GlpR Regulates the Glyoxylate Pathway and Virulence Factor Production by *Pseudomonas aeruginosa*

by

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Abstract

Pseudomonas aeruginosa infections are the leading cause of morbidity and mortality for cystic fibrosis (CF) patients. *P. aeruginosa* establishes life-long infection in the CF lung by utilizing various adaptation strategies to cause a chronic infection including alterations in central metabolic activities. The glyoxylate pathway is utilized by bacteria to grow on acetate or fatty acids as a sole carbon source to replenish intermediates of the tricarboxylic acid cycle and it appears to play a role in *P. aeruginosa* persistence in the CF lung. Son *et. al.* (2007) demonstrated that the genes encoding for the glyoxylate pathway enzymes, *aceA* and *glcB* which encode for isocitrate lyase (ICL) and malate synthase respectively, are upregulated in *P. aeruginosa* growing in the CF lung. In addition, we determined that this pathway becomes permanently upregulated in some CF adapted isolates of *P. aeruginosa* (Hagins *et. al.* 2010, 2011; Lindsey *et. al.* 2008). The occurrence of these isolates suggests deregulation of the glyoxylate pathway may benefit *P. aeruginosa* growing within the CF lung. However, the mechanism(s) responsible for alterations in the pathway have yet to be elucidated. GlpR is a transcriptional repressor that regulates the genes responsible for glycerol metabolism in *P. aeruginosa.* I demonstrate in this body of work that GlpR also regulates the glyoxylate pathway. To date, regulators of the glyoxylate pathway in *P. aeruginosa* have not been identified with the exception of RpoN, which plays an indirect role in the regulation of this pathway. GlpR's role in the regulation of the glyoxylate pathway provides a novel perspective into the interplay between fatty acid and glycerol metabolism in *P. aeruginosa*. Finally, I show that glycerol metabolism is

altered in a CF adapted isolate of *P. aeruginosa* compared to an acute isolate and that production of alginate is influenced by growth on glycerol. Alginate is an important virulence determinant produced by *P. aeruginosa* during infection of the CF lung. These results suggest the carbon sources present in the CF infection environment impact virulence factor production by *P. aeruginosa*.

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Chapter 1

Literature Review

Introduction

Some bacterial pathogens are able to establish life-long chronic infections subsequent to an initial acute infection. The ability of many bacterial pathogens to survive during chronic infections is contingent upon their ability to adapt to the environment within the host. Bacteria capable of establishing persistent infections have developed unique strategies that allow them to overcome environmental stress caused by antibiotics, the immune response, limited nutrients, or oxygen availability. The mechanisms used by bacteria to cause chronic infections differ from those used during acute infections, however little is known about the mechanisms used by bacteria to persist during long-term infections. In order to treat chronic infections we need a better understanding of how persistent infections are established and maintained by these organisms.

Pseudomonas aeruginosa is a bacterium that is notorious for establishing decade long infections in cystic fibrosis (CF) patients and it is also an important component of some persistent wound infections. Therefore, *P. aeruginosa* can establish persistent infections at multiple infection sites and it appears to utilize multiple strategies for this process (Kukavica-Ibrulj *et. al.* 2008; Mathee *et. al.* 2008). Hence, *P. aeruginosa* is a good model system for studying chronic infection mechanisms. In addition, it is easy to grow and to genetically

manipulate in the laboratory. Our overall goal is to use *P. aeruginosa* as a model system to identify global bacterial persistent strategies. One strategy that may be used by *P. aeruginosa* to persist during chronic infection is upregulation of the glyoxylate pathway. This pathway is required by many microorganisms for pathogenesis and is also required for some bacteria to cause persistent infections. It has been implicated in the ability of *P. aeruginosa* to cause persistent infections, and therefore, is the focus of my dissertation research. My initial studies centered on understanding regulation of the glyoxylate pathway by the alternative sigma factor RpoN (Hagins 2009, 2011). However, the bulk of my dissertation is characterization of GlpR as an additional regulator of the glyoxylate pathway. GlpR is a transcriptional regulator that represses the genes responsible for glycerol metabolism in *P. aeruginosa.* During the course of my studies, I discovered that some CF isolates, including the paradigm of chronic CF isolate FRD1, are able to utilize glycerol more efficiently as a carbon source than non-CF isolates. This suggests that this phenotype evolved in the CF lung and may provide some benefit to *P. aeruginosa*. Glycerol in the CF lung is likely derived from hydrolysis of host cell membranes by *P. aeruginosa* produced phopholipases that liberates fatty acids and glycerol from phopholipids. The concomitant availability of both fatty acids and glycerol in the CF lung suggests that *P. aeruginosa* may regulate metabolism of both carbon sources in a coordinate manner. The specific goals of this study were to determine whether GlpR regulates the glyoxylate pathway in *P. aeruginosa* and characterize glycerol utilization by both CF and non-CF isolates of *P. aeruginosa.*

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a facultative anaerobic, gram negative, rod-shaped bacterium that is ubiquitously distributed in the environment (Lee *et. al.* 2006; Stover *et. al.* 2000). The

genome of *P. aeruginosa* contains a large number of genes devoted to regulation, catabolism, transport, and efflux of organic compounds which enable this bacterium to quickly respond and adapt to a wide range of environmental conditions (Lee *et. al.* 2006; Stover *et. al.* 2000). *P. aeruginosa* is commonly found in soil and water but has a broad environmental range. Its diverse repertoire of genes involved in metabolism gives it the flexibility to colonize and infect different hosts, including plants, insects, nematodes, fungi, and animals (Kukavica-Ibruli *et. al.* 2008). In humans, *P. aeruginosa* is an opportunistic pathogen and is the leading gram-negative bacterial cause of nosocomial infections (Kukavica-Ibruli *et. al.* 2008). *P. aeruginosa* causes many acute infections, including nosocomial pneumonia, respiratory infections, urinary tract infections from catheter use, infections in burn patients, and is the dominant cause of microbial keratitis (Twining *et. al.* 1993; Willcox *et. al.* 2008). *P. aeruginosa* is also responsible for causing devastating, chronic respiratory infections in cystic fibrosis (CF) patients. One reason *P. aeruginosa* has become an incredibly successful pathogen is because it is equipped with an arsenal of both cell-associated and extracellular virulence factors (Lee *et. al.* 2006).

Cell-associated virulence factors include fimbriae, flagellae, lipopolysaccharide, a type III secretion system, and alginate (Choi *et. al*. 2002). Exoproducts such as exotoxin A, exoenzyme S, elastase, and alkaline protease production have been shown to be important in establishing *P. aeruginosa*-associated infections (Delden and Iglewski, 1998). These exoproducts degrade complement components and interfere with other innate defenses like interleukin 1 and 2, natural killer cells, polymorphonuclear leukocyte chemotaxis, and tumor necrosis factor (Twining *et. al.* 1993). Exoenzyme S, a cytotoxin secreted by *P. aeruginosa*'s type III secretion system, contributes to tissue damage, and exoenzyme A inhibits protein synthesis by inhibiting elongation factor 2 (Sadikot *et. al.* 2004; Twining *et. al.* 1993).

P. aeruginosa also produces several diffusible pigments: pyocyanin, pyoverdine, and pyochelin (Govan *et. al.* 1996; Sokol and Woods, 1998). During infection iron can be a limiting factor for bacteria (Sadikot *et. al.* 2004). Pyochelin and pyoverdine are siderophores that acquire iron from the host and transport it to the bacteria (Sadikot *et. al.* 2004). Pyocyanin is a blue-green, redox-active phenazine compound produced by *P. aeruginosa* (Fothergill, 2007; Lau, 2004). This pigment generates reactive oxygen species and induces apoptosis of neutrophils in host cells (Fothergill, 2007; Lau, 2004). In mice, pyocyanin mediates damage and necrosis in lung epithelial tissue and is required for airway infection (Lau, 2004). All of the virulence factors produced by

P. aeruginosa contribute to establishing infection at one or more sites (Kukavica-Ibrulj *et. al.* 2008; Lindsey *et. al.* 2008; and Mathee *et. al.* 2008). Some of these defense strategies allow *P. aeruginosa* to cause life threatening infections in cystic fibrosis patients and allow this organism to evade clearance by the host immune system, resist treatment with antibiotics, and persist in the lung during the patient's lifetime.

Cystic fibrosis

Cystic fibrosis (CF) is an inherited, multi-system, autosomal recessive disorder. Most cases of CF are caused by a deletion of phenylalanine at position 508 (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Welsh *et. al.* 1996 and 2001). Normally, CFTR functions in the transport of small molecules in epithelial cells (Welsh *et. al.* 1996 and 2001). Defective transport of small molecules caused by altered CFTRs produces multiple symptoms in CF patients including salty sweat, pancreatic insufficiency, intestinal obstruction, male infertility and severe pulmonary disease (Welsh *et. al.* 1996 and 2001).

Consequently CF life expectancy is limited due to a progressive loss of lung function caused by chronic microbial colonization.

Microbial colonization of the CF lung

During early childhood most CF patients are colonized by a mixture of microbes, including various bacterial pathogens (Lyczak 2002). Defects in the CFTR protein inhibit proper mucociliary clearance of microbes from lung, and as a result, these organisms are able to colonize and establish infections within the lung (Lyczak 2002). Some of the microbes persist in the CF lung throughout the patient's entire life (Kukavica *et. al.* 2008; Mahenthiralingam *et. al.* 1996; Smith *et. al.* 2006). The most common microbes detected during early infection of the CF lung include *Burkholderia cepacia*, *Staphylococcus aureus*, *Haemophilus influenza*, and *Streptococcus pneumonia*. Co-infections from two or more bacterial species are extremely common in the CF lung (Gilligan 1991; Harrison 2007). The CF lung represents a diverse community of bacterial species that coexist and interact both synergistically and antagonistically (Gilligan, 1991; Harrison, 2007). Aggressive antibiotic therapy has been effective in clearing or delaying some of these infections. However, most CF patients are eventually colonized with recalcitrant variants of *P. aeruginosa*.

P. aeruginosa and cystic fibrosis

P. aeruginosa is the most common and clinically important pathogen in patients with CF. Chronic lung infections caused by *P. aeruginosa* are the leading cause of lung deterioration and mortality for cystic fibrosis (CF) patients (Chamber *et. al.* 2005). *P. aeruginosa* is acquired from the environment and is maintained permanently within the CF lung. During colonization, *P. aeruginosa* converts to the mucoid phenotype, which is caused by the overproduction of the

exopolysaccahride alginate (Smith *et. al.* 2006). The mucoid phenotype contributes to the formation of biofilms by *P. aeruginosa*, prevents the penetration of antibiotics, inhibits phagocytosis and inhibits the activation of complement (Govan and Deretic, 1996). As a result, *P. aeruginosa* is able to resist clearing by therapeutic measures and the host immune system (Govan and Deretic, 1996; Kukavica *et. al.* 2008; Mahenthiralingam *et. al.* 1996; Smith *et. al.* 2006). Eventually, copious amount of alginate accumulate in CF sputum, which contributes to airway blockage, chronic lung infections and a decline of pulmonary functions (Kukavica *et. al.* 2008; Mahenthiralingam *et. al.* 1996; Smith *et. al.* 2006). Unfortunately, an effective *P. aeruginosa* vaccine is currently unavailable.

Adaptations of *P. aeruginosa* to the CF lung

Very few studies have addressed the microevolution of *P. aeruginosa* within the CF lung and the mechanisms it uses to persist in this environment. The study of *P. aeruginosa* is complicated by genotypic alterations this organism undergoes over the course of chronic infection in the lungs of CF individuals (Mena *et. al.* 2008). The harsh environment of the CF lung triggers mutations in the *P. aeruginosa* genome and selects for variants that are better equipped to survive (Ciofu *et. al.* 2010; D'Argenio *et. al.* 2007; Hoffman *et. al.* 2010). Consequently, the *P. aeruginosa* population over time differs genotypically and phenotypically from the initial infecting strain (Kukavica *et. al.* 2008; Mahenthiralingam *et. al.* 1996; Smith *et. al.* 2006). *P. aeruginosa* strains isolated during the course of chronic lung infection in patients with CF are altered for a variety of phenotypes including motility, acquisition of the mucoid phenotype, reduced virulence, antibiotic resistance, and mutations in *lasR,* a major transcriptional activator of virulence genes and biofilm formation (Bragonzi et. al. 2009; Hogardt and Hessemann, 2010; Mena *et. al.* 2008; Oliver, 2011; Smith *et. al.* 2006). One of the most

significant alterations that occur is the conversion to the mucoid phenotype which results from the overproduction of alginate (Schobert and Jahn, 2006; Xie *et al.* 1996). Mucoid strains of *P. aeruginosa* assist *P. aeruginosa* in improved biofilm formation in the CF lung (Stapper *et. al.* 2004). The biofilm communities found within the lung have increased resistance to antimicrobials (Spoering and Lewis, 2004). Hydrogen cyanide has also been detected in CF sputum where *P. aeruginosa* bacterial communities reside (Ryall *et. al.* 2008). Hydrogen cyanide is a poisonous gas that interferes with cellular respiration (Ryall *et. al.* 2008). Mucoid strains of *P. aerugionsa* have been shown to upregulate genes responsible for hydrogen cyanide and produce seven-fold more hydrogen cyanide than nonmucoid strains (Carterson *et. al.* 2004; Firoved and Deretic, 2003). This suggests that hydrogen cyanide is an important virulence factor for *P. aeruginosa* growing within the CF lung. Many of the phenotypic variations detected in CF *P. aeruginosa* are the result of hypermutations within the genome. The high mutation rate is largely caused by oxidative stress encountered by *P. aeruginosa* during chronic CF infection (Ciofu *et. al.* 2005). Hypermutable variants selected for within in the lung are thought to play a role in supporting persistence and antibiotic resistance (Mena *et. al.* 2008; Oliver *et. al.* 2000). *P. aeruginosa* also undergoes extensive metabolic changes during chronic respiratory infection that can include the constitutive expression or deregulation of genes responsible for carbon catabolism. Amino acid auxotrophs of *P. aeruginosa* commonly arise in the lung (Barth and Pitt, 1995). The selection for auxotrophic variants suggests that some amino acid biosynthetic genes may no longer be required because amino acids are freely available in the lung and appear to be a key carbon source for the bacterium (Barth and Pitt, 1996). The loss of several other regulatory mechanisms has been reported in chronic isolates. These alterations can include the upregulation of *zwf*, which encodes for glucose-6-phosphate dehydrogenase and is required for optimal

alginate production (Silo-Suh *et. al.* 2005). High expression of *zwf* could possibly be important for the survival of *P. aeruginosa* growing within the CF lung (Silo-Suh *et. al.* 2005). A transcriptome study by Son *et. al.* in 2007 revealed that some of the genes constitutively expressed during chronic infection are involved in central metabolic pathways including fatty acid and amino acid degradation and glycerol metabolism (Son *et. al.* 2007). Constitutive expression of these genes is likely due to the high availability of fatty acids and amino acids present in the lung which can serve as nutrients to fuel *P. aeruginosa* growth (Son *et. al.* 2007). Carbon sources utilized by *P. aeruginosa* during CF infection include lipids obtained from lung surfactant, fatty and amino acids (Son *et. al.* 2007). Long chain fatty acids are generated when lipases hydrolyze phosphatidylcholine (PC) from host lipid membranes and are eventually metabolized via β-oxidation. As a result, these fatty acids can become one the most abundant nutrients in the CF lung. Two genes that are induced by the presence of fatty acids and are constitutively upregulated in CF *P. aeruginosa* are *aceA* and *glcB*. These two genes are unique to the glyoxylate pathway and they encode for isocitrate lyase (ICL) and malate synthase (MS), respectively (Hagins *et. al.* 2010, 2011).

The glyoxylate pathway

The glyoxylate shunt is an anabolic pathway of the tricarboxylic acid (TCA) cycle that allows for growth on C_2 compounds by bypassing the CO_2-generating steps of the TCA cycle (Figure 1) (Dunn *et. al.* 2009). The first unique step of the glyoxylate cycle is catalyzed by ICL and involves the cleavage of isocitrate to glyoxylate and succinate (Dunn *et. al.* 2009; Gui *et. al.* 1996). During the next step, glyoxylate condenses with acetyl-CoA to form malate. This conversion is catalyzed by MS. The end result of the glyoxylate pathway is the production of

TCA cycle intermediates that can be used for gluconeogenesis or other biosynthetic reactions (Dunn *et. al.* 2009; Gui *et. al.* 1996).

The glyoxylate pathway is induced by growth on acetate or fatty acids that are degraded by β-oxidation (Dunn *et. al.* 2009; Gui *et. al.* 1996). Isocitrate lyase and isocitrate dehydrogenase utilize the same substrate, isocitrate. When TCA cycle intermediates are present in the growth medium, isocitrate dehydrogenase becomes dephosphorylated and isocitrate is directed towards the TCA cycle (Dunn *et. al.* 2009; Gui *et. al.* 1996). When acetate or fatty acids are present in high concentrations in the growth medium, isocitrate dehydrogenase is inactivated by phosphorylation and the glyoxylate pathway is induced (Dunn *et. al.* 2009; Gui *et. al.* 1996). The glyoxylate pathway occurs in many microorganisms and plants, but is absent in humans, which makes it an attractive target for therapy (Dunn *et. al.* 2009; Gui *et. al.* 1996; Hagins *et. al.* 2010 and 2011). Understanding the mechanisms by which pathogens use ICL and MS to infect a host will provide better insight on the role these enzymes play in pathogenesis.

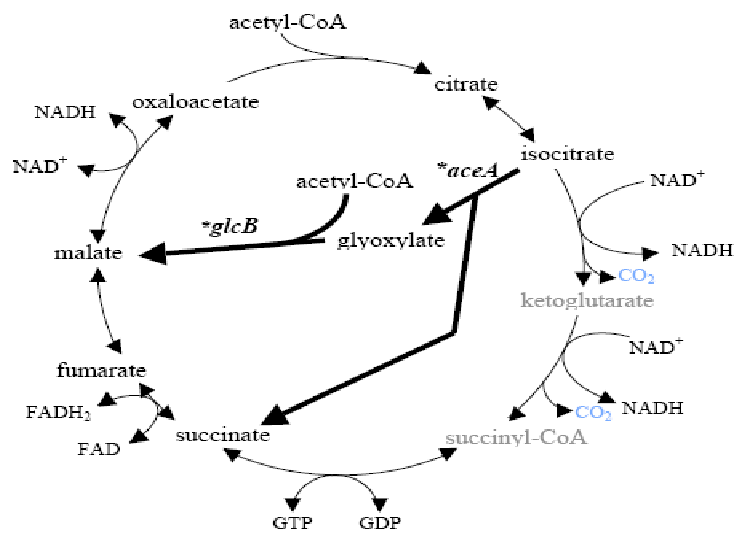

Figure 1.1 The Glyoxylate Pathway is marked by bold arrows

The role of the glyoxylate pathway in pathogenesis.

The glyoxylate pathway is required by diverse microbes to cause disease in a variety of hosts (Dunn *et. al.* 2009). Mutations in genes that encode for isocitrate lyase and malate synthase lead to reduced virulence in microorganisms such as *P. aeruginosa, Mycobacterium tuberculosis*, *Cryptococcus neoformans, Rhodococcus equi, Salmonella enterica, and Candida albicans* (Cramer *et. al.* 2007; Dunn *et. al.* 2009; Hagins *et. al.* 2010; Lindsey *et. al.* 2008; Munoz-Elias *et. al.* 2005; Wall *et. al.* 2005). More importantly, bacterial pathogens including *M. tuberculosis*, *Salmonella enterica* serovar Typhimurium and *Burkholderia pseudomallei* also rely on the glyoxylate pathway for persistence in animal models of infection (Fang *et. al.* 2005; McKinney *et. al*. 2000; Schaik *et. al.* 2009). In *B. pseudomallei*, mutations in *aceA* prevent the bacterium from entering the persistent mode of infection and force it to remain in an acute state, which is much easier to treat with antibiotics (Schaik *et. al.* 2009). Furthermore, in *M. tuberculosis*, loss of *aceA* results in the build-up of a toxic intermediate, which is lethal to the bacterium (McKinney *et. al.* 2000).

We previously showed that isocitrate lyase (ICL) is required for an acute isolate of *P. aeruginosa* to cause disease in the rat lung model of infection (Lindsey *et. al.* 2008). Moreover, ICL is required for optimal production of two important virulence determinants, hydrogen cyanide and alginate, by a chronic isolate of *P. aeruginosa* (Hagins *et. al.* 2009; Lindsey *et. al.* 2008). These data suggest that *P. aeruginosa* benefits from high ICL activity in the CF lung either by utilization of certain compounds as carbon sources or for optimal production of virulence determinants.

