Glycerol Metabolism Promotes Biofilm Formation by Pseudomonas aeruginosa

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Glycerol Metabolism Promotes Biofilm Formation by *Pseudomonas aeruginosa*

Jessica Scoffield\(^1\) and Laura Silo-Suh\(^2\)

Running Title: glycerol metabolism impacts biofilms

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Abstract

*Pseudomonas aeruginosa* causes persistent infections in the airways of cystic fibrosis (CF) patients. Airway sputum contains various host-derived nutrients that can be utilized by *P. aeruginosa*, including phosphotidylcholine, a major component of host cell membranes. Phosphotidylcholine can be degraded by *P. aeruginosa* to glycerol and fatty acids to increase the availability of glycerol in the CF lung. In this study, we explored the role that glycerol metabolism plays in biofilm formation by *P. aeruginosa*. We report that glycerol metabolism promotes biofilm formation by both a chronic CF (FRD1) and wound isolate (PAO1) of *P. aeruginosa*. Moreover, loss of the GlpR regulator, which represses the expression of genes involved in glycerol metabolism, enhances biofilm formation in FRD1 through the upregulation of Pel polysaccharide. Taken together, our results suggest that glycerol metabolism may be a key factor that contributes to *P. aeruginosa* persistence by promoting biofilm formation.

**Keywords:** *Pseudomonas aeruginosa*, glycerol, biofilms
Introduction

*Pseudomonas aeruginosa* is a dominant pathogen in the airways of cystic fibrosis (CF) patients (Chamber et al. 2005; Lyczak et al. 2002). The development of a chronic *P. aeruginosa* pulmonary infection typically correlates with a decline in lung function and mortality in cystic fibrosis (CF) patients (Paganin et al. 2015). In order to adapt and persist in the CF lung environment, *P. aeruginosa* alters the expression of metabolic pathways and virulence genes (Bragonzi et al. 2009; Ciofu et al. 2010; Lindsey et al. 2008), which is largely triggered by stress or nutritional conditions present in the CF lung environment. Some of these phenotypic and genetic modifications include increased antibiotic resistance, the overproduction of the exopolysaccharide alginate, and loss-of-function mutations (Bragonzi et al. 2009; Ciofu et al. 2010). The microevolution exhibited by *P. aeruginosa* CF isolates has proven to be a successful strategy to not only evade the host immune response and avoid clearance from the lung, but to also utilize readily available nutrients present in the host environment.

Airway sputum supports the growth and nutritional requirements of *P. aeruginosa* during CF infection (Palmer et al. 2005 and 2007). CF sputum is comprised of both host-derived and bacterial related products, including the major lung surfactant, phosphatidylcholine (PC) (Bernhard et al. 1997). Phospholipase C and lipases produced by *P. aeruginosa* cleave phosphatidylcholine into phosphorylcholine, fatty acids, and glycerol, and as a result, liberated glycerol can be used as a potential nutritional source for *P. aeruginosa* (Son et al. 2007). Moreover, studies have demonstrated that the *glp* regulon [glycerol uptake facilitator gene (*glpF*), regulator gene (*glpR*), glycerol kinase (*glpK*), glycerol-3-phosphate dehydrogenase (*glpD*) and the glycerol-3-phosphate transporter gene (*glpT*)] (Schweizer et al. 1996 and 1997) is constitutively expressed in some *P.*
aeruginosa CF isolates and is required for in vivo degradation of PC (Son et al. 2007). Furthermore, several studies have suggested that PC may support high cell density replication and maintenance of P. aeruginosa within the CF airway (Son et al. 2007; Sun et al. 2014). Our laboratory previously reported that the chronic CF isolate, FRD1, has a growth advantage on glycerol compared to the acute isolate, PAO1. In addition, we demonstrated that glycerol-3-phosphate (g/lpD) is required for the production of some P. aeruginosa virulence factors, including alginate (Daniels et al. 2014), which has been shown to play an important role in biofilm architecture and protection from antimicrobials (Hentzer et al. 2001). Not surprisingly, 80% of chronic infections are associated with microbial biofilm formation (Costerton et al. 1999). Due to the established importance of glycerol metabolism for P. aeruginosa nutrition and virulence, we sought to explore the role glycerol metabolism has on biofilm formation, which is a critical virulence strategy for many recalcitrant pathogens (Bjarnsholt 2013).

