit{algC}::Tn with a plasmid construct containing wild-type \textit{algC} (Table 1) resulted in the transformant becoming resistant to PS80, providing further support for the conclusion that disruption of \textit{algC} alters the response of \textit{P. aeruginosa} to PS80 (Figure 1).

We considered the possibility that the mutation of \textit{algC} could result in reduced growth in the presence of PS80 and this might affect subsequent biofilm formation. Planktonic growth kinetics were assessed in the presence and absence of PS80 and we found that the presence of PS80 in the culture medium did not shown any discernible growth inhibition to \textit{algC} mutants (data not shown). These data indicate that mutation of \textit{algC} induces susceptibility to PS80 mediated biofilm inhibition rather than through altered growth.

\textbf{Mutation of \textit{galU} and \textit{rmlC} render PAO1 and PA738 sensitive to PS80-mediated biofilm inhibition}
AlgC is a bifunctional enzyme with phosphoglucomutase and mannophosphomutase activities, which catalyze specific steps in the pathways for the biosynthesis of lipopolysaccharide, rhamnolipids, alginate and Psl (Figure 2) (19). Despite the large genome size of *P. aeruginosa*, no other genes with products of similar function to AlgC have been found, thus deletion of *algC* abrogates the biosynthesis of many important polysaccharides (19). Because of these broad effects, we tested mutants of PAO1 with disruption of genes whose products have been shown to catalyze metabolic steps downstream of AlgC in each of the pathways outlined in Figure 2. These experiments are crucial for determining if the effects we were observing in the *algC* mutants were the result of the disruption of a specific polysaccharide biosynthetic pathway or due to overall changes in *P. aeruginosa* cell physiology in the absence of the AlgC function.

Mutations in genes for enzymes in biosynthetic pathways which utilize mannose-1-phosphate, including *algD, wbpW, gmd* and *rmd*, (Table 1) did not render the bacteria susceptible to PS80-mediated biofilm inhibition. These results indicated that neither common polysaccharide antigen (also known as A-band LPS) nor alginate appeared to participate in PS80 resistance. In contrast, mutation of genes that encode enzymes that utilize glucose-1-phosphate as substrate, for instance, *galU* and *rmlC* (Table 1), resulted in the mutant bacteria producing diminished biofilms in the presence of PS80 (Figure 2B).

*Mutation of the *rhlC* gene in the rhamnolipid synthetic pathway does not promote PS80 sensitivity*
Glucose-1-phosphate is utilized in the synthesis of rhamnolipids, LPS core and Psl (2, 19). Our mutant analysis suggested that LPS core and Psl are likely candidates to play a role in PS80 resistance. GalU and RmlC contribute to the synthesis of the substrates, UDP-D-glucose and dTDP-L-rhamnose, required for Psl and LPS core biosynthesis, respectively. dTDP-L-rhamnose, but not UDP-D-glucose, also contributes to rhamnolipid synthesis; however, disruption of the production of either of these nucleotide-sugar precursors resulted in reduced biofilms when the mutant bacteria were grown in the presence of PS80. RhlC is a rhamnosyltransferase required specifically for the production of di-rhamnolipid, and this particular step of rhamnolipid synthesis is contingent on the completion of the production of dTDP-L-rhamnose in a 4-step biosynthesis pathway involving RmlA, RmlB, RmlC and RmlD (7, 30). The mutant containing an rhlC deletion (Table 1) (30) was able to form biofilms in the presence or absence of PS80, further indicating that rhamnolipids are not involved in PS80 resistance (Figure 2B).