Regulation of *aceA* and *glcB* in *P. aeruginosa.*

Few studies have examined the regulation of *aceA* and *glcB* in *P. aeruginosa.* Based on observations from other bacteria, regulators of *aceA* and *glcB* in *P. aeruginosa* are predicted to be encoded by genes that respond to acetate, fatty acids and possibly amino acids in the growth medium. In *Corynebacterium glutamicum, aceA* is positively and negatively regulated by RamA and RamB, respectively, and is dependent on acetate availability (Cramer *et. al.* 2007). In *Escherichia coli, aceA* is negatively regulated by FadR and IclR and responds to the presence of acetate or fatty acids (Gui *et. al.* 1996). However, these genes do not regulate *aceA* or *glcB* in *P. aeruginosa* (Hagins *et. al.* 2010). We previously demonstrated that in an acute isolate of *P. aeruginosa*, *aceA* and *glcB* are negatively regulated by RpoN (Hagins *et. al.* 2010 and unpublished data). RpoN is an alternative sigma factor that regulates several virulence genes in *P. aeruginosa*, and therefore, is required for virulence under certain conditions (Hendrickson *et. al.* 2001). However, in chronic CF isolates of *P. aeruginosa*, RpoN does not appear to regulate *aceA* and *glcB* (Hagins *et. al.* 2010 and unpublished data) probably because both genes are permanently upregulated in this isolate. Previous predictive and non-predictive approaches have failed to identify transcriptional regulators of *aceA* and *glcB* in *P. aeruginosa* suggesting the presence of novel regulators of these genes. In an effort to better understand *aceA* and *glcB* regulation, we characterized gene expression in response to various carbon sources (Hagins 2010, Hagins 2011). For example, *aceA* and *glcB* have been shown to be induced in *P. aeruginosa* cultures grown on leucine. However, the regulatory mechanisms involved in these processes have yet to be identified (Hagins *et. al.* 2010; Diaz Perez *et. al.* 2007). Due to the predominance of nutrients in the CF lung that require both the glyoxylate pathway and the *glp*

regulon for catabolism, we questioned whether glycerol metabolism might overlap with fatty acid metabolism in *P. aeruginosa* in the regulation of the glyoxylate pathway.

Glycerol metabolism and regulation

In *P. aeruginosa,* glycerol metabolism is controlled by GlpR, a negative transcriptional regulator that controls expression of the genes in the *glp* regulon (Schweizer *et. al.* 1996). The *glp* regulon encodes for a membrane-associated glycerol diffusion facilitator (GlpF), a glycerol kinase (GlpK*)*, a membrane protein involved in alginate biosynthesis (GlpM*)*, and a glycerol-3-phosphate dehydrogenase (GlpD) (Figure 2). Glycerol is transported into the cell via GlpF and is phosphorylated to glycerol 3-phosphate (G3P) by GlpK, whereas exogenous glycerol 3-phosphate is transported into the cell via the GlpT transporter system (Schweizer *et. al.* 1996 and 1997). Importantly, G3P induces the *glp* regulon by binding to GlpR (Schweizer *et. al.* 1996). Ultimately, the products of glycerol metabolism can be used by the cell as a source of energy or for the synthesis of alginate (Schweizer *et. al.* 1996 and 1997). Glycerol metabolism in *P. aeruginosa* is similar to other proteobacteria. However, in *E. coli* the *glp* genes are arranged as operons in three different loci on the chromosome: *glpTQ*/*glpABC, glpEGR*/*glpD* and *glpFKX*. These operons are all controlled by the *glp* repressor, GlpR (Danilova *et. al.* 2003; Zeng *et. al.* 1996). In *E. coli* G3P can be oxidized anaerobically or aerobically the products of *glpA* or *glpD*, respectively (Iuchi *et. al.* 1990). Interestingly, two of the most abundant products generated from the microaerobic metabolism of glycerol in *E. coli* are ethanol and acetate (Durnin *et. al.* 2009), which would require induction of the glyoxylate pathway.

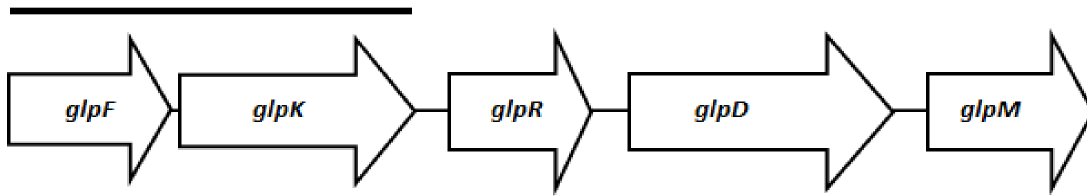

Figure 1.2. Organization of the *glp* regulon in *P. aeruginosa.*

Nutrient acquisition and metabolism via the glyoxylate pathway and the *glp* regulon in *P. aeruginosa*

During lung infection, *P. aeruginosa* secretes phospholipases that cleave host lipid membranes (e.g. phosphatidylcholine and phosphatidylethanolamine) yielding free fatty acids and glycerol, which become available to *P. aeruginosa* for use as carbon sources (Figure 3). Phosphatidylcholine (PC) derived nutrients require the glyoxylate pathway and *glp* regulon for metabolism. *In vivo* expression studies reveal that *P. aeruginosa* recovered from the CF lung show higher constitutive expression of genes that encode for lipases and phospholipases compared to non-CF *P. aeruginosa* (Son *et. al.* 2007). In addition, two genes involved in glycerol metabolism, *glpD* and *glpK*, are also constitutively expressed under the same conditions in *P. aeruginosa* (Son *et. al.* 2007). These results correlate upregulation of genes involved in fatty acid and glycerol metabolism in *P. aeruginosa* during infection of the CF lung. Furthermore, the entire *glp* regulon is induced in an acute isolate of *P. aerugionsa* when grown on PC (Son *et. al.* 2007), and it is likely that this regulon also responds to fatty acids derived from PC. Another study revealed that the expression of the glyoxylate pathway genes, *glcB* and *aceA*, is induced two and five-fold, respectively, during chemotaxis towards the membrane lipid, phosphatidylethanolomine (Miller *et. al.* 2008). In the same study, expression of *glpF* and *glpK*

was also induced, as well as the transcriptional regulator, GlpR (Miller *et. al.* 2008). Taken together, the glyoxylate pathway and the *glp* regulon appear to play a crucial role in acquiring nutrients for *P.aeruginosa* growing within the CF lung. Our current understanding of how the glyoxylate pathway and the *glp* regulon might contribute to the ability of *P. aeruginosa* to cause chronic disease is extremely limited. It is possible that both pathways share some regulatory element, particularly because both respond to nutrients found within the CF lung. Unfortunately, little is known about the regulation of both pathways in *P. aeruginosa* and the relationship between the glyoxylate pathway and *glp* regulon has never been examined. A better understanding of the glyoxylate pathway, as well as the *glp* regulon, in chronic CF isolates of *P. aeruginosa* will provide valuable insight into the CF lung environment.

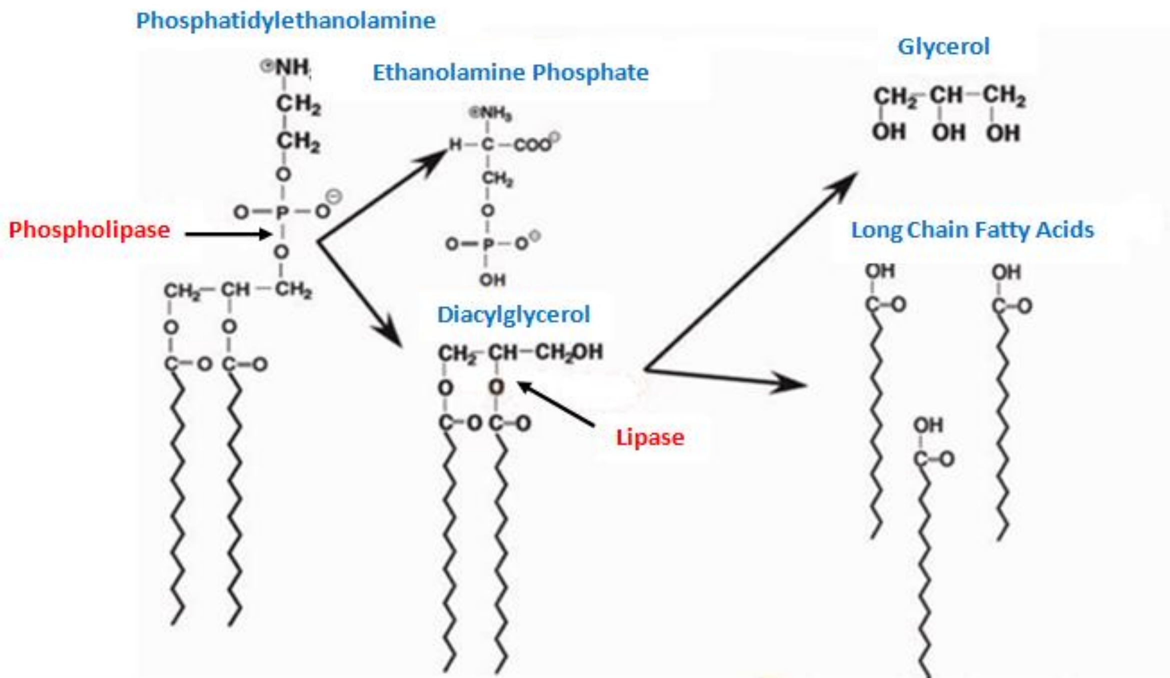

Figure 1.3. Degradation of phophatidylethanolamine to yield glycerol and fatty acids via the action of *P. aeruginosa* phospholipases and lipases.

Summary

The constitutive expression of genes responsible for glycerol and fatty acid metabolism in the CF lung likely provides an advantage to *P. aeruginosa*. Efficient catabolism of these carbon sources may require coordination of the glyoxylate and glycerol pathways. Therefore my thesis project focused on the role of GlpR in regulating the glyoxylate pathway and characterized glycerol catabolism by a CF isolate of *P. aeruginosa*. This research provides novel insight into the regulatory interchange involved in glycerol and fatty acid metabolism in *P. aeruginosa,* and promotes our understanding of how these two networks enable *P. aeruginosa* to establish and maintain chronic infections.

Understanding the regulation of genes specific to the glyoxylate pathway will advance our knowledge of how bacterial pathogens cause disease and provide clues on how to treat CF lung infection, as well as move us a step closer to understanding how *P. aeruginosa* adapts to the CF lung. The glyoxylate pathway is an attractive therapeutic target because it is not present in humans.

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Chapter 2

The Role of GlpR in the Regulation of the Glyoxylate Pathway in *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa infections are the leading cause of morbidity and mortality for cystic fibrosis (CF) patients. *P. aeruginosa* establishes life-long infection in the CF lung by utilizing various adaptation strategies to cause a chronic infection. One of these strategies includes the upregulation of the genes encoding for the glyoxylate pathway enzymes, *aceA* and *glcB* which encode for isocitrate lyase (ICL) and malate synthase (MS), respectively. The glyoxylate pathway allows certain bacteria to grow on acetate or fatty acids as a sole carbon source to replenish intermediates of the tricarboxylic acid cycle, a pathway that is required by many microorganisms for pathogenesis. We determined previously that the glyoxylate pathway becomes deregulated in some isolates of *P. aeruginosa* adapted to the CF lung, including FRD1. The occurrence of these isolates suggests deregulation of the glyoxylate pathway may benefit *P. aeruginosa* growing within the CF lung. However, the mechanism(s) responsible for regulation of the glyoxylate pathway have yet to be elucidated. GlpR is a transcriptional repressor that regulates the genes responsible for glycerol metabolism in *P. aeruginosa.* We determined that GlpR also plays a role in regulating the glyoxylate pathway. Disruption of *glpR* in PAO1, an acute isolate of *P. aeruginosa*, resulted in high ICL and MS activity. This activity was correlated with increased expression of *aceA* and *glcB,* which encode for ICL and MS respectively. GlpR's

role in the regulation of the glyoxylate pathway provides a novel perspective into the interplay between fatty acid and glycerol metabolism in *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is the major etiologic agent of chronic pulmonary infections in cystic fibrosis (CF) patients (Bragonzi *et. al*. 2009). *P. aeruginosa* is acquired early in life by the patient and persists within the lung for decades (Bragonzi *et. al*. 2009; Mahenthiralingam *et. al*. 1996). During infection of the CF lung *P. aeruginosa* acquires several mutations that facilitate its survival in the CF lung environment (Chambers *et. al*. 2005; Hoffman *et. al*. 2009; Smith *et. al.* 2006). Some of the alterations that promote the survival of *P. aeruginosa* include the overproduction of alginate, loss of flagella, and differential expression of genes responsible for virulence and catabolism (Chambers *et. al*. 2005; Smith *et. al.* 2006; Son *et. al.* 2007). These alterations facilitate *P. aeruginosa* to evade clearance by the host immune system and are important for the acquisition and metabolism of nutrients obtained from CF sputum.

Sputum is the most likely source of nutrition for *P. aerugionsa* living within the CF lung (Son *et. al*. 2007). Sputum contains a complex mixture of host and bacterial derived products including amino acids and lipids (Palmer *et. al.* 2005). In addition, carbon sources such as glycerol and fatty acids are liberated by the hydrolysis of membrane lipids by secreted bacterial phospholipases (Lyczak *et. al*. 2002; Terry *et. al.* 1992**;** Williams *et. al.* 1994). Given the relative abundance of these particular carbon sources in the CF lung, it would be advantageous for *P. aeruginosa* persisting in that environment to adopt strategies for more efficient utilization of these nutrients including altering regulation of the required metabolic pathways.

In previous studies, we showed that *aceA* and *glcB* are constitutively upregulated in the chronic CF isolate of *P. aeruginosa*, FRD1 (Lindsey *et. al.* 2008, Hagins *et. al.* 2010). We also demonstrated that *aceA* is essential for infection in the alfalfa seedling and rat lung models of infection, and is required for optimal production of alginate and hydrogen cyanide (Lindsey *et. al.* 2008, Hagins *et. al.* 2009 and 2010). *aceA* and *glcB*, which encode for isocitrate lyase and malate synthase, respectively, are specific to the glyoxylate pathway (Dunn *et. al*. 2009), which is required by certain microorganisms for growth on acetate or fatty acids as the sole carbon source (Dunn *et. al.* 2009). The mechanism of deregulation of *aceA* and *glcB* in FRD1 is unknown. We previously showed that *aceA* and *glcB* are negatively regulated by RpoN in PAO1. However, RpoN is not responsible for the deregulation of *aceA* and *glcB* in FRD1 (Hagins *et. al.* 2010). Due to the availability of fatty acids and glycerol in the CF lung, catabolism of both carbon sources may require coordination of several metabolic pathways, including the glyoxylate pathway. In this study, we focused on the contribution of GlpR to regulation of the glyoxylate pathway in both chronic (FRD1) and wound (PAO1) isolates of *P. aeruginosa*. GlpR controls glycerol metabolism in *P. aeruginosa* by negative regulation of the *glp* regulon (Schweizer *et. al*. 1996). The *glp* regulon encodes for a membrane-associated glycerol diffusion facilitator (GlpF), a glycerol kinase (GlpK), a membrane protein involved in alginate biosynthesis (GlpM), and glycerol-3-phosphate dehydrogenase (GlpD) (Schweizer *et. al*. 1996). Glycerol is transported into the cell via the GlpT transporter system, which is separate from the *glp* operon and is not regulated by GlpR. The *glp* regulon is induced by the presence of glycerol-3-phosphate (G3P) or glycerol in the growth medium (Schweizer *et. al*. 1996). We reasoned that fatty acid and glycerol catabolism might be coordinately regulated since both

carbon sources are liberated from host membranes. Therefore, we tested the effect of a *glpR* null mutation on the glyoxylate pathway in *P. aeruginosa.*

Materials and Methods

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this study are listed in Table 2.1. Unless otherwise indicated, bacteria were cultured in L-broth (LB) or on L-agar at 37°C. No-carbon-E minimal medium (NCE) supplemented with 0.1% (w/v) casamino acids (CAA) was used to assay for growth on minimal media (Davis *et al.*, 1980). Glycerol was used at a concentration of 20 mM. UV-Vis absorption spectra were recorded on a Shimadzu UV-1601 Spectrophotometer using 1 cm path length cells. 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 (Amp) ml^{-1} for *E. coli*; 100 μg carbenicillin (Cb) ml^{-1} for *P. aeruginosa*; 20 μg gentamicin (Gm) ml^{-1} for *E. coli* and 200 μg for *P. aeruginosa*; 20 μg tetracycline (Tet) ml^{-1} for *E. coli*; 100 μg ml^{-1} for *P. aeruginosa,* and 50 μg kanamycin ml^{-1} for *E. coli*. To examine growth over a 24 hour period, cultures were grown in 24 well microtiter plate and monitored at A_{600} with a BioTek Synergy HT plate reader (BioTek, Winooski, VT).

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. 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 pLS1554 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 (m*oriT*) of RP4 on a ~230 bp *Hin*dIII fragment (Suh *et al.*, 2004). pLS1554 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 *glpR101*::*aacC1* allele was verified by PCR analysis.

Construction of the *glpR* complemented strains.

To complement the *glpR* mutation, 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) to produce pLS1950. The resulting plasmid (pLS1950) was digested with *Hind*III and the m*oriT* 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 carbenicillin resistant colonies. *In cis* complementation was verified by PCR analysis and the complemented PAO1 *glpR* isolates were designated PAO1 *glpR* + (JS146).

Construction of *glpD* and *glpR* transcriptional fusions and biochemical assays.

The *glpD*::*lacZ* and *glpR*::*lacZ* transcriptional fusions were constructed using the *glpD* and *glpR* gene fragments isolated from PAO1 via PCR using *Pfu*. The fragments, which included 500 bp upstream from the coding sequence, were cloned into the *Sma*I site of pSS223 (Suh *et al.,* 2004). The plasmids (pLS1954 and pJS149), containing the 5' coding sequence for *glpD* and *glpR*, respectively, in the proper orientation, were verified by PCR and restriction digest. The plasmids containing the fusions were conjugated into FRD1 and PAO1 via triparental mating and plasmid integration events were selected by carbenicillin resistance.

Biochemical assays.

β-galactosidase assays were preformed as described by Miller (Miller, 1972). Isocitrate lyase activity was measured according to the Sigma Aldrich protocol (EC 4.1.3.1), with minor modification. *P. aeruginosa* cells were harvested from stationary cultures and washed with saline. The cells were resuspended in Tris-EDTA (TE) Buffer pH 6.9 and broken open via ultrasonification with Fisher Scientific, Model 100 Sonic dismembrator using a microtip. Following centrifugation, the total protein in the cell free extracts was quantified using the Bradford method (Bio-Rad, Hercules, CA). The cell free extract was added to a mixture of imidazole buffer, magnesium chloride, isocitrate, and phenylhydrazine per the Sigma protocol. The increase in absorbance at A_{324} was monitored for 5 minutes at room temperature and activity

was expressed as ΔA_{324} min^{-1} (mg protein)$^{-1}$ in which the rate of ΔA_{324} was determined using only the linear part of the reaction. Malate synthase activity was determined according to the Sigma Aldrich protocol (EC 4.1.3.2), with slight modifications: *P. aeruginosa* cells were harvested from stationary phase cultures and washed with saline. The cells were resuspended in TE Buffer pH 8.0 and sonicated. Following centrifugation, the total protein in the cell free extracts was quantified using the Bradford method (Bio-Rad). The cell free extract was added to a mixture of imidazole buffer, magnesium chloride, acetyl-CoA, glyoxylic acid, and dithionitrobenzoic acid per the Sigma protocol. The increase in absorbance at A_{412} was monitored for 5 minutes and activity was expressed as ΔA_{412} min^{-1} (mg protein)$^{-1}$ in which the rate of the ΔA_{412} min^{-1} was determined using the linear rate of the reaction.

Overexpression and purification of his-tagged GlpR from *P. aeruginosa.*

A PCR product containing the *glpR* coding region was cloned between the *Nco*I/*Eag*I site of the expression vector pET28-b (Novagen) and electroporated into DH10B, creating a His-tag at the C-terminus. Following confirmation of a positive clone the plasmid was subsequently electroporated into BL21 (DE3). The *E. coli* BL21 (DE3) strain harboring the His-tagged GlpR plasmid was induced with 1mM IPTG (Isopropyl β-D-1 thiogalactopyranoside) in L-broth and induced at 28° C for 4 hours. The cells were harvested, washed, re-suspended and sonicated (Sonic Dismembrator 100, Fisher Scientific) in a lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole. The recombinant His-tagged GlpR protein was purified on Ni-NTA resin according to the Qiaexpressionist protocol.

Gel mobility shift assay.

DNA promoter fragments (60 bp) of *aceA* and *glcB* were synthesized with a 5' biotin end label (Integrated DNA Technologies). Binding reactions were performed according to the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) instructions. Briefly, promoter fragments were incubated with various concentrations of purified GlpR in a mixture of 1X Binding Buffer, 2.5% glycerol, 5mM $MgCl_2$, 50 ng/uL Poly dI·dC, and 0.05% NP-40 % in a final volume of 20 µL (Thermo Scientific). The reaction was incubated for 20 min at room temperature and separated on a native 5 % acrylamide gel. After migration the gel was transferred to a nylon membrane and developed using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific). The gel shift was visualized using the ImageQuant 4000 (GE Healthcare).