Materials and Methods

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were cultured in L-broth (LB) or on L-agar and grown at 37°C. Glycerol, glucose, and succinate were used at a concentration of 20 mM. A 1:1 mixture of L-agar and Pseudomonas Isolation Agar (PIA) was used to select for P. aeruginosa transconjugants and to counter select for E. coli following triparental mating. Media were solidified with 1.5% (w/v) Bacto Agar (Difco). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations in this study: 100 µg ampicillin mL^{-1} for E. coli; 100 µg carbenicillin mL^{-1} for P. aeruginosa; 20 µg gentamicin mL^{-1} for E. coli and 180 µg for P. aeruginosa; and 50 µg kanamycin mL^{-1} for E. coli and 1000 µg mL^{-1} for P. aeruginosa.
DNA manipulations, transformations, and conjugations. *E. coli* strain DH10B was routinely used as a host strain for cloning. DNA was introduced into *E. coli* by electroporation and into *P. aeruginosa* by conjugation as previously described (Suh et al. 2004). Plasmids were purified with QIAprep Spin Miniprep columns (Qiagen, Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Either *Pfu* from Stratagene (La Jolla, CA) or *Taq* from New England Biolabs were used for PCR amplification of DNA. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

**Construction of *P. aeruginosa* glpR mutants.** To generate glpR mutants of *P. aeruginosa*, the suicide plasmid pLS1954 was constructed: a DNA sequence containing approximately 400 bp upstream and 430 bp downstream of the *glpR* coding sequence was PCR amplified from PAO1 cells with *Pfu* and cloned into the *Sma*I site of pBluescript K(+) . The resulting plasmid was digested with *Sph*I and the internal 1.3 kb fragment of the *glpR* coding sequence was removed and replaced with the *aacC1* gene encoding gentamicin resistance as a *Sma*I fragment (Schweizer 1993). This was followed by introduction of an origin of transfer (*moriT*) of RP4 on a 230 bp *Hind*III fragment (Suh et al. 2004). pLS1954 was introduced into *P. aeruginosa* strains FRD1 and PAO1 by triparental mating, and potential *glpR* mutants were isolated as gentamicin-resistant, carbenicillin-sensitive colonies, indicating a double crossover event. Replacement of the wild-type *glpR* gene with the *glpR*101::*aacC1* allele was verified by PCR analysis.

**Construction of the glpR complemented strains.** To complement the *glpR* mutation, a PCR product containing the *glpR* coding region was cloned between the *Nco*I/EagI sites of the expression vector pJSGLP. The resulting plasmid was digested with *Hind*III and the *moriT* was inserted to
allow for mobilization of the plasmid into *P. aeruginosa*. The plasmid was mobilized into *P. aeruginosa* by triparental mating and potential complemented strains were isolated as kanamycin resistant colonies. *In cis* complementation was verified by PCR analysis.

**Construction of the mucA complemented strains.** To complement the *P. aeruginosa* FRD1 strain with *mucA*, the wild-type gene was PCR amplified from PAO1 using *Pfu*. The resulting fragment was cloned into the *SmaI* site of a plasmid which contains a regulatable promoter upstream of the multiple cloning site (Silo-Suh et al. 2005). The resulting plasmid was digested with *Hind*III and the *oriT* was inserted to allow for mobilization of the plasmid into *P. aeruginosa*. The plasmid (pJSM1) was mobilized into FRD1 by triparental mating and potential complemented strains were isolated as carbenicillin resistant colonies. *In cis* complementation was verified by PCR analysis.