**Mutation of genes essential for Psl biosynthesis render PAO1, PA715 and PA738 sensitive to PS80-mediated biofilm inhibition**

Having found evidence that disruption of genes downstream of algC in its role as a phosphoglucomutase could promote biofilm formation in PS80, we turned our attention to the Psl biosynthetic pathway (Figure 3). Interestingly, Psl is produced by the PS80-resistant strain PAO1, but not by PS80-sensitive strain PA14 providing a preliminary indication that Psl might be related to PS80 resistance (34).
In support of this model, a PAO1 strain containing a deletion of the \textit{psl} promoter (WFPA800) (Table 1) was unable to form biofilms in the presence of PS80 (Figure 3A) (2). Moreover, mutations of individual \textit{psl} genes previously shown to be required for Psl synthesis (14) also resulted in PS80-mediated sensitivity (Fig. 3A). Similar results with WFPA800 were found with biofilms formed both on polyvinyl chloride (PVC) or polystyrene and in different culture media, LB and M63, indicating that these effects were not surface or culture medium-dependent (data not shown). PA738 and PA715, both PS80-resistant clinical isolates, were also rendered PS80 sensitive by a deletion of the \textit{psl} promoter (Table 1 and Figure 3B). It is important to note that we evaluated biofilms at 24 hours at which time we observed diminished but not the absence of biofilms in strains with disrupted Psl production. This has been reported even in strains with little or no biofilm formation at earlier time points in the absence of Psl (4).

We considered the possibility that disruption of Psl production in PAO1 and other strains might broadly alter growth in PS80-containing media. If this were the case, apparent sensitivity to PS80-mediated biofilm inhibition might simply reflect impaired growth. However, planktonic growth of strain WFPA800 was in fact slightly enhanced in LB containing 0.1% PS80 compared to LB alone suggesting that loss of Psl specifically affects PAO1 ability to form biofilms in PS80 (Figure 3C). Similar results were obtained when WFPA800 was cultured in M63 medium (data not shown).

PAO1 strains containing individual deletions of genes in the \textit{psl} operon were tested for biofilm formation in the presence and absence of PS80, respectively. Consistent with the hypothesis that Psl is involved in PS80 resistance, disruption all 11 genes known to be essential for Psl synthesis, \textit{pslACDEFGHIJKL} (Table 1), resulted in strains which did
not form biofilms in the presence of PS80 (Figure 3A) (2). In contrast, mutants with deletions in the 3 genes that are not required for Psl synthesis, psIMNO (Table 1), continued to form robust biofilms in the culture medium supplemented with PS80. Taken together, these data indicate that disruption of Psl production and not some unrelated activity of one of the enzymes encoded in the psl operon, is responsible for the PS80 resistance associated with PAO1.

Biofilm formation in PS80 is correlated with Psl production in a psl inducible strain of PAO1

In *P. aeruginosa* WFPA801, the wild type psl promoter has been replaced with an arabinose-inducible promoter (Table 1) (2). As such, WFPA801 produces little Psl unless arabinose is added. Biofilm formation was observed at 24 h of growth in the absence of arabinose and PS80. In contrast, biofilm formation was severely reduced when PS80 was added (Figure 4). However, induction of Psl production via the addition of arabinose (0.01-0.1%) was associated both with increasing levels of Psl, as measured by an immunoblot using anti-Psl antibodies, and biofilm formation both in the presence or absence of PS80 (Figure 4). Some of the increased biofilm formation, which we observed in the presence of arabinose, is likely due to enrichment of the medium, since strain WFPA800 with a deletion in the promoter of psl also formed enhanced biofilms in the presence of increasing levels of arabinose. However, despite this enhancement of biofilms in the absence of PS80, strain WFPA800 did not form biofilms when the culture medium was supplemented with both arabinose and PS80, whereas strain WFPA801 was capable of forming biofilm under the same conditions (23).
Over expression of Pel in a ΔbifA deletion strain results in hyperbiofilm formation but does not render PA14 resistant to PS80.

Since WFPA801 is a “hyper” biofilm former under induction by high levels of arabinose, we considered the possibility that other strains known to form hyper-biofilms might display similar pattern of resistance to PS80. As previously reported, deletion of the bifA gene in PA14 resulted in over production of Pel exopolysaccharide and hyper biofilm formation (21). Experiments were performed based on this premise, and we observed that while the bifA deletion mutant did result in hyper biofilm formation in the absence of PS80, it could not produce biofilm in the presence of PS80 (Figure 5). Thus PS80 resistance does not appear to be a general phenomenon of hyper-biofilm formation or EPS over production but rather is specifically associated with Psl.