Strain or Plasmid	Genotype, relevant characteristics	Source
Strains		
FRD1	CF isolate, mucoid	Ohman *et. al. (*1981)
PAO1	Wound isolate, nonmucoid	Holloway *et al.* (1979)
FRD1*glpR* (JS134)	FRD1*glpR101::aacCI*	This study
PAO1 *glpR* (JS97)	PAO1*glpR101::aacCI*	This study
FRD1 *glpR*$^{+}$ (JS148)	FRD1 complemented for *glpR*	This study
PAO1 *glpR*$^{+}$ (JS145)	PAO1 complemented for *glpR*	This study
PAO1*glpR aceA::lacZ*	PAO1*glpR* carrying *aceA::lacZ* fusion	This study
PAO1*glpR glcB::lacZ*	PAO1*glpR* carrying *glcB::lacZ* fusion	This study
PAO1 *glpR::lacZ*	PAO1 carrying *glpR::lacZ* fusion	This study
FRD1 *glpR::lacZ*	FRD1 carrying *glpR::lacZ* fusion	This study
PAO1 *glpD::lacZ*	PAO1 carrying *glpD::lacZ* fusion	Dr. Laura Silo-Suh
FRD1 *glpD::lacZ*	FRD1 carrying *glpD::lacZ* fusion	Dr. Laura Silo-Suh
PAO1 *aceA::lacZ*	PAO1 carrying *aceA::lacZ* fusion	Lindsey *et. al.* 2009
FRD1 *glcB::lacZ*	FRD1 carrying *aceA::lacZ* fusion	Hagins *et. al.* 2011
BL21(DE3)	*glpR* His-tag expression strain	This study
Plasmids		
pLS1954	*glpR*101 in pBluescript K+	Dr. Laura Silo-Suh
pLS1968	*glpD::lacZ* transcriptional fusion in pSS223	Dr. Laura Silo-Suh
pJS149	*glpR*::lacZ transcriptional fusion in pSS223	This study
pLS1950	*glpR* complementing plasmid for PAO1 and FRD1	Dr. Laura Silo-Suh
pET28-b+*glpR*	His-Tag Plasmid	Dr. Laura Silo-Suh

Table 2.1 Bacterial strains and plasmids. Abbreviations used for genetic markers are described by Holloway *et al*. (1979). Alternate strain names are shown in parentheses.

Results

GlpR controls ICL and MS activity

To identify possible regulators of *aceA* and *glcB* in *P. aeruginosa* we measured ICL and MS activity from parental strains (FRD1 and PAO1) and their *glpR* mutant derivatives. As shown in Figures 2.1 and 2.2, disruption of *glpR* resulted in high ICL and MS activity in PAO1 which suggests that *glpR* plays a role in regulation of the glyoxylate pathway in the acute wound isolate. However, a mutation in *glpR* had no effect on ICL and MS activity in the chronic CF isolate FRD1, both of which had abnormally high activity. PAO1 strains complemented for *glpR* with a wild type copy of the gene *in cis* restored normal ICL and MS activity.

High ICL and MS activity correlate with increased *aceA* and *glcB* expression in the PAO1 *glpR* mutant

To determine if altered *aceA* and *glcB* expression were the cause of high ICL and MS activity in PAO1, *aceA*::*lacZ* and *glcB*::*lacZ* fusions were utilized to measure promoter activity. *aceA*::*lacZ* expression was induced in stationary phase cultures in the PAO1 *glpR* mutant (Figure 2.3a). In contrast, *glcB*::*lacZ* expression was induced in the PAO1 *glpR* mutant throughout the entire growth period with a slight increase during late stationary phase (Figure 2.3b). The results indicate that upregulation of ICL and MS activity in the PAO1 *glpR* mutant correlates with increased transcription of the genes encoding for the enzymes.

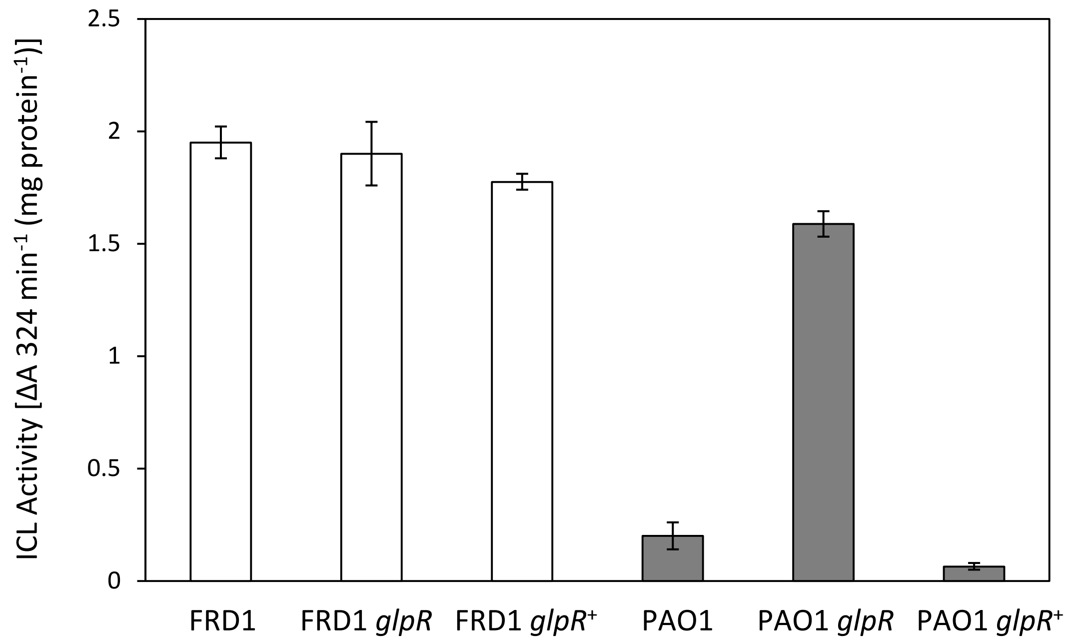

Figure 2.1 Effect of *glpR* mutation on ICL activity in PAO1. ICL activity was assayed from overnight cultures of *P. aeruginosa* grown in L-broth. Complemented strains are designated as "+" for those containing a wild-type copy of *glpR* from PAO1. Values represent the average of 2 experiments with standard error bars.

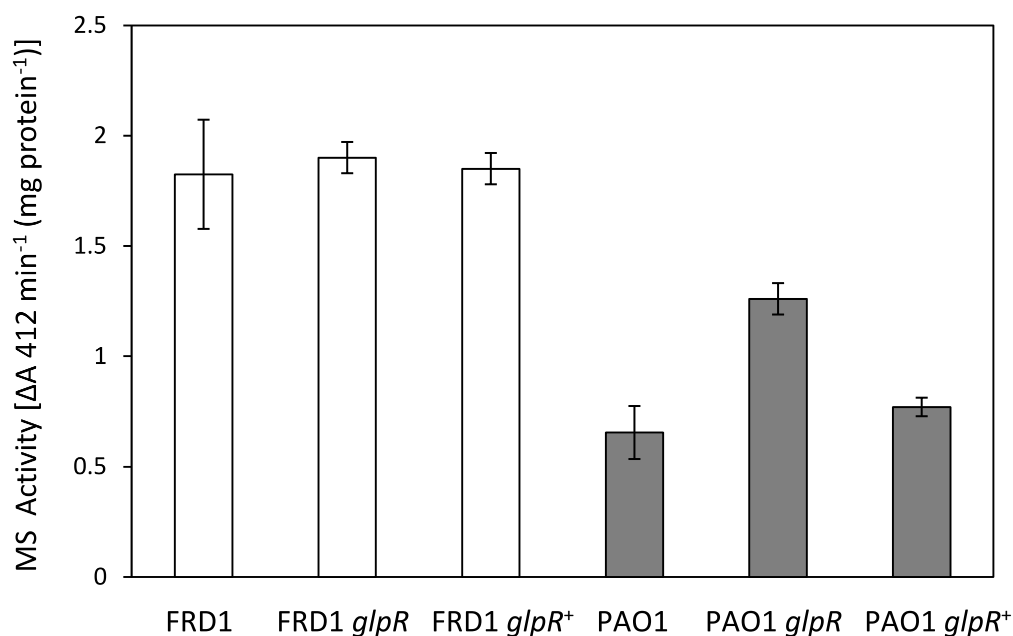

Figure 2.2 Effect of *glpR* mutation on MS activity in PAO1. MS activity was assayed from overnight cultures of *P. aeruginosa* grown in L-broth. Complemented strains are designated as "+" for those containing a wild-type copy of *glpR* from PAO1. Values represent the average of 2 experiments with standard error bars.

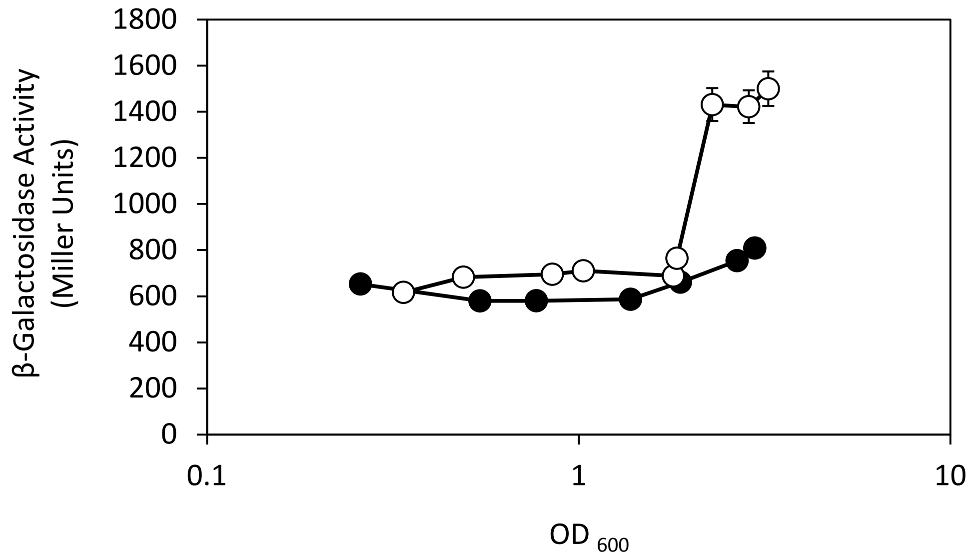

Figure 2.3a Effect of *glpR* mutation on *aceA* expression in PAO1. β-galactosidase activity is presented in Miller units. PAO1 ●; PAO1 *glpR* ○. The results are representative of 2 experiments with standard error bars.

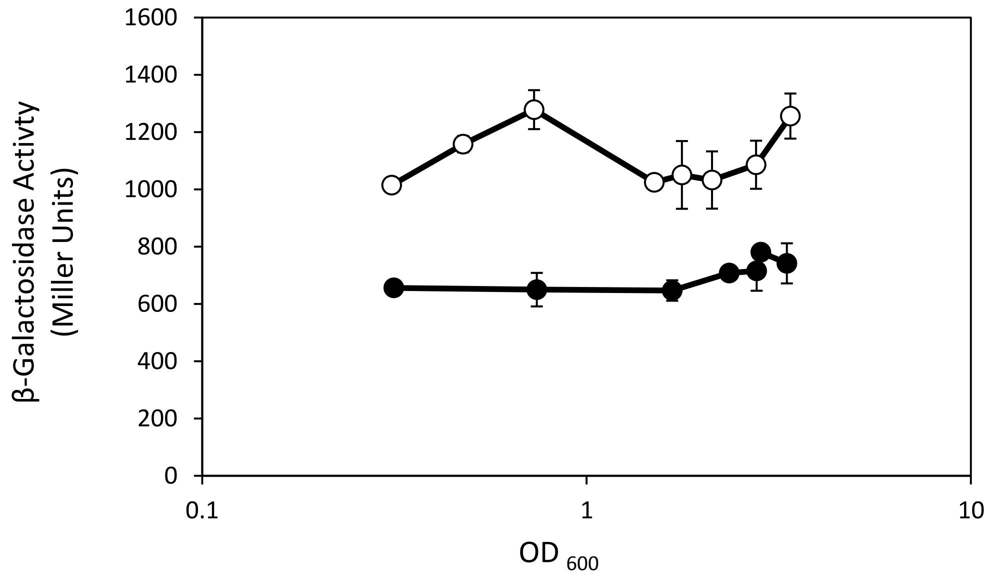

Figure 2.3b Effect of *glpR* mutation on *glcB* expression in PAO1. β-galactosidase activity is presented in Miller units. PAO1 ●; PAO1 *glpR* ○. The results are representative of 2 experiments with standard error bars.

Expression of *glpR* is higher in FRD1 compared to PAO1

As previously shown, a mutation in *glpR* had no effect on ICL and MS activity in FRD1 but resulted in increased ICL and MS activity in PAO1. A simple explanation for this observation would be an altered *glpR* in FRD1 due to a mutation. However, sequence analysis of the FRD1 *glpR* gene revealed no changes that would indicate that it is defective. To verify that *glpR* is unaltered in FRD1, *glpR*::*lacZ* and *glpD*::*lacZ* transcriptional fusions were constructed to compare the expression of these genes between FRD1 and PAO1. In L-broth, *glpR*::*lacZ* expression was increased in FRD1 compared to PAO1 and *glpD*::*lacZ* expression was decreased in FRD1 compared to the acute isolate PAO1 (Figures 2.4 and 2.5). A defective *glpR* would no longer be able to repress the expression of *glpD*. Therefore, high ICL and MS activity in FRD1 is not caused by reduced expression of *glpR*. We also considered whether high internal glycerol-3-phosphate (G3P) concentrations in FRD1 would relieve repression of the genes potentially regulated by GlpR, including *aceA* and *glcB*. However, this is not supported by the reduced expression of *glpD* in FRD1 compared to PAO1. A simple explanation for the lack of a GlpR effect on the glyoxylate pathway in FRD1 is the loss of a major regulator for this pathway in FRD1 that overshadows the small effects of GlpR.

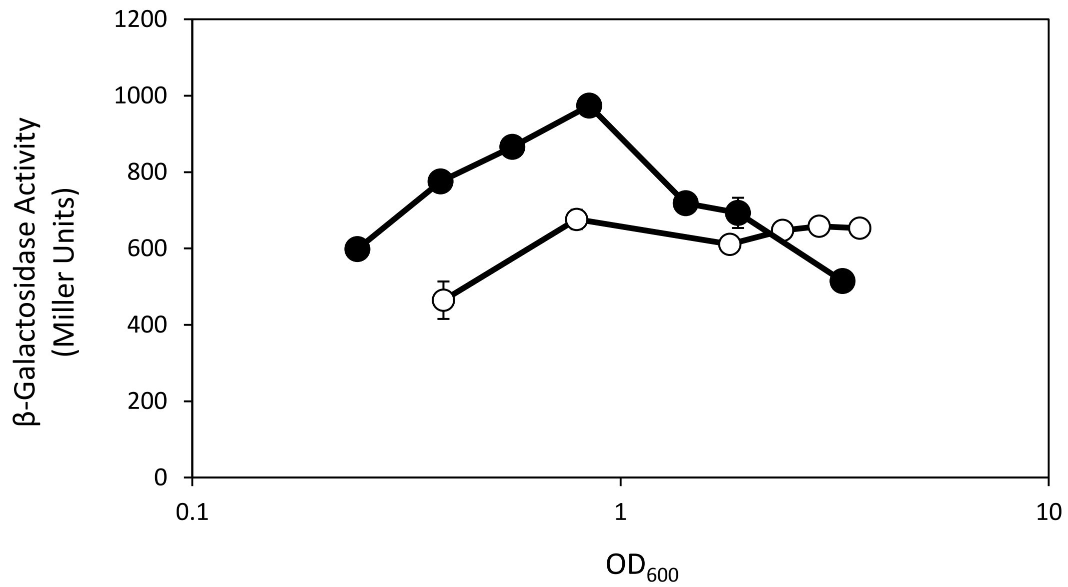

Figure 2.4 Expression of *glpR*::*lacZ* in PAO1 and FRD1. PAO1 ○; FRD1 ●. The results are representative of 2 experiments with standard error bars.

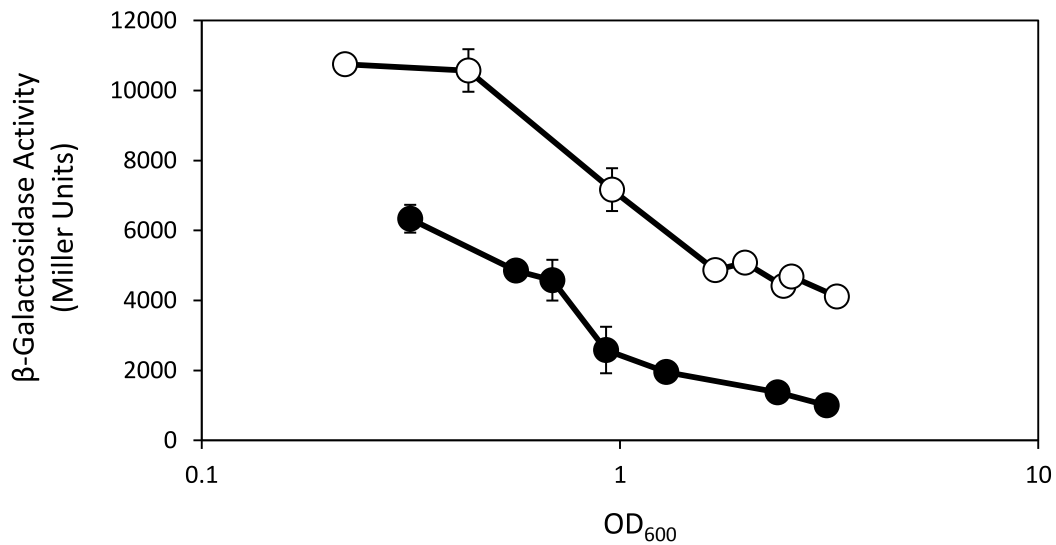

Figure 2.5 Expression of *glpD*::*lacZ* in PAO1 and FRD1. PAO1 ○; FRD1 ●. The results are representative of 3 experiments with standard error bars.

Growth on glycerol induces expression of *glcB* in PAO1

Expression of *glpR* is not substantially altered in FRD1 and does not appear to be responsible for increased ICL and MS activity in FRD1. However, GlpR does regulate the glyoxylate pathway in PAO1. Schweizer *et. al*. in 1996 previously reported that the *glp* regulon is induced by glycerol or glycerol-3-phosphate (G3P). Induction of *aceA* or *glcB* by the presence of glycerol or G3P in the growth medium would provide additional evidence of GlpR's role in the regulation of the glyoxylate pathway. We attempted to measure *aceA* expression in PAO1 grown on G3P, however, in contrast to Schweizer's findings, PAO1 was unable to utilize G3P as a sole carbon source (Figure 2.6) and the addition of G3P to L-broth had no effect on *aceA*::*lacZ* expression (data not shown). PAO1 was able to utilize glycerol as a carbon source when supplemented with 0.1% casamino acids. Therefore, we compared the expression of *aceA* and *glcB* in PAO1 grown in L-broth versus minimal medium with glycerol as a carbon source. Growth on glycerol increased expression of *glcB* in PAO1 compared to L-Broth but there was no difference in *aceA* expression between the two carbon sources (Figure 2.7). Therefore, GlpR appears to regulate *aceA* and *glcB* differently and has a more dramatic effect on *glcB* expression and MS activity.

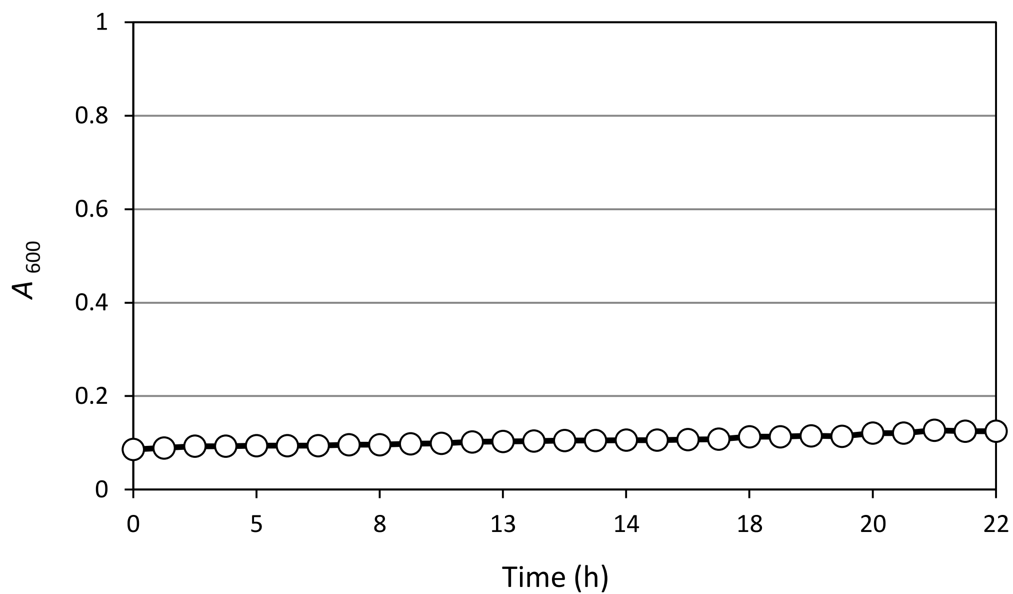

Figure 2.6 Growth of PAO1 on glycerol-3-phosphate. Cultures were Grown for 22 hours 20mM glycerol-3-phosphate.