**Biofilm assays.** Overnight cultures were sub-cultured in fresh L-broth and grown to an OD$_{600}$ of 0.5 and diluted at a ratio of 1:100 in 1X M9 salts supplemented with either 20 mM glucose, glycerol, or succinate. Biofilms were grown statically for 18 hours in 96-well polystyrene plates or in borosilicate glass. To measure biomass the planktonic cells were gently washed off and the biofilm was stained with 0.1% crystal violet and solubilized in 30% acetic acid. Biofilm biomass was measured at OD$_{562}$ using a BioTek® microplate reader and biofilm formation was calculated by normalizing for bacterial growth using OD$_{562/600}$.

**Biochemical Assays.** Pel production was assessed on L-agar plates without NaCl that contained 40 µg/mL Congo red and 15 µg/mL brilliant blue R (Sigma-Aldrich). Overnight cultures of *P. aeruginosa* cells were sub-cultured and grown to exponential phase and diluted 1:100. Ten microliters of each culture was spotted on the plate and incubated at 30°C overnight. For the alginate assays, bacteria were grown in 20 mM glycerol. Alginate was isolated from *P. aeruginosa* culture supernatants that were dialyzed against distilled water as previously
described (Suh et al. 1999), and the alginate level (i.e. uronic acid) was quantified by the carbazole method (Knutson and Jeanes 1968) using *Macrocystis pyrifera* alginate (Sigma-Aldrich) as a standard.

**Quantitative Real Time PCR.** RNA was extracted from late exponential phase cultures grown in L-broth using the Direct-zol kit (Zymo Research, Irvine, CA). Residual DNA was digested using RQ1 DNase (Promega, Madison, WI). RNA was purified with the mini-RNAeasy kit (Qiagen, Venlo, Limberg), and converted into cDNA using the iScript cDNA Synthesis kit (Bio-rad, Hercules, CA). cDNA was then used for qRT-PCR with iQ SYBR Green Supermix (Bio-rad). The qRT-PCR primer sequences are listed in Table 2.

**Results**

**Growth on glycerol and deregulation of glycerol metabolism promotes biofilm formation by FRD1**

We previously reported that the *P. aeruginosa* CF isolate, FRD1, displays a growth advantage when grown on glycerol compared to the wound isolate, PAO1 (Daniels et al. 2014). Moreover, we demonstrated that glycerol-3-phosphate dehydrogenase, *(glpD)*, is required for the optimal production of key *P. aeruginosa* virulence factors, including alginate production by FRD1, suggesting that glycerol metabolism may be an important factor that mediates persistence by some chronic isolates of *P. aeruginosa*. As a follow up to these results, we tested the role glycerol metabolism plays in biofilm formation by *P. aeruginosa*. As shown in Figs. 1A and 1B, growth on glycerol enhanced biofilm formation by FRD1 and PAO1 compared to growth on glucose or succinate. In *P. aeruginosa*, glycerol metabolism is under the control of GlpR, a transcriptional regulator that represses expression of the *glp* regulon in the absence of glycerol. Due to an increase
in biofilm formation by *P. aeruginosa* when grown on glycerol we reasoned that a mutation in *glpR*, which results in constitutive expression of the *glp* genes, would also promote biofilm formation. To this end, we tested biofilm formation by *glpR* mutants in both the FRD1 and PAO1 backgrounds. In FRD1, loss of *glpR* resulted in an a four-fold increase in biofilm formation when grown on glucose and succinate, and a five-fold increase when grown on glycerol compared to wild-type, however, biofilm formation was not increased in the PAO1 *glpR* mutant compared to wild-type PAO1 (Fig. 1A). Taken together, these data suggest that biofilm formation is differentially modulated in response to glycerol or loss of *glpR* in FRD1 and PAO1.