In strains known to produce both Psl and Pel, ZK2870 and MJK8, loss of Psl but not Pel restores PS80 mediated biofilm inhibition.

Since Psl in strains such as PAO1 is known to be important for biofilm formation, we hypothesize that loss of Psl might render the bacteria susceptible to PS80 because of an overall reduction in their facility to form biofilms rather than the loss of a specific mechanism of resistance to PS80. To address this hypothesis, we investigated P. aeruginosa strains ZK2870 and MJK8, which produce two distinct forms of EPS, Psl and Pel (10, 20). Disruption of Pel synthesis was accomplished through deletion of pelA in both strains (Table 1). Disruption of Psl production was accomplished in ZK2870 by deletion of the psl promoter, whereas in MJK8 a deletion of the pslBCDE was tested. ZK2870 and MJK8 derivatives lacking both Psl and Pel were also evaluated.
Both wild type strains ZK2870 and MJK8 formed robust biofilms in the presence or absence of PS80. Disruption of genes essential for Psl production in both resulted in reduced biofilms in the presence of PS80 (Figure 6). In contrast, ZK2870 or MJK8 strains containing a deletion of pelA (Table 1), essential for Pel production (10), retained PS80 resistance (Figure 6). These data indicate that Psl can mediate PS80 resistance even in strains producing Pel and that in the absence of Psl, Pel producing strains are unable to form biofilms in PS80.

**Expression of Psl in the PS80 sensitive strain PA14 is sufficient to promote resistance to PS80 biofilm inhibition**

As noted earlier wild-type PA14 produces Pel, but not Psl and does not form biofilms in the presence of PS80 (34, 39). We hypothesized that production of Psl in PA14 would promote resistance to PS80. A cosmid, pMO0113050, which contained the psl operon, was moved into PA14. The empty vector pLAFR3 as a control was also transformed into PA14 (Table 1). Semiquantitative RT-PCR for pslA and rplU (as a positive control for the RT-PCR) was performed to demonstrate presence or absence of expression from the psl operon. As expected, rplU expression was observed in all strains, whereas pslA expression was only observed in PAO1 and PA14 pMO0113050 (Figure 7). PA14 pMO0113050 was able to form biofilms in 0.05% PS80 while wild-type PA14 or PA14 pLAFR3 (vector control), did not (Figure 7). These results did not appear to be surface or culture medium-dependent since similar results were obtained when the experiments were conducted when the cultures were grown on 96-well polystyrene
plates using LB medium (data not shown). Additionally, we noted that biofilm formation in the absence of PS80 was enhanced in PA14.

When we increased the PS80 concentration from 0.05% to 0.1%, PA14 pMO0113050 did not reproducibly form biofilms (data not shown). We speculate that this difference in the therapeutic window of PS80 in PA14 pMO0113050 compared to wild-type PAO1 may reflect different levels of Psl production. Alternatively, there may be differential expression of co-factors that interact with Psl and impact its ability to mediate PS80 resistance. However, since wild-type PA14 was inhibited at both concentrations of PS80, these data strongly support the hypothesis that Psl production in *P. aeruginosa* promotes biofilm formation in PS80.