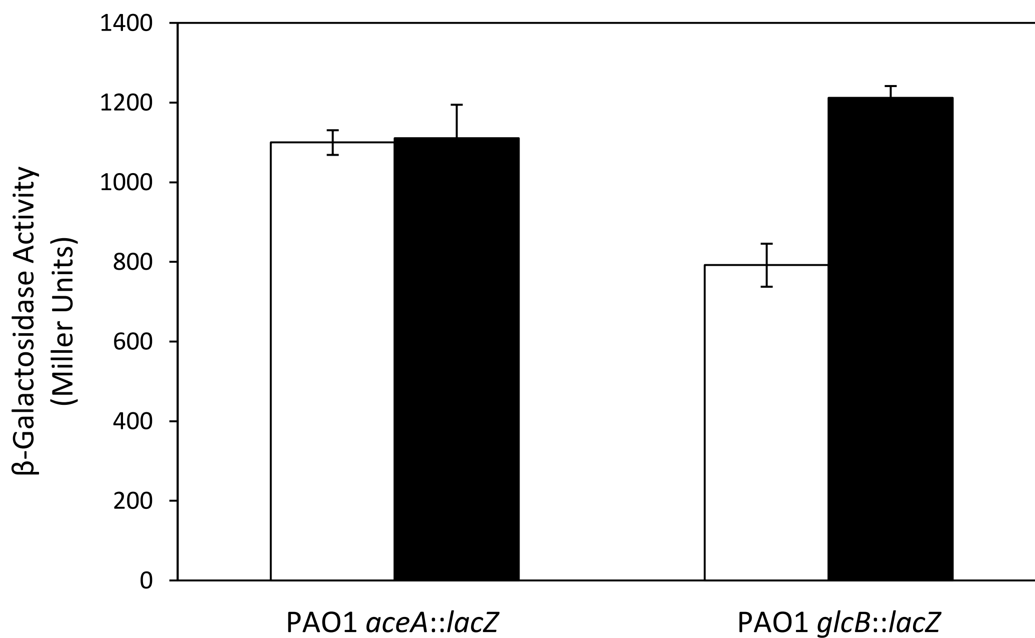

Figure 2.7 *aceA* and *glcB* expression in PAO1 in LB vs. glycerol. Cultures were grown overnight in L-Broth □ or 20mM glycerol ■ supplemented with 0.1% casamino acids.

GlpR binds to the *glcB* promoter

E. coli GlpR was shown previously to bind a consensus sequence forming an inverted repeat (Weissenborn and Larson, 1992) (Figure 2.8a). Similar sequences to the *E. coli* GlpR binding site were identified upstream of GlpR regulated genes in *P. aeruginosa* (Schweizer *et. al.* 1996). However, these sequences were never verified to bind to *P. aeruginosa* GlpR. Using a putative consensus sequence we generated from known GlpR regulated genes in *P. aeruginosa*, we identified several potential GlpR binding sites upstream of the *glcB* promoter (Fig 2.8b). To determine whether *P. aeruginosa* GlpR binds the *glcB* promoter, we fused GlpR to a hexa histidine-tag at the C-terminal end and purified the protein from *E. coli* grown in L-broth (Figure 2.9 a-b). The purified protein was tested in a gel-mobility shift assay with a 60 nucleotide fragment of the *glcB* promoter containing a putative site (Figure 2.10a). As expected the Gel-shift assay showed that GlpR binds to the *glcB* promoter and that the complex can de dissociated in a competition reaction containing 100X unlabeled DNA containing the putative GlpR binding site (Figure 2.10b). In addition, no interaction was observed between the purified GlpR and the control DNA lacking the putative GlpR binding site (2.10c), which indicates that the binding between GlpR and the *glcB* promoter is specific.

A

E. c. consensus	W	A	T	G	T	T	C	G	W	T
P. a. *glpD* 1	T	a	T	t	T	t	c	g	a	a
P. a. *glpF*	T	t	T	t	T	t	c	g	a	a
P. a. *glcB* 1	T	t	T	a	T	c	t	g	g	a
P. a. *glcB* 2	T	t	T	t	T	c	c	c	g	c
P. a. consensus	T	W	T	W	T	t/c	C/t	G/c	a/g	A/c

B *glcB* 2

TG[TTTTTAGA**TTTATCTGGA**ACAAAGTACAGTTTTTTGCGAACATTGAGCC

TGGCCAACG]TGACCGTGAAGCGTCATCCAGTCGTAACGCGACGCGTAACCA

glcB 1

CTGAT**TTTTCCCGC**GGCATCATGTAGTATGCCGCGGCTCGGACTACAAGGCC

GTGCGGCCCGGGTCCAGAGCTGGTCTAGAGCAGAGTGAGGCAAACA **ATG**

M

glpR >

Figure 2.8 Comparison of putative *glpR* binding sites. **A**. The *E. coli* consensus half site (*E.c.*) and putative half sites from GlpR regulated genes in *P. aeruginosa* (*P.a.*) are given. W=A or T. **B.** The upstream non-coding sequence of *glcB* is presented. Putative GlpR binding sites are indicated in bold. Sequence used for gel shift assay is in brackets.

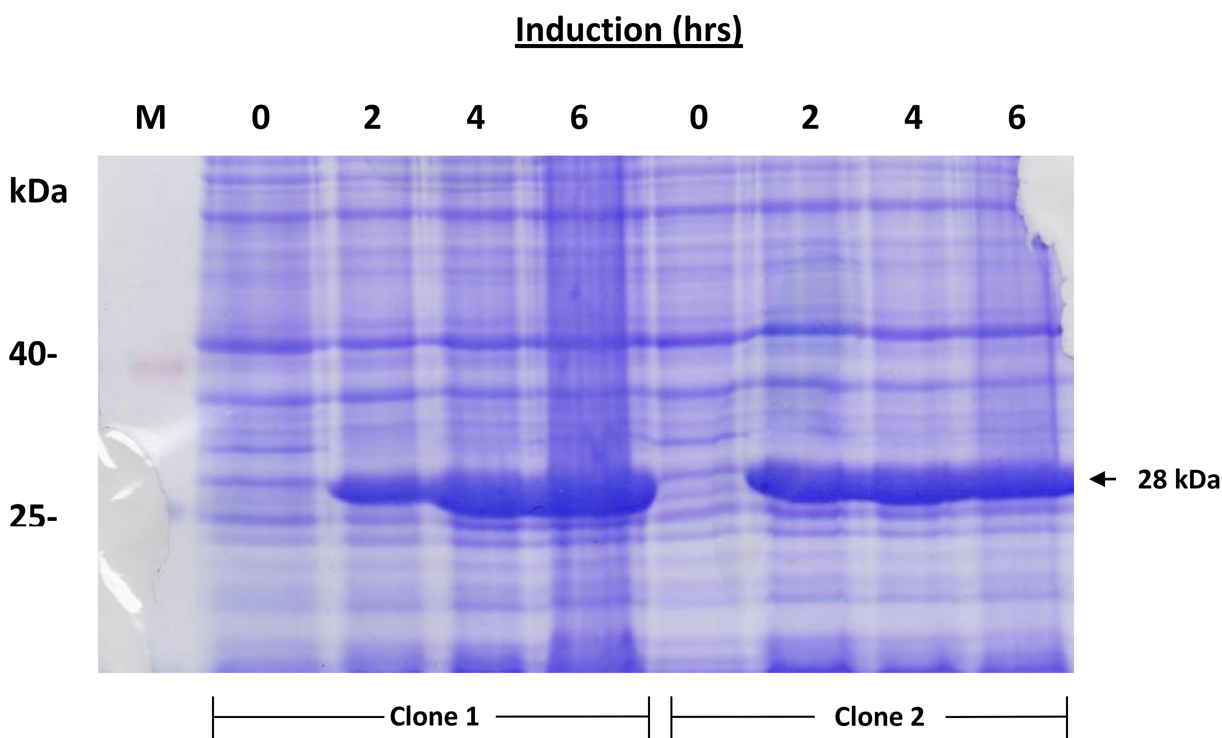

Figure 2.9a Overexpression of the 28 kDa protein GlpR in *E. coli.* GlpR from *P. aeruginosa* was overexpressed in *E. coli* using the T7 vector pET28-b (Merck, Rockland, MA) in two different clones. Cultures were grown in L-broth and induced with 1mM IPTG. Proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue.

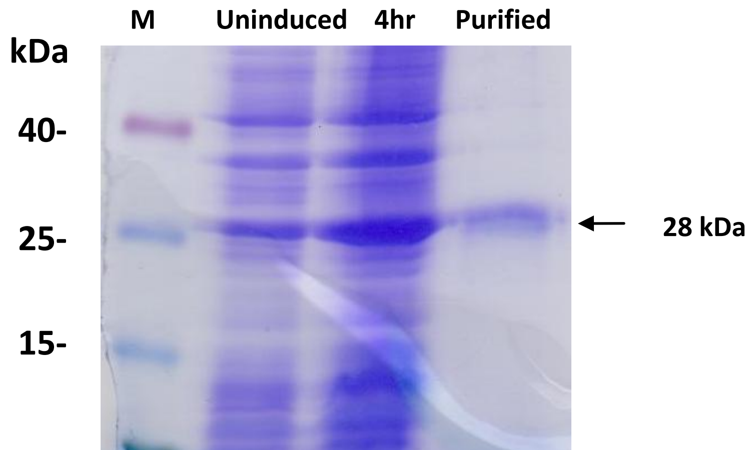

Figure 2.9b Purification of the 28 kDa protein GlpR in *E. coli.* GlpR from *P. aeruginosa* was overexpressed in *E. coli* using the T7 vector pET28-b. GlpR was induced for 4 hours.

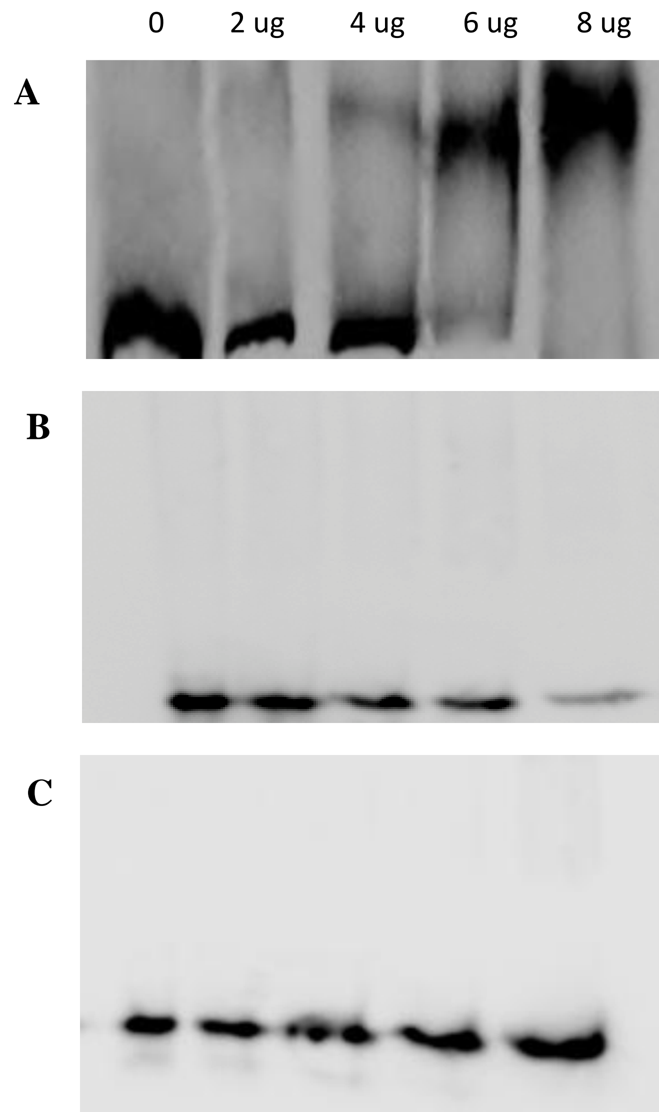

Figure 2.10 Gel shift assays using the putative GlpR binding sites. **a).** Gel shift assays using a *glcB* fragment. **b).** Competition assay using 100X unlabeled probe. **c).** Negative control. The amounts of GlpR added to the reaction mixtures are indicated above each lane.

Discussion

Understanding the mechanisms pathogens use to maintain chronic infections is necessary in order to develop successful therapeutic approaches that target these mechanisms. ICL, the first enzyme unique to the glyoxylate pathway is required for *P. aeruginosa* virulence in rat lungs (Lindsey *et. al.* 2008). ICL is also required for the optimal production of alginate and hydrogen cyanide (Lindsey *et. al.* 2008 and Hagins *et. al.* 2009). To date, transcriptional regulators of the glyoxylate pathway in *P. aeruginosa* have not been identified with the exception of RpoN's indirect role in the regulation of this pathway (Hagins *et. al.* 2010). In an effort to determine the mechanism of deregulation of ICL in FRD1, we focused on the contribution of GlpR in the regulation of the glyoxyate pathway because GlpR controls glycerol metabolism. Glycerol and fatty acids are liberated from membrane lipids by the action of phospholipases, and both compounds serve as important carbon sources for *P. aeruginosa* during infection. We predicted that efficient catabolism of both substrates may require coordination of some metabolic pathways including the glyoxylate pathway.

As shown in this study, ICL and MS activity were induced in a PAO1 *glpR* mutant of *P. aeruginosa*. Increased enzymatic activity of ICL and MS in PAO1 correlated with increased expression of *aceA* and *glcB*, respectively. These results suggest that GlpR plays a role in negative regulation of the glyoxylate pathway similar to its effect on the *glp* regulon in the absence of glycerol or glycerol-3-phosphate.

To determine if GlpR regulated *glcB* directly we conducted an electrophoretic mobility gel shift assay using a His-tagged GlpR and promoter sequences derived from *glcB*. As expected, GlpR binds to the *glcB* promoter that contains putative GlpR binding sites. In contrast, we were unable to identify a GlpR consensus site in the *aceA* promoter. Therefore, GlpR

regulates *glcB* directly and *aceA* via another mechanism. These results are reflected by expression of the *glcB* and *aceA* genes in a *glpR* mutant. While *glcB*::*lacZ* shows increased expression throughout a growth cycle in the *glpR* mutant compared to the parental strain, *aceA*::*lacZ* expression is only affected in late stationary phase. Soh *et. al*. (2001) also noted high levels of ICL activity in late cultures of *Streptomyces clavuligerus* during growth on 0.5% - glycerol, which may suggest that ICL plays an important physiological role when oxygen availability is limited (Soh *et. al.* 2001). In addition, Wayne *et. al.* (1981) reported a five-fold increase in ICL activity in anaerobic *Mycobacterium tuberculosis* cultures that were grown over a 28 day period. Results from our lab (Chapter 3) demonstrated that FRD1 shows a growth advantage on glycerol compared to PAO1. This growth advantage correlated with the overproduction of the exopolysaccharide alginate which provides an oxygen-limited growth environment for FRD1. The CF lung is comprised of various oxygen rich and poor niches. Therefore, *P. aeruginosa* within the CF lung would have to adapt to utilizing available nutrients under these conditions, including limited oxygen availability (Hoffman *et. al.* 2010). Taken together, these findings suggest that microanaerobic growth conditions might be necessary for the efficient catabolism of glycerol by *P. aeruginosa*. Thus, GlpR's role in *P. aeruginosa* may include activating genes during anaerobic conditions including activation of *aceA*. In addition, some bacteria use an alternate set of genes for catabolizing glycerol or G3P during anaerobic conditions. For example, the *glpA* operon in *E. coli* encodes an anaerobic glycerol-3-phosphate dehydrogenase that is activated during anaerobic growth of G3P (Iuchi *et. al.* 1990). However, a *glpA* homolog in *P. aeruginosa* has yet to be identified. In addition, anaerobic catabolism of glycerol by *P. aeruginosa* has not been demonstrated or characterized to date.

In summary, we present evidence that GlpR, a transcriptional regulator involved in glycerol utilization in *P. aeruginosa*, also regulates malate synthase, an enzyme unique to the glyoxylate pathway. We suggest that coordinate regulation of glyoxylate and glycerol may be advantageous to *P. aeruginosa* during catabolism of lipids. However, further investigation is required to reveal the benefits of this process.

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Chapter 3

The Chronic Cystic Fibrosis Isolate, FRD1, is Enhanced for Growth on Glycerol

Abstract

Pseudomonas aeruginosa is the major etiologic agent of chronic pulmonary infections in cystic fibrosis (CF) patients. During establishment of chronic infections, the pathogen develops various adaptation strategies including redirecting metabolic pathways to utilize readily available nutrients present in the host environment. The airway sputum contains various host-derived nutrients that can be utilized by *P. aeruginosa* including phosphotidylcholine, a major component of host cell membranes. *P. aeruginosa* can degrade phosphotidylcholine to glycerol and fatty acids to increase the availability of glycerol in the CF lung. The goal of this study was to characterize and compare glycerol metabolism between an acute and a chronic isolate of *P. aeruginosa.* We show here that the chronic CF isolate, FRD1, displays a growth advantage on glycerol compared to the acute isolate PAO1. The enhanced ability of FRD1 to metabolize glycerol is correlated with alginate overproduction because FRD1 *algT* and *algD* mutants were unable to grow on glycerol. In addition, the alginate producing PAO1 *mucA* mutant showed increased growth on glycerol compared to the parent strain. Thus, alginate appears to be important for optimal glycerol utilization by *P. aeruginosa.* Finally, the addition of glycerol to L-broth enhanced alginate production by PAO1 suggesting that the CF lung may provide a

nutritional environment that promotes alginate production by *P. aeruginosa* even before the bacteria convert to the mucoid phenotype.

Introduction

Pseudomonas aeruginosa is the leading cause of lung dysfunction and death in cystic fibrosis (CF) patients (Bragonzi *et. al*. 2009). CF patients acquire *P. aeruginosa* from the environment at an early age and this bacterium establishes persistent lung infections during the patient's lifetime (Bragonzi *et. al*. 2009; Mahenthiralingam *et. al*. 1996). *P. aeruginosa* produces several virulence factors that facilitate the survival of *P. aeruginosa* during infection, including phospholipases, lipases, proteases, and exotoxins. During chronic infection in the CF lung, *P. aeruginosa* undergoes several phenotypic and genetic adaptations to persist in the lung and evade clearance by the host immune system and antibiotic therapy. Some of these adaptations include loss-of-function mutations, deregulation of metabolic genes, loss of motility, antibiotic resistance, and overproduction of the exopolysaccharide alginate (Chambers *et. al*. 2005; Hagins *et. al.* 2011; Hoffman *et. al*. 2009; Lindsey *et. al*. 2008; Silo-Suh *et. al.* 2005; Smith *et. al.* 2006). These adaptations appear to not only be necessary for avoiding clearance in the lung, but also for the acquisition and catabolism of nutrients found within the CF lung (Son *et. al*. 2007).

Current evidence suggests that *P. aeruginosa* and other bacteria that colonize the CF lung grow within the airway sputum (Palmer *et. al.* 2005; Son *et. al.* 2007). CF sputum contains a complex mixture of host secretions, inflammatory components, dead host and bacterial cells, nucleic acids, and bacterial products, and acts as a surface for biofilm development. Sputum also serves as a source of nutrition for colonizing bacteria (Palmer *et. al.* 2005). In the lung, nutrients

such as glycerol become available carbon sources due to the degradation of host cell membranes. Phosphotidylcholine, a major component of both membranes and lung surfactant, is degraded by *P. aeruginosa* to 1,2-diacylglycerol and phosphorylcholine using phospholipase C (Lyczak *et. al*. 2002; Terry *et. al.* 1992**;** Williams *et. al.* 1994). 1,2-diacylglycerol is then hydrolyzed to glycerol and fatty acids by lipases. The action of phospholipase C and lipases increases the availability of glycerol to *P. aeruginosa* as a potential carbon source (Lyczak *et. al*. 2002; Palmer *et. al.* 2005; Son *et. al.* 2007; Terry *et. al.* 1992**;** Williams *et. al.* 1994).