**Alginate production represses the promotion of biofilm formation by glycerol**

Unlike PAO1, the chronic CF isolate, FRD1, displays a mucoid phenotype due to an overproduction of the exopolysaccharide alginate, which is the result of a mutation in *mucA*. Alginate has been shown to delay attachment of *P. aeruginosa* alginate overproducers to abiotic surfaces and to alter biofilm structure (Hentzer et al. 2001). Although the FRD1 *glpR* mutant appears mucoid on L-agar plates, we analyzed whether alginate production was altered in this isolate. As shown in Figure 2A, the FRD1 *glpR* mutant is significantly reduced for alginate production compared to wild-type FRD1 when grown in the presence of glycerol. For comparison, we included the FRD1 *mucA* complemented strain, which is non-mucoid due to the lack of alginate production. This result suggests that alginate does not contribute to the promotion of biofilm formation in the FRD1 *glpR* mutant. We also examined biofilm formation by the FRD1 *mucA* complemented strain and a PAO1 derivative that overproduces alginate, PDO300 (PAO1 with a *mucA22* mutation). As expected, the production of alginate in the presence of glycerol correlated with reduced biofilm formation in both the chronic and acute isolates of *P. aeruginosa* (Fig. 2B).
Loss of GlpR increases *pelA* expression and Pel production in the CF isolate FRD1

Alginate, Pel, and Psl are polysaccharides produced by *P. aeruginosa* that have been shown to play varying roles in biofilm development (Ghafoor et al. 2011). In an effort to elucidate the mechanism involved in increased biofilm formation by the FRD1 *glpR* mutant, we analyzed the expression of *algD*, *pelA*, and *pslA*, which are essential for the biosynthesis of alginate, Pel, and Psl, respectively. As shown in Fig. 3, *algD* expression was slightly reduced in the FRD1 *glpR* mutant compared to wild-type FRD1, which provides an explanation for the reduced alginate production by this mutant. There was no change in *pslA* expression in the FRD1 *glpR* mutant, whereas *pelA* expression increased ten-fold in the FRD1 *glpR* mutant compared to wild-type FRD1. In contrast, *pelA* and *pslA* expression decreased in the PAO1 *glpR* mutant compared to wild-type PAO1. These results support our earlier observation that biofilm formation is differentially modulated in FRD1 and PAO1.

The high expression of *pelA* in the FRD1 *glpR* mutant suggests that Pel is produced in abundance in this strain. To test this hypothesis, we performed a Pel binding experiment using the Congo Red assay, which is routinely used to measure Pel production (Colvin et al. 2013; Ghafoor et al. 2011). The FRD1 *glpR* mutant displayed more Congo Red binding compared to wild-type FRD1 and the *glpR* complemented strain. However, there was no phenotypic difference in Congo red binding between PAO1, the PAO1 *glpR* mutant, and complemented strains (Fig. 4). In summary, our data demonstrate that glycerol metabolism in the chronic CF isolate, FRD1, may play a significant role in modulating the production of polysaccharides involved in biofilm formation, particularly Pel.
**Discussion**

*P. aeruginosa* genes that are devoted to glycerol metabolism have been shown to be constitutively expressed during chronic CF infection (Son et al. 2007), suggesting that glycerol may be an important nutritional source present during CF pulmonary infections and could possibly contribute to *P. aeruginosa* persistence. In this study, we sought to clarify the role that glycerol metabolism plays in the ability of *P. aeruginosa* to form a biofilm. We demonstrate that growth on glycerol promotes biofilm formation by both a chronic CF (FRD1) and wound isolate (PAO1) of *P. aeruginosa*. In addition, loss of the glycerol repressor, GlpR, results in the upregulation of Pel polysaccharide in FRD1, which may be the mechanism by which biofilm formation is promoted in this background. Taken together, our results show that glycerol metabolism may support the maintenance of *P. aeruginosa* chronic infections, and secondly, chronic and acute isolates may harbor distinct mechanisms that regulate biofilm formation in response to nutritional cues.