**DISCUSSION**

Our data indicate that Psl production promotes biofilm formation of *P. aeruginosa* in PS80. Psl-mediated PS80 resistance does not appear to involve inactivation of PS80 unlike our previously reported findings of cleavage of PS80 by the lipase LipA. Psl is known to be an important component of the biofilm formed by strains such as PAO1 and we considered the possibility that loss of Psl resulted in strains which were susceptible to PS80 because of an overall reduction in their facility to form biofilms rather than the loss of a specific mechanism of resistance to PS80. However, mutational analysis of strains ZK2870 and MJK8 which produce both Psl and Pel and our data from PA14 in which Psl is expressed from a cosmid argue strongly against this hypothesis. Both wild type strains ZK2870 and MJK8 form biofilms in the presence of PS80. However, disruption of Psl but not Pel production resulted in strains with diminished biofilms in
PS80. Unlike disruption of Psl synthesis in PAO1, disruption of Psl in ZK2870 and MJK8 does not leave these strains devoid of EPS, since they still produce Pel. This ability to produce Pel is reflected in relatively intact biofilm formation in the absence of PS80 of both strains in which either Pel or Psl production had been disrupted, whereas the double mutants had markedly reduced biofilms. PA14 forms robust biofilms in the absence of PS80 and produces Pel, but not Psl. However, when Psl was expressed in PA14 from a cosmid, PA14 was able to form biofilms in PS80. Thus we have provided genetic evidence, through deletion of psl in PS80 resistant strains (PAO1, PA738, PA715, ZK2870 and MJK8) and expression of Psl from a cosmid in PS80 sensitive strain PA14, that Psl promotes biofilm formation despite the presence of PS80 in the growth medium. It is worth noting that disruption of Psl conferred sensitivity to PS80-mediated biofilm inhibition in 5 PS80 resistant strains including 3 clinical isolates, suggesting that Psl plays a major role in resistance to PS80. Pel overproduction in a non-Psl producing strain, as shown by the PA14 bifA mutant, is not sufficient to overcome PS80-mediated biofilm inhibition. This demonstrates that the sheer amount of EPS overproduction and hyper-biofilm forming ability are not sufficient to mediate PS80 resistance. These data strongly suggest a specific ability of Psl to promote biofilm formation of P. aeruginosa when treated with PS80.

We propose three mechanisms to explain how Psl may promote biofilm formation of P. aeruginosa when treated with PS80. First, Psl may limit contact of PS80 with cells or the abiotic surface where biofilm formation occurs either by competitive binding of PS80 or by creation of a barrier, which excludes PS80. Second, Psl-dependent pathways to biofilm formation, unlike those associated with Pel, may be not
be targeted by interaction with PS80. For instance, surfactants such as PS80 have been reported to sequester antibacterial agents in micelles, thus reducing their antimicrobial effects (17, 18). It is possible that PS80 sequesters molecules involved in Pel dependent biofilm formation, which are not required for Psl dependent biofilms. Finally, it has been suggested that the ability of a surfactant to reduce biofilm formation is correlated with its ability to reduce surface tension (41). If this is the case, it is possible Psl alters PS80 ability to function as a surfactant and thus reduce surface tension. All these possibilities will be important avenues to explore in future investigations.

Interaction between PS80 and Psl in vitro may mirror more complex interactions between bacteria and the many surfactants produced by microbes in the environment. Such an interaction was reported by Mireles and colleagues who noted that a surfactant produced by Bacillus subtilis, surfactin, inhibits biofilm formation of Salmonella enterica (26). One can speculate that differing responses to PS80 associated with the type of EPS produced may reflect adaptations of P. aeruginosa to interactions with similar environmental microbial surfactants (3, 40).

A better understanding of the interactions between PS80 and Psl will provide crucial information towards development of PS80 derivatives active against Psl producing strains and provide clues to the mechanism of action by which PS80 inhibits biofilms. A biofilm inhibitor based on PS80 would be attractive because PS80 is well tolerated on human tissues at concentrations above those used in this paper. Rinsing or modifying surgical materials and devices with a PS80 derivative before implantation could be a strategy to reducing biofilm formation and thus infection in patients. Finally, PS80 will
be a useful reagent in studying the differences between Psl and Pel pathways in *P. aeruginosa* biofilm formation.