Transcriptome studies reveal that amino acids and lipids are probable growth substrates for chronic *P. aeruginosa* isolates growing within CF sputum, while acute wound isolates primarily use amino acids during growth on CF sputum (Palmer *et. al.* 2005; Son *et. al.* 2007). Although amino acids and lipids appear to be a primary source of nutrition for *P. aeruginosa* in the lung, studies also indicate that genes responsible for glycerol metabolism are deregulated in some CF isolates of *P. aeruginosa* (Son *et. al.* 2007). Constitutive expression of genes involved in glycerol metabolism suggests that glycerol could be an important nutrient for *P. aeruginosa* during chronic infection (Son *et. al.* 2007). Unfortunately, glycerol utilization by *P. aeruginosa* is poorly understood in chronic CF infection.

In this study, we examined glycerol metabolism in a CF and non-CF isolate of *P. aeruginosa*. We determined that a CF isolate of *P. aeruginosa*, FRD1, displays a growth advantage on glycerol compared to the wound isolate, PAO1. This growth advantage on glycerol correlates with the mucoid phenotype of FRD1, which results from the overproduction of alginate. This is supported by a growth analysis of CF *P. aeruginosa* isolates recovered from the lungs of a single patient. Many of the mucoid isolates from this collection show a growth

advantage on glycerol comparable to a wound isolate of *P. aeruginosa*. The mechanism by which alginate production facilitates growth on glycerol is presently unclear.

Materials and Methods

Bacterial strains, plasmids, and media.

Bacterial strains used in this study are listed in Table 3.1. Unless otherwise indicated, bacteria were cultured in L-broth (LB) or on L-agar at 37°C. No-carbon-E minimal medium (NCE) supplemented with 0.1% (w/v) casamino acids (CAA) was used to assay for growth on minimal media (Davis *et al.*, 1980). Glycerol was used at a concentration of 20 mM with or without CAA supplementation. UV-Vis absorption spectra were recorded on a Shimadzu UV-1601 Spectrophotometer using 1 cm path length cells. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at a concentration of 200 μg gentamicin (Gm) ml^{-1}. To examine growth over a 24 hour period, cultures were grown in 24 or 96 well microtiter plate and monitored at A600 with a BioTek Synergy HT plate reader (BioTek, Winooski, VT).

Biochemical assays.

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 & Jeanes, 1968) using *Macrocystis pyrifera* alginate (Sigma-Aldrich) as a standard. β-galactosidase assays were preformed as described by Miller (Miller, 1972).

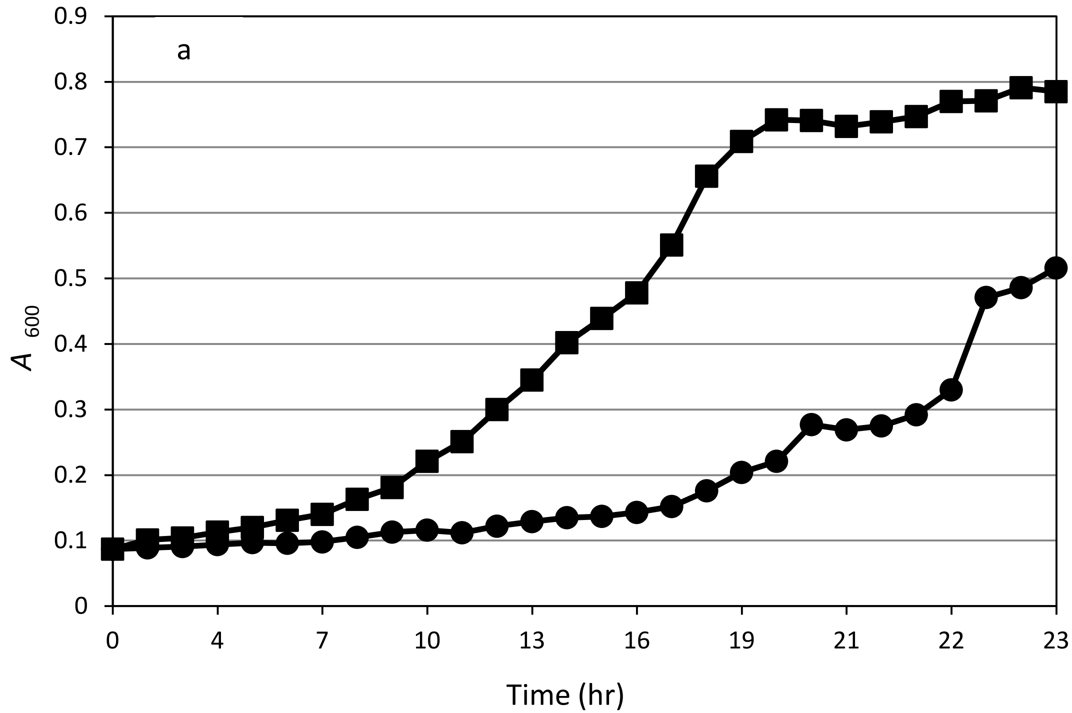
Strain or Plasmid	Genotype, relevant characteristics	Source
FRD1	CF isolate, mucoid	Ohman & Chakrabarty (1981)
PAO1	Wound isolate, nonmucoid	Holloway *et al.* (1979)
FRD1 *algT* (LS586)	FRD1 *algT101*::*aacCI*	Silo-Suh *et al.* (2002)
FRD1 *algD* (LS75)	FRD1 *algD101*::*aacCI*	Dr. Laura Silo-Suh
PAO1 *mucA* (LS856)	PAO1 *mucA101*::*aacCI*	Dr. Laura Silo-Suh
P3, P6, P13, P18-P19, P22, P24-P27	Clinical Isolates	Dr. Laura Silo-Suh
ENV2, ENV10, ENV46, ENV54	Environmental Isolates	Mahenthiralingam *et al.* (1994)
CF Isolates	Sequential Isolates	Dr. Laura Silo-Suh
FRD1 *algD*::*lacZ* (SS934)	*algD* transcriptional fusion (pSS223)	Dr. Sang-Jin Suh
PAO1 *algD*::*lacZ* (SS956)	*algD* transcriptional fusion (pSS223)	Dr. Sang-Jin Suh

Table 3.1 Bacterial strains and plasmids. Abbreviations used for genetic markers are described by Holloway *et al*. (1979). Alternate strain names are shown in parentheses.

Results

The chronic CF isolate FRD1 displays a growth advantage on glycerol compared to the acute wound isolate PAO1.

We tested the ability of FRD1 and PAO1 to use glycerol as a sole carbon source. FRD1 demonstrated an enhanced ability to grow on glycerol as a sole carbon source compared to PAO1 (Figure 3.1a). Interestingly, the addition of 0.1% casamino acids restored PAO1 growth on glycerol and boosted the growth of FRD1 (Figure 3.1b). Neither strain showed significant growth on 0.1% casamino acids alone (Figure 3.2). We considered that FRD1's enhanced growth on glycerol might involve upregulation of the *glp* operon that encodes for various proteins involved in the transport and catabolism of glycerol. However, *glpR* expression (glycerol repressor) was upregulated and *glpD* expression (glycerol dehydrogenase) was downregulated in FRD1 compared to PAO1 (Chapter 2 results), which is inconsistent with that scenario.



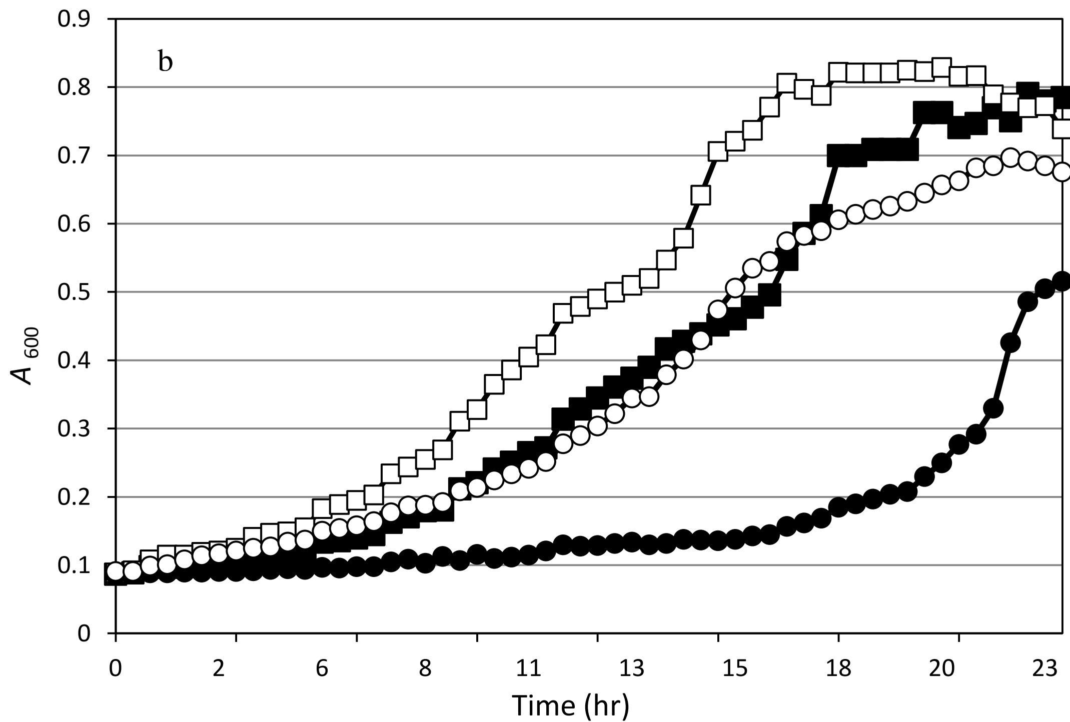
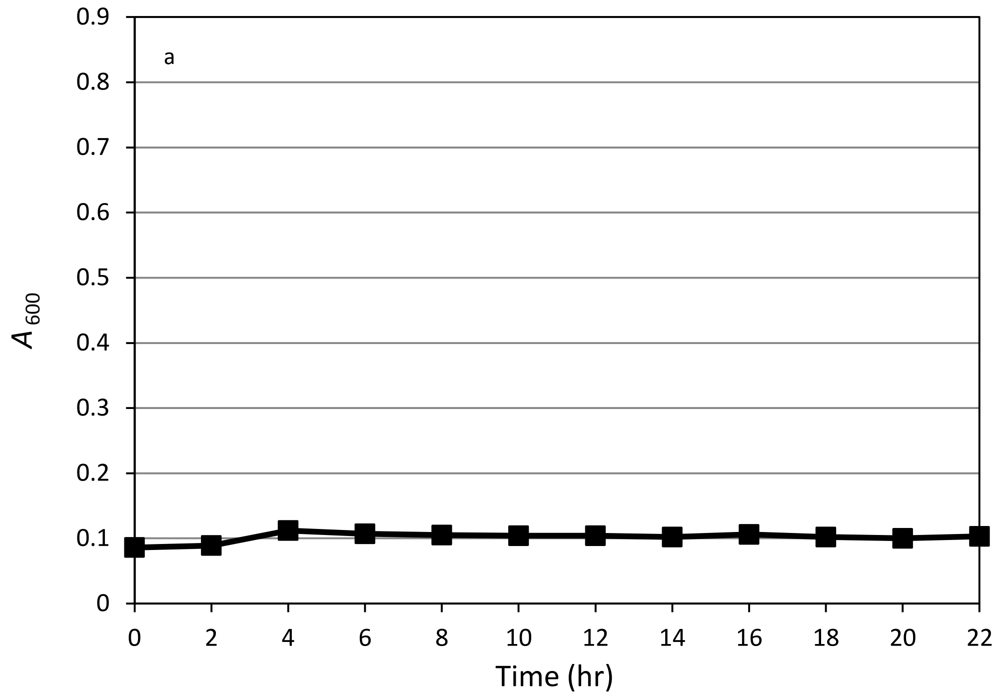
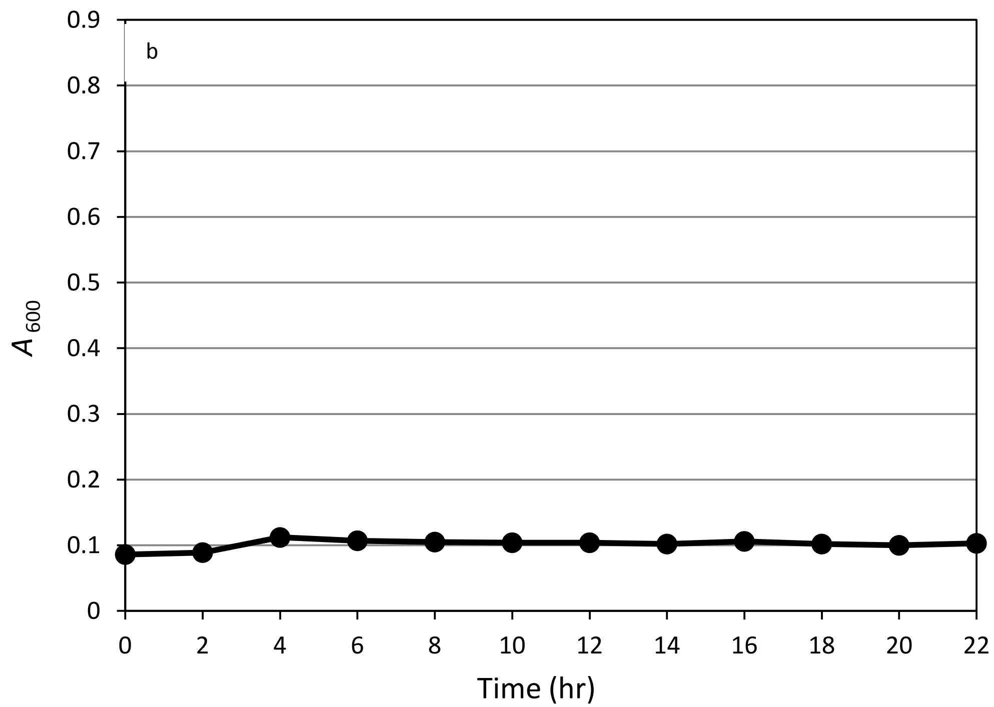


Figure 3.1 FRD1 Displays a Growth Advantage on Glycerol (a) FRD1 ■ and PAO1 ● in minimal medium with 20mM glycerol. (b) FRD1 in glycerol ■; FRD1 in glycerol + 0.1% CAA □ PAO1 in glycerol ●; PAO1 in glycerol + 0.1% CAA ○ All cultures were grown for 24 hours.





Figures 3.2a and b. FRD1 and PAO1 grown on 0.1% casamino acids.
(a) FRD1 ■ (b) PAO1 ● Cultures were grown in minimal medium with 0.1% CAA for 22 hours.

Utilization of glycerol by clinical, environmental, and sequential CF isolates of *P. aeruginosa*

To determine if enhanced growth on glycerol is common in other isolates of *P. aeruginosa*, we tested the ability of several clinical (non-CF acute clinical isolates), environmental, and sequential CF isolates of *P. aeruginosa* for the ability to use glycerol as a sole carbon source. The acute clinical and environmental isolates displayed growth similar to PAO1 on glycerol (Figure 3.3). All of the clinical and environmental isolates used in this study were non-mucoid. In contrast, some of the sequential isolates recovered from CF patients showed increased growth on glycerol at comparable levels to FRD1 (Figure 3.4) and some of these isolates were mucoid. The mucoid phenotype is caused by an overproduction of the exopolysaccharide alginate. These results suggest that the ability to efficiently utilize glycerol is an adapted phenotype that arises in chronic CF isolates of *P. aeruginosa* and may be influenced by the presence of alginate.

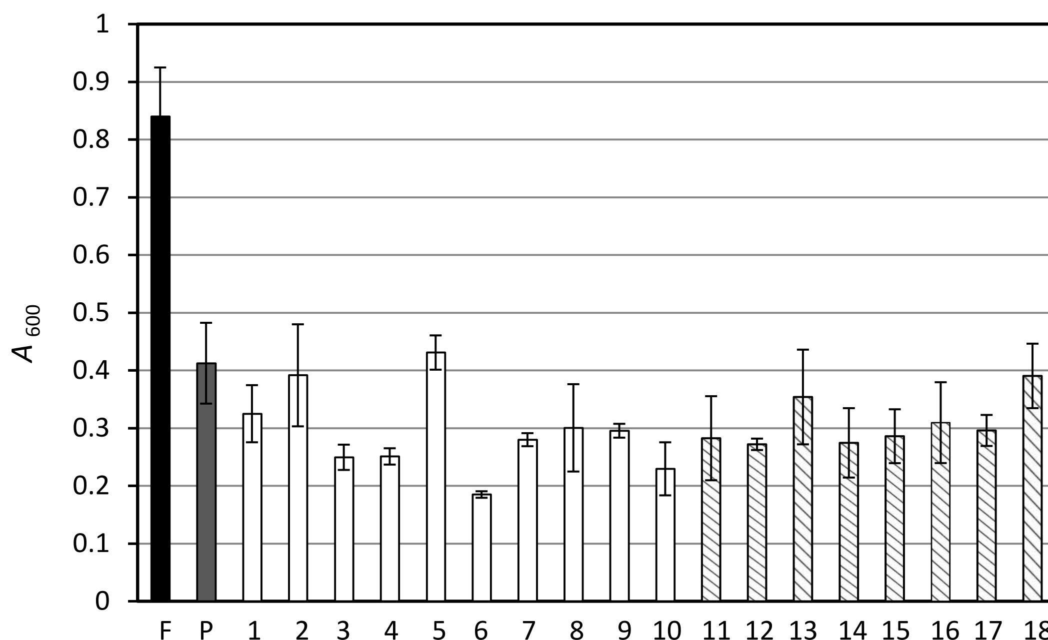


Figure 3.3 Survey of glycerol utilization in *P. aeruginosa* isolates. Cultures of *P. aeruginosa* isolates were grown in minimal medium with 20 mM glycerol for 24 hours in a 24-well microtiter plate. Values represent the average of 3 experiments. (± standard error). FRD1 ■ ; PAO1 ■; Clinical Isolates □; Environmental Isolates ▨; Lanes 1, P3; 2, P6; 3, P13; 4, P18; 5, P19; 6, P22; 7, P24; 8, P25; 9, P26; 10, P27; 11, ENV2; 12, ENV10; 13, ENV46;14, ENV54; 15, C1; 16, C2; 17, C3; 18.

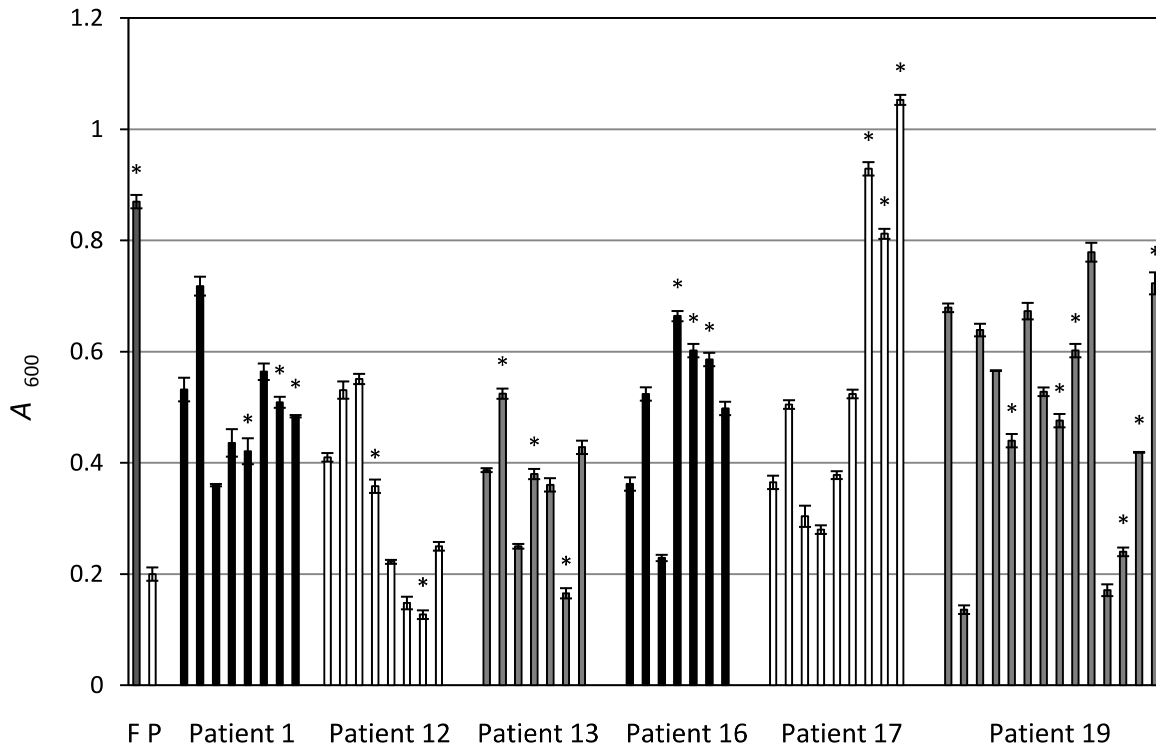


Figure 3.4 Survey of glycerol utilization in sequential *P. aeruginosa* CF isolates. Isolates were grown in minimal medium with 20 mM glycerol for 24 hours in a 24-well microtiter plate. Values represent the average of 3 experiments. (± standard error). FRD1 ■ , PAO1 □ , * = mucoid.