Glycerol metabolism plays an important role in biofilm formation by other bacteria. For example, glycerol and manganese enhance biofilm formation by *Bacillus subtilis* through histidine kinase signaling (Shemesh et al. 2013). In addition, glycerol biosynthetic genes are upregulated during the biofilm mode of growth in *Candida albicans*, and furthermore, mutations in these genes result in reduced adherence by *C. albicans* (Desai et al. 2013). Moreover, in *Salmonella enterica serovar* Typhi, biofilm cells catabolize glycerol more readily compared to cells growing in a planktonic state (Kalai et al. 2015). Glycerol metabolism has also been shown to directly impact bacterial virulence and fitness. For example, *P. aeruginosa* mutants defective in glycerol degradation display a reduction in competitive fitness in a mouse lung infection model (Sun et al. 2014), suggesting that glycerol acquisition may be important for pathogenesis during lung infection.
In *Mycoplasma pneumoniae*, GlpD (glycerol 3-phosphate dehydrogenase) and GlpQ (glycerophosphodiesterase) are required for cytotoxicity (Hames et al. 2009; Schmidl et al. 2011). Additionally, GlpD is required for the production of hydrogen peroxide, which is the major virulence factor in *M. pneumoniae* (Hames et al. 2009). Altogether, these studies highlight the importance of glycerol metabolism for bacterial pathogenesis among diverse microbes.

Extracellular polysaccharides are a major component of the biofilm matrix produced by *P. aeruginosa*. The biosynthetic pathways for these polysaccharides compete for common carbon precursors and high production of one type of polysaccharide often limits production of the others (Ma et al. 2012). Alginate appears to be the predominant exopolysaccharide in biofilms formed by mucoid isolates of *P. aeruginosa*, and Pel and Psl are the dominant polysaccharides for non-mucoid isolates (Colvin et al. 2012; Hentzer et al. 2001; Wozniak et al. 2003). While PAO1 predominantly utilizes Psl for the biofilm matrix, upregulation of Pel biosynthesis can compensate for the loss of Psl (Ghafoor et al. 2011), indicating that a back-up mechanism for biofilm matrix formation exists in this pathogen. In our study, loss of the GlpR repressor resulted in reduced alginate production and overproduction of Pel polysaccharide by the chronic CF isolate FRD1, but not by the wound isolate PAO1. Taken together, Pel appears to be the back-up polysaccharide for FRD1 during biofilm formation. Interestingly, overproduction of Pel enhances the resistance of PAO1 biofilms to aminoglycoside antibiotics (Colvin et al. 2011). Therefore, it is likely that glycerol metabolism may support the persistence of *P. aeruginosa* isolates similar to FRD1 by promoting biofilm formation through the upregulation of Pel, which could also render CF isolates more resistant to antimicrobials.

In summary, the correlation that exists between carbon metabolism and virulence suggests that bacteria rely heavily on central catabolic genes and these genes may be beneficial for the colonization or adaptation of *P. aeruginosa* in various niches, particularly during chronic infection.
In particular, glycerol, which is a host derived nutrient within the CF lung, may signal specific bacterial responses that promote colonization in vivo through the production of polysaccharides that enhance biofilm formation. Moreover, the development of therapeutics that interfere with genes related to glycerol metabolism could prove to be an effective method to reduce P. aeruginosa virulence.

Acknowledgements

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Figure Legends

**Figure 1. Growth on glycerol promotes biofilm formation by PAO1 and FRD1.** A. Normalized biofilm formation by FRD1, PAO1, and glpR mutants when grown in 1X M9 salts supplemented with either 20mM of glucose, glycerol, or succinate. B. Crystal violet staining of biofilms in borosilicate glass. The data represent the means (with standard deviations) of 3 independent experiments conducted in triplicate.

**Figure 2. The effect of alginate production on biofilm formation.** A. Alginate production by FRD1, FRD1 complemented with mucA, and the FRD1 glpR mutant during growth on M9 salts (1X) supplemented with 20mM glycerol. The data represent the means (with standard deviations) of 3 independent experiments conducted in triplicate. B. Normalized biofilm formation by FRD1, FRD1 complemented with mucA, PAO1, and the PDO300 mutant. The data represent the means (with standard deviations) of 3 independent experiments conducted in triplicate.

**Figure 3. Loss of GlpR increases pelA expression in the CF isolate FRD1.** qRT-PCR expression of algD, pelA, and pslA in FRD1, PAO1, and derivatives. Data represent the means (with standard deviations) of 3 independent experiments conducted in triplicate.

**Figure 4. Loss of GlpR increases Pel production in the CF isolate FRD1.** Congo red binding assay of FRD1, PAO1, and derivatives.
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190x254mm (96 x 96 DPI)
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