**Acknowledgements:**

Work by M.E.Z. was supported by Centers of Excellence in Biomedical Research (COBRE) grant NIH P20 RR16437 and P30 RR032136 in Molecular, Cellular and Translational Immunology and the Harmes Fellowship, Department of Surgery, Dartmouth Medical School; work by D.J.W supported by Public Health Service grants AI061396 and HL058334; work in the laboratory of J.S.L. related to *P. aeruginosa* biofilms is supported by a grant from Cystic Fibrosis Canada. J.S.L. holds a Canada Research Chair in Cystic Fibrosis and Microbial Glycobiology.
REFERENCES


<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA 14 wild type</td>
<td>wild type</td>
<td>(31)</td>
</tr>
<tr>
<td>PA14 ΔbifA</td>
<td>In-frame deletion of bifA</td>
<td>(21)</td>
</tr>
<tr>
<td>PA14 pLAFR3</td>
<td>empty pLAFR3</td>
<td>(15, 16)</td>
</tr>
<tr>
<td>PA14 pMO0113050</td>
<td>pLAFR3 with psl operon</td>
<td>(15, 16)</td>
</tr>
<tr>
<td>PA01 pLAFR3</td>
<td>wild type</td>
<td>(14)</td>
</tr>
<tr>
<td>PA01 algC::tet</td>
<td>tetracycline cassette insertion in algC</td>
<td>(5)</td>
</tr>
<tr>
<td>PA01 ΔrmlC</td>
<td>In-frame deletion of rmlC</td>
<td>(29)</td>
</tr>
<tr>
<td>PA01 galU</td>
<td>Transposon aaC1 inserted in galU</td>
<td>(6)</td>
</tr>
<tr>
<td>PA01 algD</td>
<td>Transposon Tn501-31 inserted in algD</td>
<td>(11)</td>
</tr>
<tr>
<td>PA01 Δgmd</td>
<td>In-frame deletion of gmd</td>
<td>(23, 29)</td>
</tr>
<tr>
<td>PA01 Δrmd</td>
<td>In-frame deletion of rmd</td>
<td>(29)</td>
</tr>
<tr>
<td>PA01 ΔwbpW</td>
<td>In-frame deletion of wbpW</td>
<td>(33)</td>
</tr>
<tr>
<td>PA01 ΔpslA-L</td>
<td>In-frame deletions of pslA-L</td>
<td>(2)</td>
</tr>
<tr>
<td>PA01 ΔpslMNO</td>
<td>In-frame deletion of pslMNO</td>
<td>(2)</td>
</tr>
<tr>
<td>PA01 rhlC</td>
<td>Tn5 derived transposon insertion in rhlC</td>
<td>(30)</td>
</tr>
<tr>
<td>WFPA800</td>
<td>PA01 in–frame psl promoter deletion</td>
<td>(23)</td>
</tr>
<tr>
<td>WFPA801</td>
<td>PA01 with pBAD replacing psl promoter</td>
<td>(23)</td>
</tr>
<tr>
<td>PA715</td>
<td>Clinical eye isolate</td>
<td>This study</td>
</tr>
<tr>
<td>PA715 Δpsl-pr</td>
<td>In–frame psl promoter deletion</td>
<td>This study</td>
</tr>
<tr>
<td>PA738</td>
<td>Clinical eye isolate</td>
<td>This study</td>
</tr>
<tr>
<td>PA738 algC::Tn</td>
<td>algC::Mar (Mariner inserted in algC)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 1: Strains list
<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation/Deletion</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA738</td>
<td>algC::Tn + palgC</td>
<td>algC::Mar complemented with algC</td>
<td>This study</td>
</tr>
<tr>
<td>PA738 Δpsl-pr</td>
<td>In-frame deletion of psl promoter</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>ZK2870</td>
<td></td>
<td>Clinical isolate</td>
<td>(10)</td>
</tr>
<tr>
<td>ZK2870 ΔpelA</td>
<td>In-frame deletion of pelA</td>
<td></td>
<td>(10)</td>
</tr>
<tr>
<td>ZK2870 Δpsl</td>
<td>In-frame deletion mutation psl promoter</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>ZK2870 Δpel Δpsl</td>
<td>In-frame deletion mutation of pslD + pelA</td>
<td></td>
<td>(10)</td>
</tr>
<tr>
<td>MJK8</td>
<td></td>
<td>Small colony variant of PA01</td>
<td>(21)</td>
</tr>
<tr>
<td>MJK8 ΔpelA</td>
<td>pelA in-frame deletion mutation</td>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>MJK8 ΔpslBCDE</td>
<td>In-frame deletion of psl promoter</td>
<td></td>
<td>(21)</td>
</tr>
<tr>
<td>MJK8 Δpsl/pel</td>
<td>In-frame deletion of pslBCD + pelA</td>
<td></td>
<td>(38)</td>
</tr>
</tbody>
</table>
Table 2: Primers used to generate constructs.