Alginate enhances *P. aeruginosa* growth on glycerol

FRD1 *algT* and FRD1 *algD* mutants were tested for growth on glycerol to determine if AlgT or alginate plays a role in glycerol metabolism. *algT* encodes for an alternative sigma factor that has a global effect including activation of the alginate biosynthetic genes. The alginate biosynthetic genes are located in a single operon beginning with *algD,* which encodes for GDP-mannose dehydrogenase. Disruption of *algT* or *algD* results in the loss of alginate and the mucoid phenotype by FRD1. Alternatively, deletion of *mucA*, the anti-sigma factor of AlgT, produces a mucoid phenotype in PAO1. As shown in Figure 3.5, the FRD1 *algT* and *algD* mutants were defective for growth on glycerol compared to the parental strain. In contrast, activation of *algT* in the PAO1 *mucA* mutant enhanced its growth on glycerol compared to wild-type PAO1 (Figure 3.6). These results suggest that alginate influences *P. aeruginosa* growth on glycerol. A known environmental factor that affects glycerol metabolism in bacteria is oxygen availability. Some studies suggest that glycerol might be better metabolized during anaerobic or microaerophilic growth conditions compared to aerobic growth (Durin *et. al.* 2009). The presence of alginate in the growth medium may provide an oxygen-limited environment for FRD1 and enhance glycerol catabolism. It is tempting to speculate that overproduction of alginate in the CF lung helps *P. aeruginosa* catabolize glycerol in this environment.

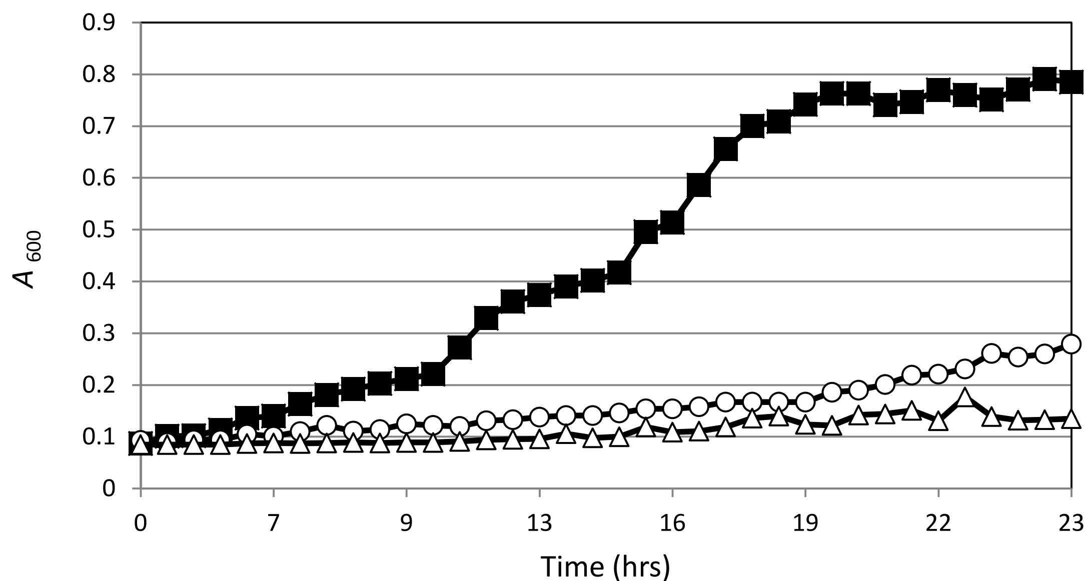


Figure 3.5 *algD* and *algT* are required by FRD1 for optimal growth on glycerol. FRD1 ■ ; FRD1 *algD* ○; FRD1 *algT*△. Cultures were grown in minimal medium with 20 mM glycerol for 24 hours.

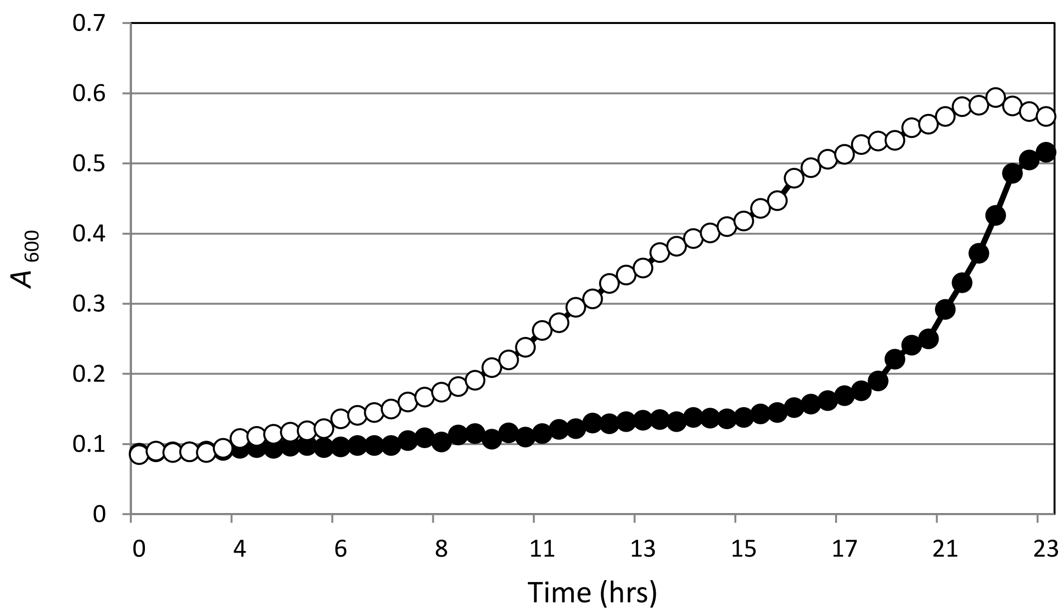


Figure 3.6 PAO1*mucA* mutants and wild-type PAO1. PAO1 ●; PAO1 *mucA* ○. Cultures were grown in minimal medium with 20 mM glycerol for 24 hours.

Glycerol promotes alginate production by PAO1

To further study the connection between glycerol metabolism and alginate, we determined whether the addition of glycerol to L-broth would increase the production of alginate by PAO1. Comparatively, FRD1 produces copious amount of alginate when grown in L-broth (Chapter 4), whereas PAO1 produces negligible amounts. In this experiment, we grew FRD1 and PAO1 on L-broth with the addition of various amounts of glycerol. Increased alginate production correlated with the addition of glycerol for both isolates (Figures 3.7 and 3.9). These results show that glycerol promotes the production of the virulence determinant, alginate in FRD1 and PAOl. While growth on glycerol would likely produce precursors for alginate production, such as DHAP and fructose-6-phosphate (Figure 3.11), efficient utilization of F-6-P for alginate production would require activation of the alginate biosynthetic operon. Therefore, we compared the expression of an *algD*::*lacZ* fusion in FRD1 and PAO1 in the presence and absence of glycerol in L-broth. As shown in Figures 3.8 and 3.10, *algD*::*lacZ* expression increased with the addition of glycerol in FRD1 and PAO1.

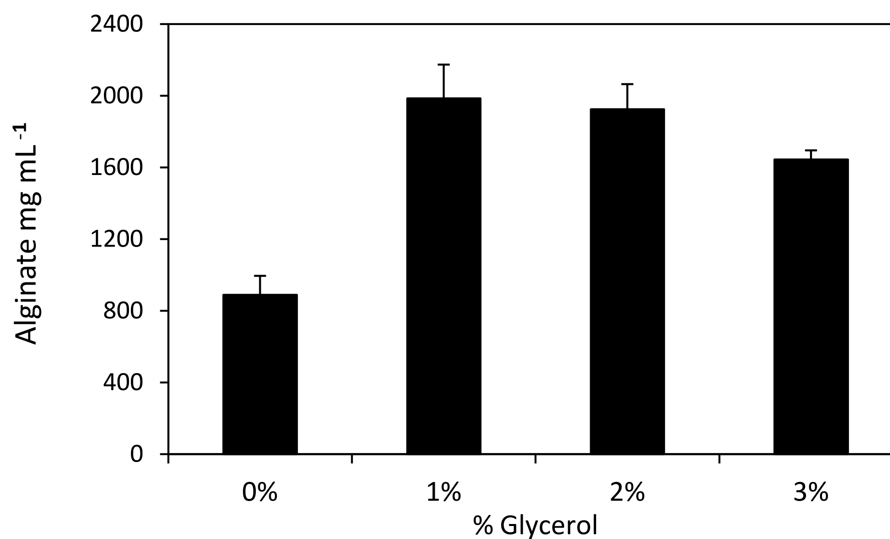


Figure 3.7 Glycerol increases alginate production in FRD1. Cultures were grown overnight in L-broth with the addition of varying concentration of glycerol. Values represent the average of 3 experiments. (± standard error).

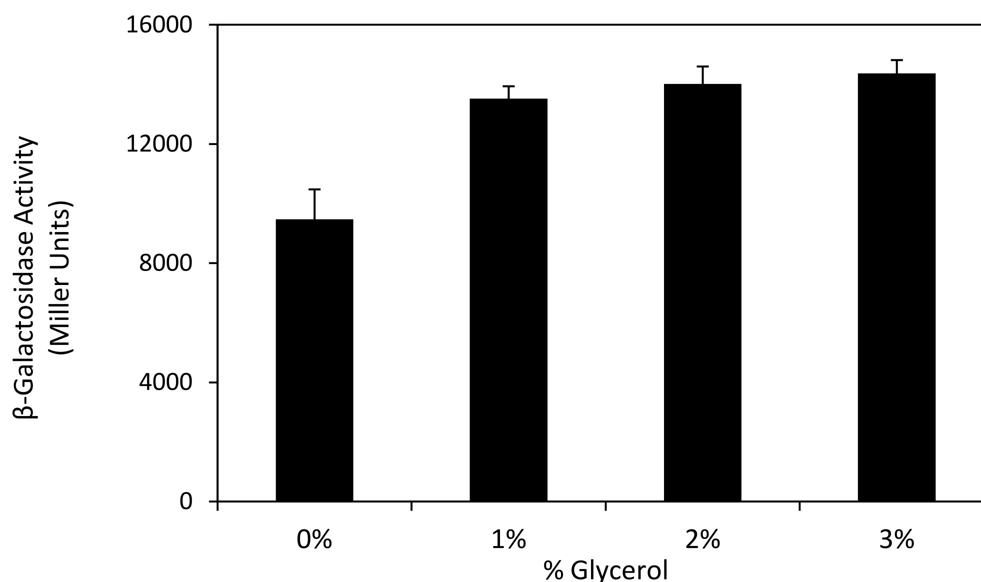


Figure 3.8 Expression of *algD* in FRD1. Cultures were grown overnight in L- broth with the addition of varying concentrations of glycerol. β-galactosidase activity is presented in Miller units. Values represent the average of 3 experiments. (± standard error).

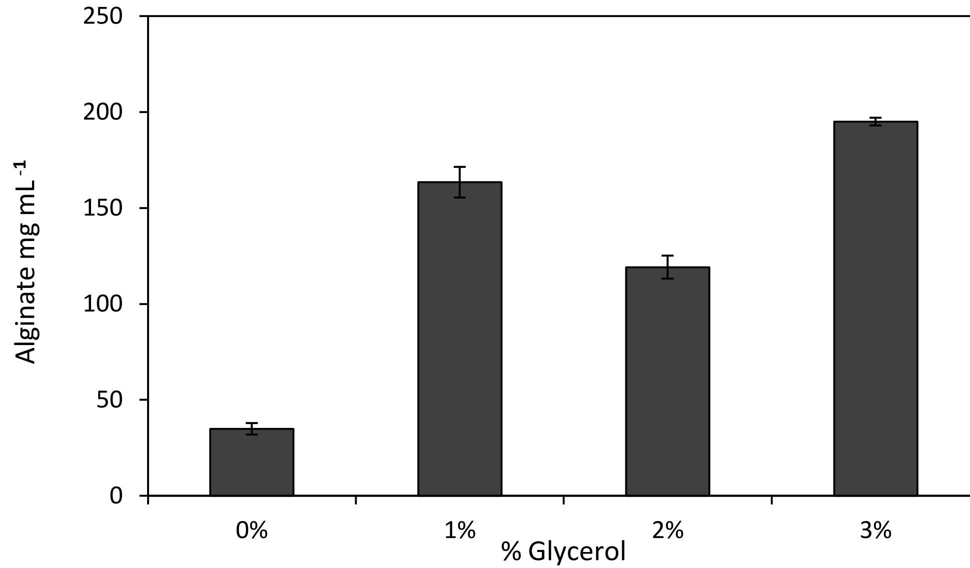


Figure 3.9 Glycerol increases alginate production in PAO1. Cultures were grown overnight in L-broth with the addition of varying concentrations of glycerol. Values represent the average of 3 experiments. (± standard error).

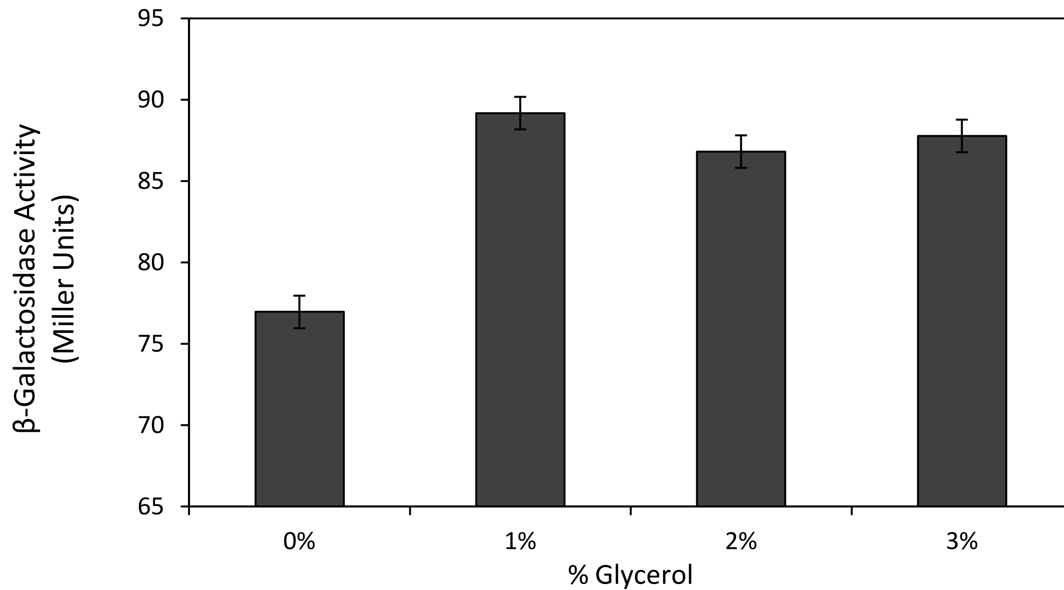


Figure 3.10 Expression of *algD* in PAO1. Cultures were grown overnight in L- broth with the addition of varying concentrations of glycerol. β-galactosidase activity is presented in Miller units. Values represent the average of 3 experiments. (± standard error).

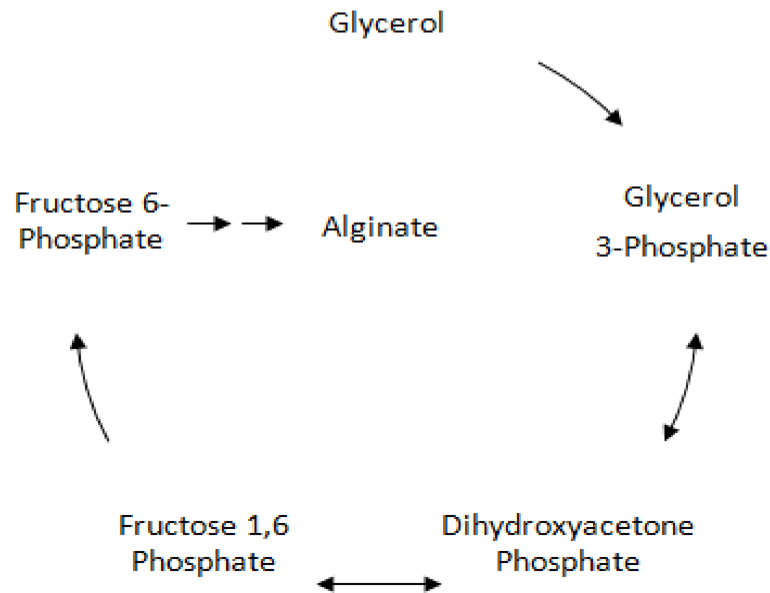


Figure 3.11 Pathway for alginate biosynthesis from glycerol.

Discussion

The ability to acquire nutrients from the host *in vivo* is essential for chronic *P. aeruginosa* isolates growing within the CF lung. CF sputum contains various nutrients that are potential carbon sources, including glycerol (Son *et. al.* 2007). In this study, we tested the ability of several *P. aeruginosa* isolates to utilize glycerol as a sole carbon source. The chronic CF isolate, FRD1, displayed a growth advantage on glycerol compared to the wound isolate, PAO1. The environmental and clinical isolates tested in this study were also deficient in their ability to use glycerol as a sole carbon source. However, several *P. aeruginosa* isolates recovered from CF patients displayed an enhanced ability to grow on glycerol, similar to that of FRD1. The ability to grow efficiently on glycerol correlated with the mucoid phenotype associated with chronic CF

isolates. Therefore, the overproduction of alginate may provide an advantage to chronic *P. aeruginosa* isolates for glycerol catabolism. In addition, glycerol catabolism can produce carbon precursors for alginate production by *P. aeruginosa*.

The conversion of *P. aeruginosa* to the mucoid phenotype in the CF lung, which results from alginate overproduction, is associated with decreased lung function and an improved ability to resist antibiotics and phagocytosis (Bragonzi *et. al*. 2009; Mahenthiralingam *et. al*. 1996). We show here that alginate facilitates the acquisition of nutrients like glycerol, and in turn, glycerol fuels the production of alginate. The CF airway contains microenvironment pockets ranging from aerobic to anaerobic (Hassett *et. al.* 2009; Schobert *et. al.* 2010). *P. aeruginosa*, which normally prefers aerobic respiration, is able to persist in anaerobic environments because it can utilize NO_3^- or NO_2^- as terminal electron acceptors, and these nitrogen sources are abundant in the CF airway (Hoffman *et. al.* 2010; Schobert *et. al.* 2010). Under these conditions, *P. aeruginosa* is able to produce alginate, which also contributes to hypoxic conditions by restricting the influx of oxygen in the airway (Hasset, 1996).

The efficient utilization of glycerol by *P. aeruginosa* appears to be dependent upon the presence of amino acids, as demonstrated by the presence of casamino acids in the growth medium in PAO1, or the production of alginate in FRD1. Although we did not measure oxygen concentrations, we speculate that PAO1 cultures grown with the addition of glycerol become microaerophilic due to the increased amount of alginate produced (Hasset, 1996). In addition to glycerol, other nutrients may be acquired anaerobically in the CF lung including arginine, and the arginine fermentation pathway is induced in this environment (Palmer *et. al.* 2005; Son *et. al.* 2007).

CF sputum contains an abundant source of fatty acids and catabolism of these nutrients would produce high concentrations of glycerol (Son *et. al.* 2007). *P. aeruginosa* derivatives that are capable of utilizing glycerol would have a growth advantage over other bacteria present in the CF lung. Interestingly, some of the genes responsible for glycerol metabolism are upregulated in *P. aeruginosa* isolates that have been recovered from the CF lung (Son *et. al.* 2007). In that study, GlpD (glycerol 3-phosphate dehydrogenase), was required for *in vivo* degradation of phosphatidylcholine (PC), which is the source of many host derived nutrients. Furthermore, *glpD* and other *glp* genes (glycerol uptake facilitator gene (*glpF*), regulator gene (*glpR)*, kinase gene (*glpK*), and the glycerol-3-phosphate transporter gene (*glpT*)), were induced when PAO1 was grown on PC (Son *et. al.* 2007).

Efficient utilization of host nutrients is essential for *P. aeruginosa* isolates growing within the CF lung. This study demonstrates that alginate helps facilitate the acquisition of certain host derived nutrients by *P. aeruginosa* and assigns another role for alginate in *P. aeruginosa* virulence. Further studies that analyze the metabolic capabilities of CF *P. aerugionsa* will provide insight as to how to target these adaptation strategies.