| Primers used to generate *psl* promoter clean deletions  
<table>
<thead>
<tr>
<th>(PA715(<em>\Delta)psl-pr, PA738(</em>\Delta)psl-pr and ZK2870(_\Delta)psl-pr).</th>
<th>Replicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pMQ30(_{\text{upper-psl-pro_Fwd}})</strong></td>
<td>GACCGCTTCTGCCTGTTATTTAATCTGTATCAGGCTGACCTCTCCGGCTTGGAGGACG</td>
</tr>
<tr>
<td><strong>(\Delta\text{psl-promoter_OLR})</strong></td>
<td>GCAGTCCATTGAGTGATAGGAGAGGAGCAAGGCG</td>
</tr>
<tr>
<td><strong>(\Delta\text{psl-promoter_OLF})</strong></td>
<td>GGCACAGGCGCTGCAGGATGGCGCGCTTGGCTGCTTCTCCTATCAGCTGGAAGCCGG</td>
</tr>
<tr>
<td><strong>pMQ30(_{\text{Lower-psl-pro_Rev}})</strong></td>
<td>TTTGAGCGGATAAACATTTACACACAGGAAACAGCTATGC</td>
</tr>
</tbody>
</table>

| Primers used to generate *algC* complementation construct, *palgC* |
|-----------------------------|-------------|
| **pDPM73\(_{\text{algC cc_insert\_Fwd}}\)** | ATACCCGTTTTTTGGGCTAGCCCAAGGAAGC ACAAAGGCGACCGGCAAGCTGCTGGGAACC | 2644 bp |
| **pDPM73\(_{\text{algC cc_insert\_Rev}}\)** | TTAATCTGTATCAGGCTTATTTAACACCTCTCCATCCGCTGAGCGGCAGCGA | |

| Primers used to check plasmid insertions |
|-----------------------------|-------------|
| **pMQ30\(_{\text{insert\_check\_Fwd}}\)** | GGGGTCAAGGACGGGACCACC | 436 bp |
| **pMQ30\(_{\text{insert\_check\_Rev}}\)** | CAGGCTTTACACTTATGCTTCCGGCTC | 715 bp |
| **pDPM73\(_{\text{insert\_check\_Fwd}}\)** | GTTTTCGGGCTAGCCTAAAGGAAGC | |
| **pDPM73\(_{\text{insert\_check\_Rev}}\)** | TCTGTATCAGGCTGAAATCTCTCTCATCC | |

| Empty vector with *psl* homology fragment | |
| Empty vector Plus *algC* fragment | |
### Primers used to check *psl* promoter deletion

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>psl_promoter_check_Fwd</td>
<td>CGTCATGGAACCGCAGGCGCAT</td>
<td>788 bp</td>
</tr>
<tr>
<td>psl_promoter_check_Rev</td>
<td>GAACCAGAAGATCACCAGCTTGCTGC</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Biofilm formation of PA738 in PS80. Quantification of crystal violet staining associated with biofilm formation at 24 h of PA738, a PS80-resistant clinical isolate, PA738 algC::Tn (PA738 with the Mariner transposon inserted in algC) and PA738 algC::Tn + palgC (PA738 algC::Tn transformed with a plasmid containing the wild type algC). Black column = LB, Grey Column= LB+ 0.01% PS80. Representative wells stained with crystal violet are shown below. Values on the Y-axis result from solubilization of crystal violet with 50% acetic acid and quantified at OD550. Error bars represent standard deviation.
Figure 2: Biofilm formation in the presence of 0.1% PS80 in strains with mutations in genes functioning downstream of AlgC in polysaccharide biosynthesis.

A. Enzyme names represented in black indicates genes for which PAO1 deletion mutants were found be unable to form biofilms in PS80. Grey indicates strains in which deletion of the gene for the corresponding enzymes were able to form biofilms in PS80. Some enzymes involved in these biosynthetic pathways have been omitted from this figure, because their deletion mutants were not tested.

B. Quantification of crystal violet staining associated with a representative 24-h biofilm assay of PA14 and PAO1 as controls and PAO1 strains containing transposon insertions or deletions in genes algC, rmlC, galU, wbpW, algD and rhlC. Black column = M63, Grey Column= M63 + 0.1% PS80. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.
Figure 3. Biofilm formation in PS80 and planktonic growth of *psl* operon mutants in strains PAO1, PA715 and PA738.

A. Quantification of crystal violet staining associated with a representative 24-h biofilm assay of PA14 and PAO1 as controls and PAO1 strains containing non-polar deletions of genes in the *psl* operon. Black column = M63, Grey Column = M63 + 0.1% PS80. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.

B. Quantification of biofilm formation, representative images of biofilm wells in PA14, PA715, PA715_D *psl-pr* (PA715 with polar deletion of *psl* promoter), PA738 and PA738_D *psl-pr* (PA738 with polar deletion of *psl* promoter). Black column = M63, Grey Column = M63 + 0.1% PS80. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.

C. OD$_{600}$ of planktonic growth curve WFPA800 with and without 0.1% PS80 in LB. Error bars represent standard deviation.
Figure 4. Biofilm formation in PS80 and Psl production of WFPA801, a PAO1 strain with an arabinose inducible psl promoter.

Quantification of 24h biofilm formation and representative images of biofilm wells in PA14, PAO1, WFPA800 (PAO1 with a deletion in the psl promoter) and WFPA801 (PAO1 with an arabinose inducible psl promoter). Black column = M63, Grey Column= M63 + 0.1% PS80. Percent arabinose is indicated below wells. Dot blots of polysaccharide extracts from PAO1, WFPA800, and WFPA801 grown with the noted arabinose concentrations on nitrocellulose, probed with anti-Psl polysaccharide antibodies and detected by chemiluminescence using a HRP-conjugated secondary antibody are shown beneath the corresponding biofilm wells. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.
Figure 5. Biofilm formation of PA14 with a deletion in *bifA* in PS80. 
Quantification of crystal violet staining and representative images of biofilm 
wells associated with 24-h biofilm formation of PA14 and PA14 with a non 
polar deletion of *bifA*. Black column = M63, Grey Column = M63 + 0.1% PS80. 
Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. 
Each histogram is the mean of four replicates, and error bars indicate standard 
deviation.
Figure 6. Biofilm formation of ZK2870 and MJK8 in PS80.
Quantification of crystal violet staining associated with 24h biofilm formation of PA14, ZK2870 and MJK8 with mutations disrupting Psl or Pel biosynthesis as noted. Black column = M63, Grey Column= M63 + 0.1% PS80. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.
Figure 7. Biofilm formation in PS80 of PA14 expressing Psl. Quantification of crystal violet staining associated with 24h biofilm formation and representative well images of PAO1, PA14, pLAFR3 (PA14 containing the empty cosmid) and pMO0113050 (PA14 containing cosmid with psl operon). Underneath each well is RT PCR for pslA and rplU (as a control) to demonstrate presence or absence of expression from the psl operon. Black column = M63, Grey Column= M63 + 0.1% PS80. Paired histograms have a P-value less than 0.05, but * indicates when P>0.05. Each histogram is the mean of four replicates, and error bars indicate standard deviation.