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Chapter 4

GlpR is Required for Virulence in a Chronic Isolate of *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa is the major etiologic agent of chronic pulmonary infections in cystic fibrosis (CF) patients. During establishment of chronic infections, the pathogen develops various strategies, including altering the expression of virulence determinants, to adapt to the infection niche. Virulence factor production is controlled by a network of transcriptional regulators or alternative sigma factors. Factors that mediate the acquisition of certain carbon sources may also play a role in the expression of key virulence determinants that are involved in chronic CF infection. For this study we focused on characterizing the role of GlpR in virulence factor production by an acute (PAO1) and a chronic (FRD1) isolate of *P. aeruginosa*. GlpR is a transcriptional repressor that is required for glycerol metabolism in *P. aeruginosa.* The FRD1 *glpR* mutant was decreased for production of pyocyanin, pyoverdine and rhamnolipids compared to the parent strain. In the alfalfa seedling infection assay, the FRD1 *glpR* mutant was severely decreased in its ability to cause disease. Interestingly, the *glpR* mutation had no significant effect on PAO1 virulence. Finally, our data indicate that GlpR is involved in the emergence of persister cells. In summary, GlpR plays several important roles in the pathogenesis of CF *P. aeruginosa*.

Introduction

Chronic infections caused by *Pseudomonas aeruginosa* are the major cause of lung dysfunction and mortality in cystic fibrosis (CF) patients. The ability of *P. aeruginosa* to cause or maintain an infection is dependent upon a large number of regulatory genes that control the expression of virulence determinants (Carterson *et. al.* 2003; Heurlier *et. al.* 2003; Juhas *et. al.* 2004; Suh *et. al.* 1999). *P. aeruginosa* produces several cell-associated and extracellular virulence factors (e.g., exotoxin A, exoenzyme S, cytotoxin, proteases, lipases, pyocyanin, rhamnolipids and phospholipases) that contribute to pathogenesis. However, the expression of these genes varies between acute and chronic infections (Bragonzi *et. al.* 2009; Govan, 1996; Nguyen *et. al.* 2006). Nutrient acquisition can also influence virulence factor production suggesting that some virulence factors may be coordinately regulated by nutritional sources. For example, *Cryptococcus neoformans* mutants that are unable to utilize glucose are severely reduced for virulence (Price *et. al.* 2011). Furthermore, genes responsible for arginine acquisition are required for the expression of several virulence genes in *Streptococcus pneumonia* (Kloosterman and Kuipers, 2011).

As shown in Chapter 3, *P. aeruginosa* variants that efficiently catabolize glycerol arise and persist within the CF lung. Catabolism of glycerol enhances alginate production suggesting virulence of CF *P. aeruginosa* may depend upon the presence of this nutrient within the CF lung. In fact, glycerol metabolism can play a role in virulence factor production and virulence by bacteria. For example, the *glpD* gene, which encodes glycerol-3-phosphate oxidase, is required for toxicity and the production hydrogen peroxide by *Mycoplasma pneumoniae* (Hames *et. al.* 2008). In the same study, *glpF* and *glpK*, which encode for a glycerol facilitator and the glycerol kinase, respectively, were also necessary for pathogenicity (Hames *et. al.* 2008). GlpR is a

transcriptional regulator that negatively controls the genes required for glycerol metabolism in *P. aeruginosa*, which are located in the *glp* regulon (Schweizer *et. al.* 1996). The FRD1 *glpR* mutant displayed a white colony phenotype on agar plates in contrast to the blue-green color normally seen with the parental strain (data not shown). This suggested a loss of pyocyanin (blue fluorescence) and pyoverdine (green fluorescence) production. Therefore, we examined the role of GlpR in the production of various virulence determinants by *P. aeruginosa.*

Materials and Methods

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this study are listed in Table 4.1. Unless otherwise indicated, bacteria were cultured in L-broth (LB) or on L-agar at 37°C. UV-Vis absorption spectra were recorded on a Shimadzu UV-1601 Spectrophotometer using 1 cm path length cells. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations in this study: Gentamicin at 200 μg/ml and ofloxacin at 10 μg/ml for *P. aeruginosa*.

Alfalfa seedling infection assay.

Seeds of alfalfa variety 57Q77, a wild-type strain not bred for pest resistance, were provided by Pioneer Hi-Bred International. The alfalfa seedling infection assay was conducted as previously described (Silo-Suh *et al.*, 2002) with the following modifications: FRD1 and derivatives were inoculated onto wounded alfalfa seedlings using ~10^5 colony forming units (CFU) per seedling while PAO1 and derivatives were inoculated using ~10^4 CFU per seedling. Water agar plates containing inoculated seedlings were sealed with Parafilm and placed in a

30°C incubator without light. Disease symptoms were scored 6–7 days following inoculation by visual inspection. Seedlings with symptoms of infection were scored positive. FRD1, FRD1 *glpR*, PAO1 and PAO1 *glpR* were tested on 50 seedlings for each experiment. Data were expressed as the mean ± standard error and analyzed for significance using an ANOVA (InStat; Graph Pad Software). A value of $P<0.05$ was considered significant.

Biochemical assays.

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 & Jeanes, 1968) using *Macrocystis pyrifera* alginate (Sigma-Aldrich) as a standard. Pyocyanin was purified and measured from 20 h cultures as described by Essar *et al.* (1990). Pyoverdine was measured as previously described by Suh *et al.*, 1999 with several modifications. Briefly, *P. aeruginosa* was grown in Kings B medium for 16 to 17 h at 37°C with aeration. The culture supernatants were serially diluted in 10 mM Tris-HCl (pH 7.5) and excited at 400 nm, and the emission at 460 nm was recorded using Black 96 welled Costar plates read in a BioTek Synergy HT. Cyanide was assayed according to Carterson *et al.* (2004), as previously reported (Hagins *et al.* 2009), and normalized to CFU of bacteria recovered from each Pseudomonas Isolation Agar plate. Cyanide levels were quantified by comparison with KCN standards using the same protocol and presented as micromoles per 10^9 CFUs. Rhamnolipid was purified and measured as previously described (Du Plessis, 2005).

Persister cell assay.

Persistence was measured by determining survival upon exposure to antibiotics in a time dependent manner as described by Mulchaly *et. al.* (2010). Briefly, 16 hour cultures of *P. aeruginosa* derivatives were washed, diluted, and inoculated into 3 mL's of Mueller-Hinton Broth. Prior to exposure with oflaxicin, samples were washed and plated on Mueller-Hinton agar plates to calculate CFUs. Samples exposed to oflaxicin for 8 hours were treated in the same manner. Samples were allowed to grow on Mueller-Hinton plates for 48 hours before CFUs were calculated.

Strain or Plasmid	Genotype, relevant characteristics	Source
FRD1	CF isolate, mucoid	Ohman *et. al.* (1981)
PAO1	Wound isolate, nonmucoid	Holloway *et al.* (1979)
FRD1 *glpR* (JS134)	FRD1*glpR101*::*aacCI*	Chapter 2
PAO1 *glpR* (JS97)	PAO1*glpR101*::*aacCI*	Chapter 2

Table 4.1 Bacterial strains and plasmids. Abbreviations used for genetic markers are described by Holloway *et al*. (1979). Alternate strain names are shown in parentheses.

Results

GlpR is required for optimal pyoverdine and pyocyanin production by FRD1

Pyocyanin and pyoverdine are two quorum sensing-regulated virulence factors that are important for establishing infection (Govan *et. al.* 1996). We tested the effect of the *glpR* mutation on pyocyanin and pyoverdine production in PAO1 and FRD1. There was a three-fold

decrease in pyoverdine production and a two-fold decrease in pyocyanin production by the FRD1 *glpR* mutant compared to the parental strain (Figures 4.1 and 4.2). However, the PAO1 *glpR* mutant was slightly enhanced for the production of both products compared to its parental strain (Figures 4.1and 4.2). Therefore, both virulence factors are differentially regulated by GlpR in the chronic and acute isolates of *P. aeruginosa*.

GlpR is required for optimal rhamnolipid production but not hydrogen cyanide production by FRD1 and PAO1

Rhamnolipids are quorum sensing regulated virulence factors that promote infiltration and adherence of *P. aeruginosa* in the CF airway (Zulianello *et. al.* 2006). Glycerol can provide intermediates for biosynthesis of rhamnolipids (Figure 4.5). However, in the absence of glycerol, upregulation of the biosynthetic genes for glycerol metabolism may divert intermediates away from rhamnolipid production. This scenario is consistent with our observation that the FRD1 *glpR* mutant did not produce detectable rhamnolipid under the conditions tested while the PAO1 *glpR* mutant was decreased two-fold for rhamnolipid production compared to the parental strain (Figure 4.3). Hydrogen cyanide (HCN) production is enhanced in some *P. aeruginosa* isolates recovered from the CF lung and HCN production is associated with decreased cellular functions (Ryall *et. al.* 2008; Hagins *et. al.* 2009). In contrast to the other virulence factors tested, there was a moderate increase in hydrogen cyanide production by the FRD1 *glpR* and PAO1 *glpR* mutants compared to their parental strains (Figure 4.4).

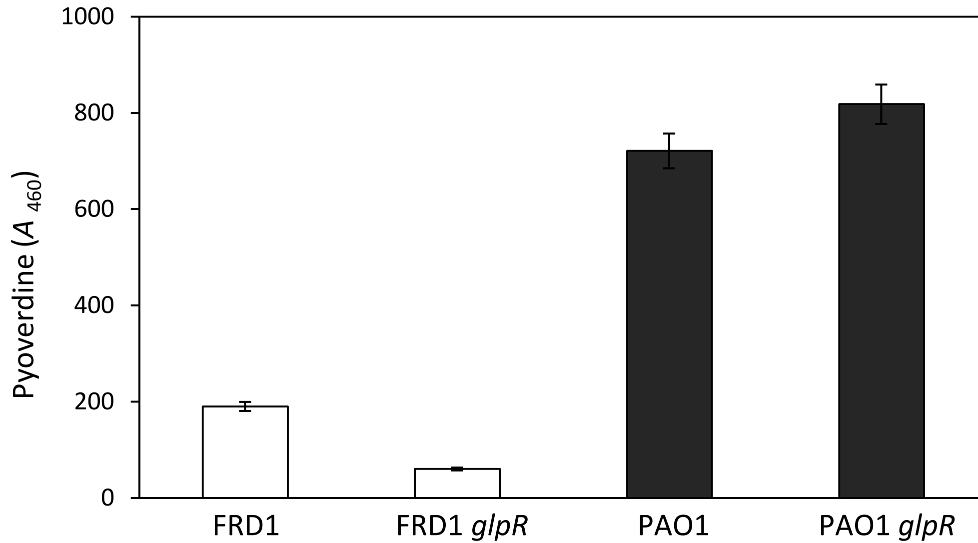

Figure 4.1 The effect of *glpR* on pyoverdine production in FRD1 and PAO1. Cultures were grown in L-broth overnight and assayed for pyoverdine production. Data represent the average of 3 experiments ± standard error.

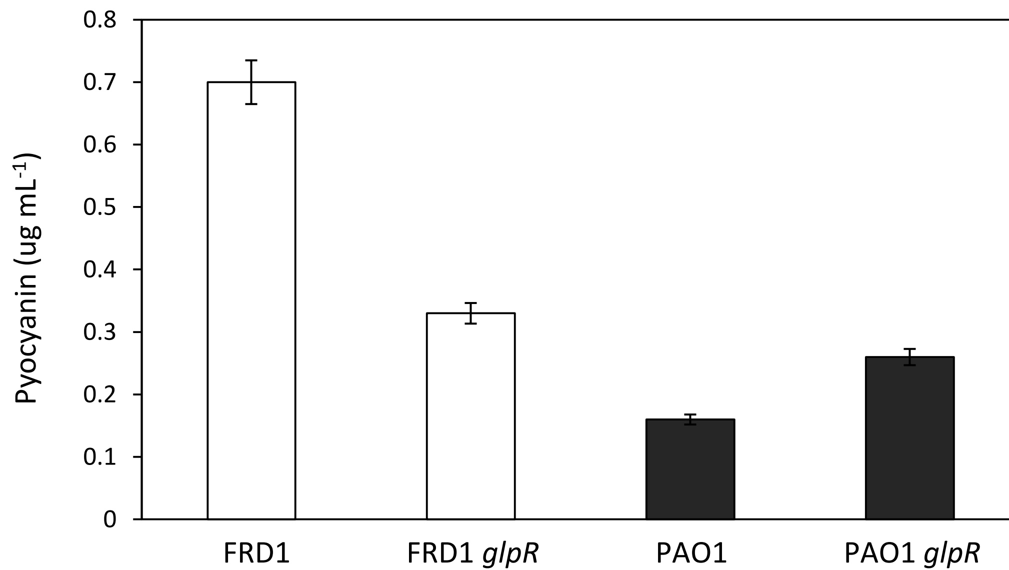

Figure 4.2 The effect of *glpR* on pyocyanin production in FRD1 and PAO1. Cultures were grown in L-broth overnight and assayed for pyocyanin. Data represent the average of 3 experiments ± standard error.

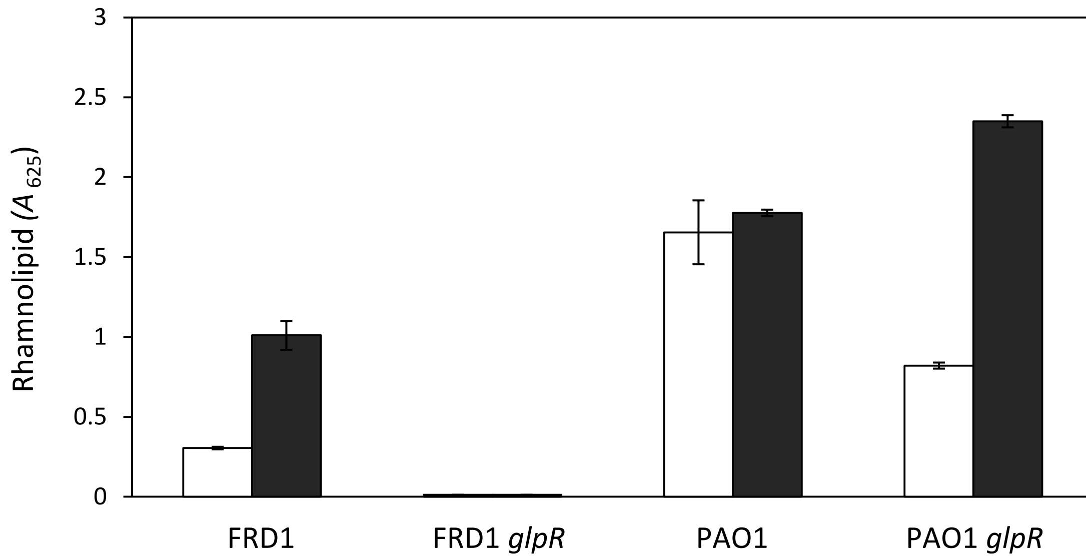

Figure 4.3 The effect of *glpR* on rhamnolipid production in FRD1 and PAO1. Cultures were grown in L-broth □ or L-broth + 1% glycerol ■ overnight and assayed for rhamnolipid production. Data represent the average of 3 experiments ± standard error.

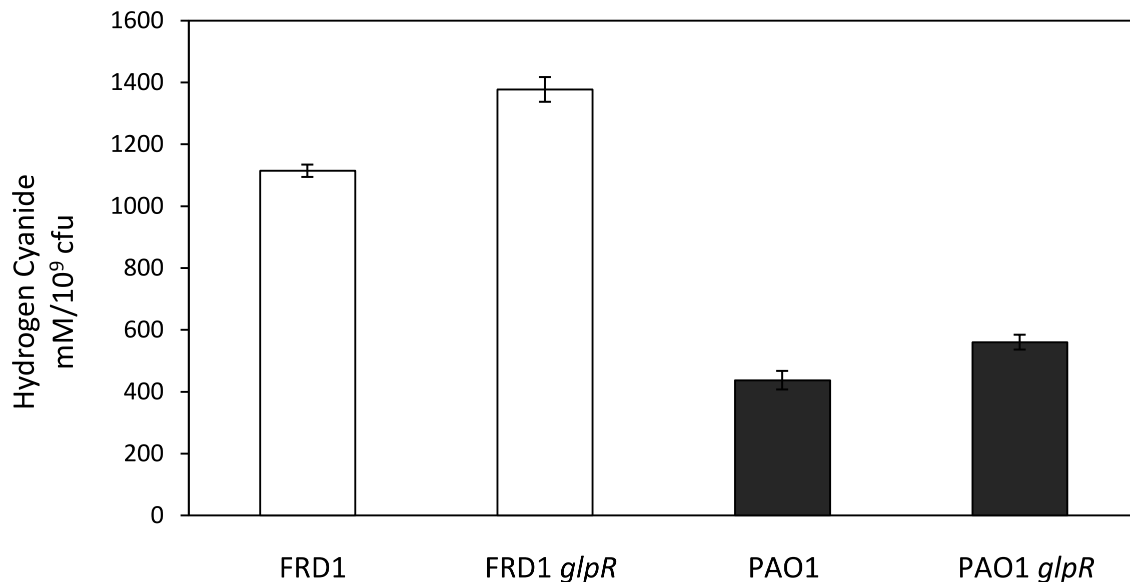

Figure 4.4 The effect of *glpR* on hydrogen cyanide production in FRD1 and PAO1. Cultures were grown in L-broth overnight and assayed for hydrogen cyanide production. Data represent the average of 3 experiments ± standard error.

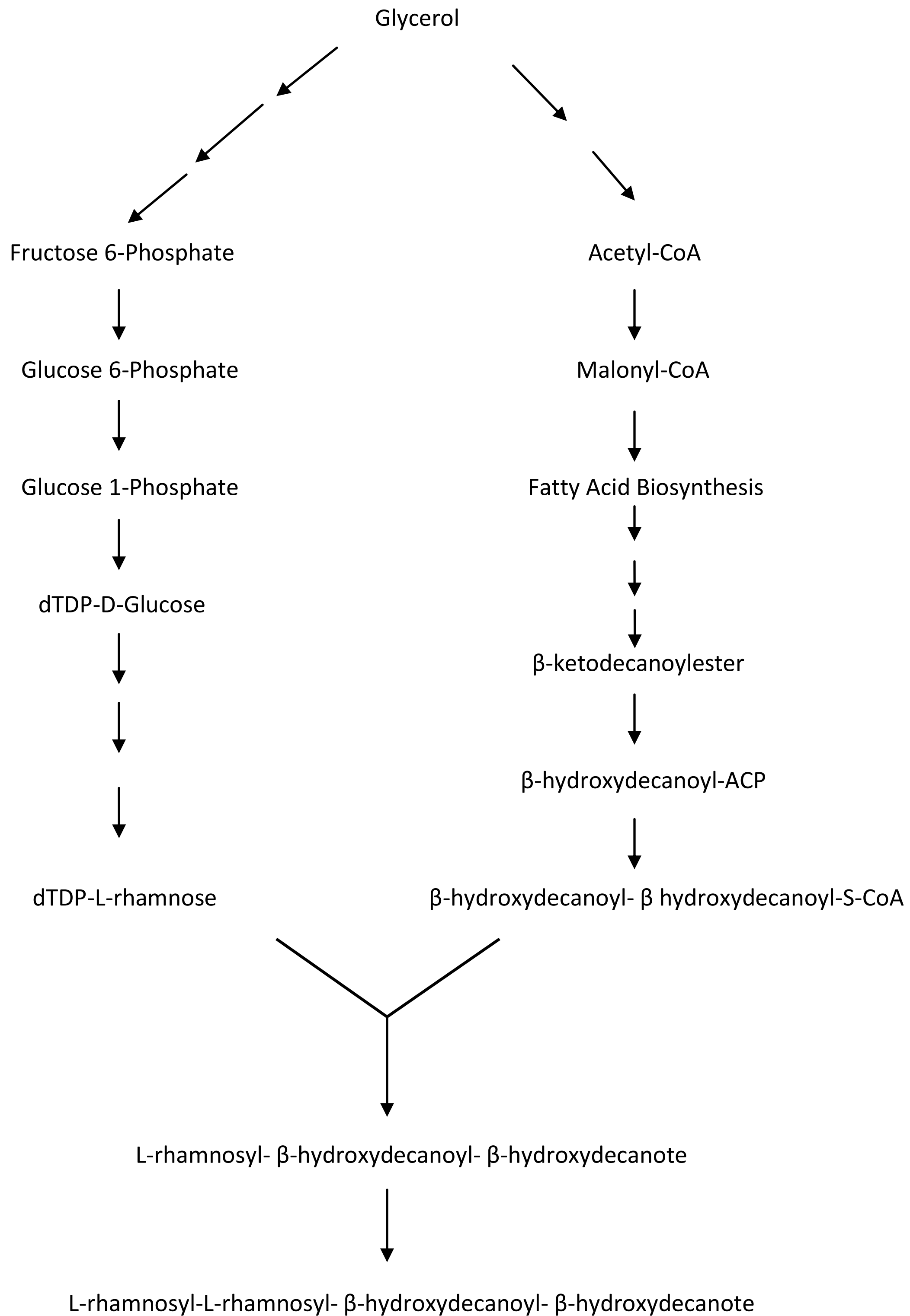

Figure 4.5. Rhamnolipid biosynthesis from glycerol. (Template courtesy of Dr. Sang-Jin Suh)

The FRD1 *glpR* mutant overproduces alginate

Alginate is an exopolysaccharide that is often produced by CF *P. aeruginosa* isolates, including FRD1. Alginate protects *P. aeruginosa* in a variety of ways during infection such as inhibiting the penetration of antibiotics and promoting the formation of biofilms in the CF lung (Govan *et. al.* 1996). It was previously reported that some genes in the *glp* regulon are required for alginate production. Schweizer *et. al.* (1995) reported that insertions in *glpD* had a polar effect on *glpM,* which abolished alginate production in *P. aeruginosa* when grown on certain carbon sources. In our study, a mutation in *glpR* resulted in an increase in alginate production by the FRD1 *glpR* mutant (Table 4.2).

GlpR is required for FRD1 virulence on alfalfa

We questioned whether reduced virulence factor production by the FRD1 *glpR* mutant affected its ability to cause disease. As shown in Table 4.2, *glpR* is required for FRD1, but not PAO1, virulence on alfalfa.

Strain[1]	**% Alfalfa Seedling Infection**	**Alginate (mg·mL^{-1})**
FRD1	90±4	782±98
FRD1 *glpR101*::*aacCI*	29±4	1004±56
PAO1	93±3	NA
PAO1 *glpR*::*aacCI*	83±1	NA

Table 4.2 GlpR is required for optimal infection of CF isolates on alfalfa seedlings and alginate is increased in the FRD1 *glpR* mutant. 1. *aacCI* gentamicin resistant cassette. Values represent the number of seedlings showing maceration symptoms and is the average of 3 experiments with 50 seedlings each. ± Standard error.

Loss of GlpR increases persister cell formation by FRD1 and PAO1

Persister cells are near-dormant bacterial populations that are resistant to killing by antibiotics and contribute to the recalcitrance of many chronic infections (Lewis 2010). In this study, the FRD1 *glpR* and PAO1 *glpR* mutants showed increased resistance to killing by oflaxacin compared to the parent strains. These results are consistent with a previous study that showed overexpression of *glpD*, which encodes for a glycerol 3-phosphate dehydrogenase, resulted in increased tolerance to ampicillin and oflaxacin in *E. coli* (Spoering *et. al.* 2006). Loss of GlpR would relieve repression of the *glp* regulon and activate *glpD* expression in *P. aeruginosa*. Previous studies show that persister cells arise in the lung and are a common occurrence among late chronic isolates of *P. aeruginosa* (Mulcahly *et. al.* 2010). However, in contrast to published reports, the CF adapted isolate FRD1 did not produce more persister cells than PAO1 (Figure 4.6). Taken together, the results suggest that catabolism of glycerol in the CF lung may facilitate persister formation by *P. aeruginosa*.

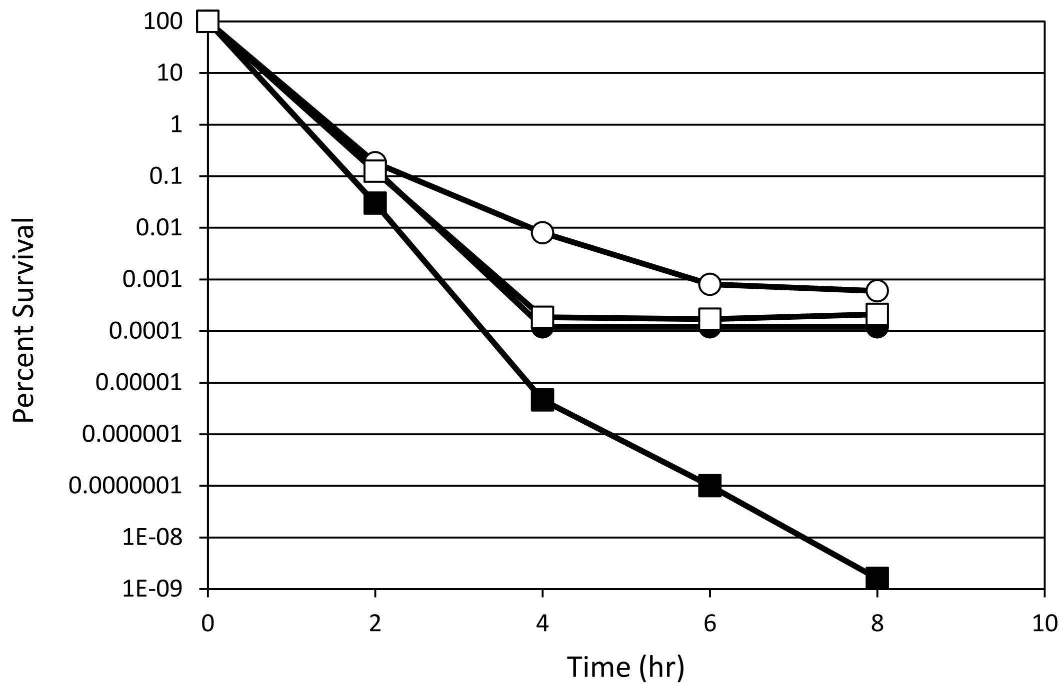

Figure 4.6 The Effect of *glpR* on Persister Cell Formation by FRD1 and PAO1. Data represent the average of 4 experiments. FRD1 ■ FRD1 *glpR* □ PAO1 ● PAO1 *glpR* ○

Discussion

In this study, we examined the contribution of GlpR to virulence by both a chronic CF and an acute wound isolate of *P. aeruginosa*. Loss of GlpR affected the production of several virulence determinants with a more pronounced effect on FRD1 compared to PAO1. This was reflected in the significant loss of virulence by the FRD1 *glpR* mutant in the alfalfa seedling model of infection compared to the parent strain and to PAO1. Further analysis will have to determine whether virulence factors such as pyoverdine or pyocyanin are regulated at the level of gene expression or by the re-routing of carbon sources.

The virulence factors regulated by GlpR are predicted to be affected by growth of *P. aeruginosa* on glycerol (Figure 4.7). Interestingly, the given profile mimics the known phenotype of CF adapted *P. aeruginosa* isolates in that some virulence factors are downregulated and others such as alginate, HCN production and persister cell formation are upregulated. Therefore, initiation of these phenotypes within the CF lung may begin in the early non-adapted bacteria by the presence of glycerol as a nutritional source in this environment. This begs the question of whether the CF adapted phenotype is merely the consequence of isolates adapted to use glycerol more efficiently as a carbon source.

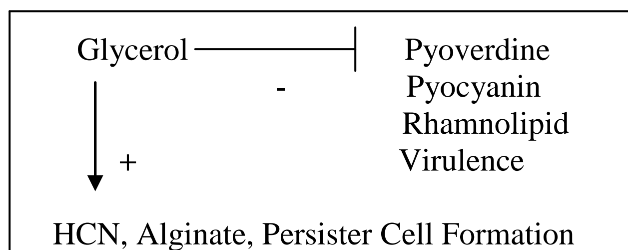

Figure 4.7. **Summary of virulence phenotypes regulated by GlpR and glycerol catabolism**.

This is the first study to characterize the role of GlpR in virulence of *P. aeruginosa*. The results from this study show that GlpR appears to be required for the expression of several acute virulence determinants, particularly by FRD1. This was not an unexpected results based on the requirement for GlpR by other bacteria for virulence. Expression of *glpR* is upregulated almost three-fold during otitis media infection by *Haemophilus influenza* (Mason *et. al.* 2003). In *Mycoplasma pneumoniae*, GlpD (glycerol 3-phosphae dehydrogenase) and GlpQ (glycerophosphodiesterase) are required for cytotoxicity (Hames *et. al.* 2009; Schmidl *et. al.* 2011). Furthermore, glycerol metabolism results in the production of hydrogen peroxide, which is the major virulence factor in *M. pneumonia*e (Halbedel *et. al.* 2007).

GlpR controls the expression of genes involved in glycerol catabolism and may regulate virulence factors such as alginate and rhamnolipids by affecting carbon intermediates. In this study, the addition of glycerol to the growth medium was able to alleviate the effect of a *glpR* mutation on rhamnolipid production by the acute isolate PAO1. Interestingly, the addition of glycerol to growth medium was able to diminish the effects of stress and compromised fitness in *prfA* mutants in *Listeria monocytogenes* (Bruno and Freitag, 2001). PrfA is global transcriptional regulator of virulence in *L. monocytogenes* (Bruno and Freitag, 2001). Similar to the FRD1 *glpR* mutant, *prfA* mutants are severely attenuated in virulence (Leimeister-Wachter *et. al.* 1990).

The expression of virulence factors appears to be strongly modulated by carbon source availability, particularly in the host (Milenbachs *et. al.* 1997; Stoll *et. al.* 2008; Weir *et. al.* 2008). For example, sulfate and phosphates have an effect on alginate and exotoxin A production in *P. aeruginosa* (Weir *et. al.* 2008). In addition, increasing concentrations of iron

reduce the production of elastase, toxin A, pyocyanin, and other extracellular virulence determinants produced by *P. aeruginosa* (Sokol *et. al.* 1982). In the CF lung, glycerol, amino acids, and fatty acids are major nutritional sources for *P. aeruginosa*, as evident by the constitutive expression of genes involved in the metabolism of these carbon sources (Palmer *et. al.* 2005; Son *et. al.* 2007). During infection, the ability to metabolize certain host derived nutrients appears to signal specific bacterial responses that influence the differential expression of virulence genes or pathogenicity. For example, loss of *dadA*, a gene involved in alanine catabolism in *P. aeruginosa*, exhibits reduced competitive fitness in a rat lung model of infection (Boulette *et. al.* 2009). In addition, functional histidine catabolic genes are required for optimal infection of eukaryotic hosts and expression of Type III secretion genes in *P. aeruginosa* (Rietsch *et. al.* 2004). Moreover, the regulator involved in fatty acid metabolism, FadR, is required for *Vibrio vulnificus* to cause disease in mammalian hosts (Brown and Gulig, 2008).

The correlation that exists between carbon metabolism and virulence suggests that bacteria rely heavily on central catabolic genes to initiate or persist during an infection. The overlap in regulatory networks that govern the expression of virulence determinants and carbon metabolism appears to be beneficial for the adaptation of *P. aeruginosa* in various niches, particularly during chronic infection.

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Chapter 5

This chapter contains published and unpublished miscellaneous results

Introduction

We determined previously that the glyoxylate pathway becomes deregulated in some isolates of *P. aeruginosa* that have adapted to the CF lung, including FRD1. The occurrence of these isolates suggests deregulation of the glyoxylate pathway may benefit *P. aeruginosa* growing within the CF lung. However, the mechanism(s) responsible for the deregulation of this pathway have yet to be elucidated. This chapter, outlined in three data sections, details some of the preliminary studies that lead to the search for the mechanism of deregulation of the glyoxylate pathway.

1. Expression of *rpoN* in FRD1 and PAO1

Published in: Hagins, J. M., **J. A. Scoffield**, S.-J. Suh, and L. Silo-Suh. 2010. Influence of RpoN on isocitrate lyase activity in *Pseudomonas aeruginosa*. *Microbiology* (Reading, England) **156**:1201-10.

Summary

We previously reported that RpoN negatively regulated *aceA* expression and ICL activity in the acute isolate PAO1. Expression of *rpoN* was analyzed in FRD1 and PAO1 to support the regulation data.

2. Preliminary Characterization of Malate Synthase in *P. aeruginosa*

Published in: Hagins, J., and **J. Scoffield**. 2011. Malate synthase expression is deregulated in the *Pseudomonas aeruginosa* cystic fibrosis isolate FRD1. *Canadian Journal of Microbiology* **195**:186-195.

Summary

P. aeruginosa establishes life-long chronic infections in the CF lung by utilizing various strategies for adaptation. Some of these strategies include upregulation of *glcB*, which encodes for malate synthase (MS). However, regulation of *glcB* expression is poorly understood. The goal of this analysis was to better understand the regulation of *glcB* in order to provide clues to its role (s) in *P. aeruginosa* pathogenesis.

3. Unpublished Data

Summary

This section discusses the attempt to identify the mechanism of *aceA* deregulation in FRD1. We first measured *aceA* expression in *P. aeruginosa* derivatives that were disrupted for known regulators of ICL or carbon catabolism.

Materials and Methods

Bacterial strains and plasmids used in this study are listed in Table 5.1. Leucine was used at 1%, arabinose at 20mM, and Palmitic acid was dissolved in 10% (w/v) Brij 58 solution and used at 2.5 mM (pH with 1M KOH), Heptanoic acid was used at 5 mM. PA1015, PA3508, PA3604, and PA4341 are transposon insertion mutants that were purchased from the University of Washington Pseudomonas mutant stock center.

Strain or Plasmid	Genotype, relevant characteristics	Source
FRD1	CF isolate, mucoid	Ohman *et. al.* (1981)
PAO1	Wound isolate, nonmucoid	Holloway *et al.* (1979)
FRD1 *glcB* (JH104)	FRD1 *glcB101*::*aacCI*	Hagins *et. al.* 2011
PAO1 *glcB* (JH105)	PAO1 *glcB101*::*aacCI*	Hagins *et. al.* 2011
FRD1 *glcB*+ (JH148)	FRD1 *glcB* complemented for *glcB*	Hagins *et. al.* 2011
PAO1 *glcB*+ (JH151)	PAO1 *glcB* complemented for *glcB*	Hagins *et. al.* 2011
FRD1 *glcB*::*lacZ* (JH133)	FRD1 carrying *glcB*::*lacZ* fusion	Hagins *et. al.* 2011
PAO1 *glcB*::*lacZ* (JH135)	PAO1 carrying *glcB*::*lacZ* fusion	Hagins *et. al.* 2011
FRD1 *aceA glcB* (LS1916)	FRD1 *glcB101*::*aacCI aceA102*::*tetA*	Hagins *et. al.* 2011
PAO1 *aceA glcB* (LS1917)	PAO1 *glcB101*::*aacCI aceA102*::*tetA*	Hagins *et. al.* 2011
PAO1 *rpoN*::*lacZ*	PAO1 carrying *rpoN*::*lacZ* fusion	Hagins *et. al.* 2011
FRD1 *rpoN*::*lacZ*	FRD1 carrying *rpoN*::*lacZ* fusion	Hagins *et. al.* 2011
PAO1 *lrp*	PAO1 *lrp101*::*aacCI*	Dr. Laura Silo-Suh
PA1015 (*iclR1*)	Mutation in a probable IclR transcriptional regulator	Univ. of Washington
PA3508 (*iclR2*)	Mutation in a probable IclR transcriptional regulator	Univ. of Washington
PA3604 (*erdR*)	Mutation in rsponse regulator *erdR*	Univ. of Washington
PA4341 (*iclR3*)	Mutation in a probable IclR transcriptional regulator (*iclR3*)	Univ. of Washington
PA1015 (*iclR1*) *aceA*::*lacZ*	PA1015 carrying *aceA*::*lacZ* fusion	This study
PA3508 (*iclR2*) *aceA*::*lacZ*	PA3508 carrying *aceA*::*lacZ* fusion	This study
PA3604 (*erdR*) *aceA*::*lacZ*	PA3604 carrying *aceA*::*lacZ* fusion	This study
PA4341 (*iclR3*) *aceA*::*lacZ*	PA4341 carrying *aceA*::*lacZ* fusion	This study

Table 5.1 Bacterial strains and plasmids. Abbreviations used for genetic markers are described by Holloway *et al*. (1979). Alternate strain names are shown in parentheses.

Results

1. Published Results

Expression of *rpoN* in PAO1 and FRD1

In Hagins *et. al.* 2010, we reported that RpoN negatively regulates *aceA* expression and ICL activity in PAO1. However, expression of *aceA*::*lacZ* was much higher in FRD1 compared with the PAO1 *rpoN* mutant (Hagins *et. al.* 2010). This suggests that deregulation of RpoN-mediated repression is not solely responsible for high expression of *aceA* in FRD1. To confirm that our data were not due to differential expression of *rpoN* between PAO1 and FRD1, we analyzed *rpoN*::*lacZ* expression in both strains. As shown in Figure 5.1, the *rpoN*::*lacZ* transcriptional fusion was expressed at a slightly lower level in FRD1 compared with PAO1 throughout a growth cycle, and therefore it is unlikely to account for the nine-fold difference in ICL activity between the two isolates.

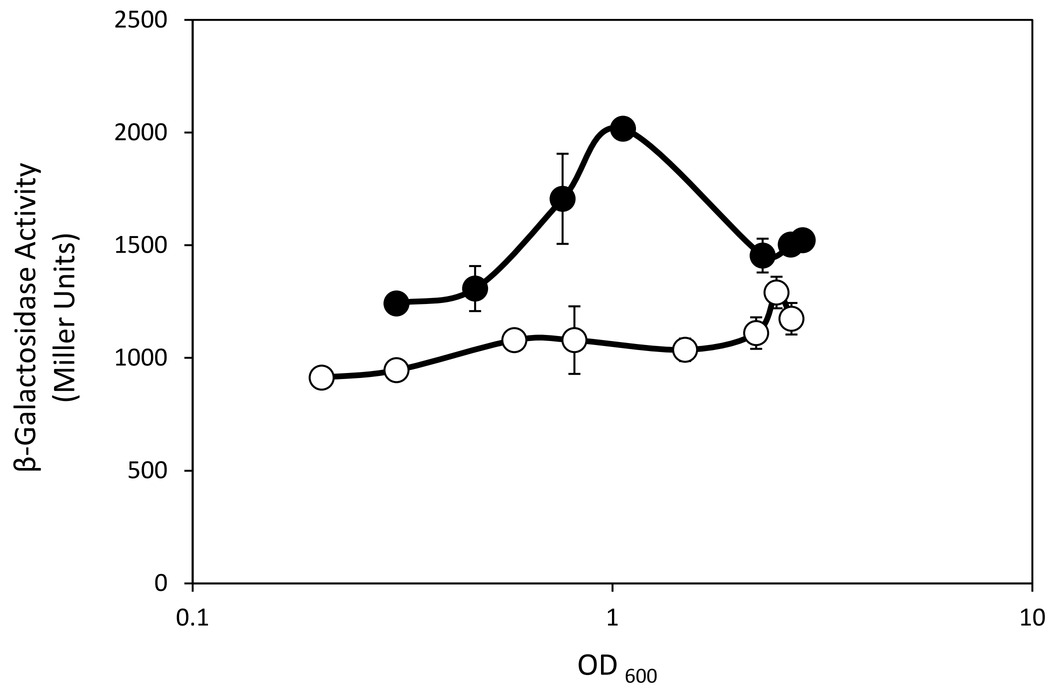

Figure. 5.1. Expression of an *rpoN*::*lacZ* transcriptional fusion. β-Galactosidase assays were conducted using cultures grown in L-broth. Activity is expressed in Miller units and is an average of 3 experiments conducted in duplicate. FRD1 ○ PAO1 ●

2. Published Results

glcB expression is deregulated in FRD1

To initiate characterization of *glcB* expression in *P. aeruginosa*, a *glcB*::*lacZ* transcriptional fusion was constructed and introduced into the FRD1 and PAO1 genomes. As shown in Figure 5.2, expression of *glcB*::*lacZ* is significantly higher in FRD1 than in PAO1 over a growth cycle when grown in L-broth. Because a peptide-rich medium such as L-broth has not previously been shown to induce *glcB* expression in bacteria, this result suggested that regulation of *glcB* is altered in FRD1. Alternatively, upregulation of *glcB* in FRD1 may be a consequence of high glyoxylate concentrations provided by increased *aceA* activity in this isolate. However, the *glcB*::*lacZ* fusion retained high expression even in the FRD1 *aceA* mutant, suggesting that *glcB* is not induced by high glyoxylate concentrations in the parental background (Figure 5.2). The promoter of *glcB* from FRD1 is identical to the published PAO1 sequence for over 200 bp upstream of the open reading frame (data not shown). Therefore, the simplest explanation for altered *glcB* regulation in FRD1 compared with PAO1 is the loss of a negative regulator. We previously demonstrated that *aceA*, which encodes for the first enzyme of the glyoxylate pathway in *P. aeruginosa*, is also deregulated in FRD1. Because *aceA* and *glcB* are both single open reading frames and located distantly from each other in the *P. aeruginosa* genome, it is likely these genes share a common regulatory mechanism that became altered following adaptation of *P. aeruginosa* to the CF lung.

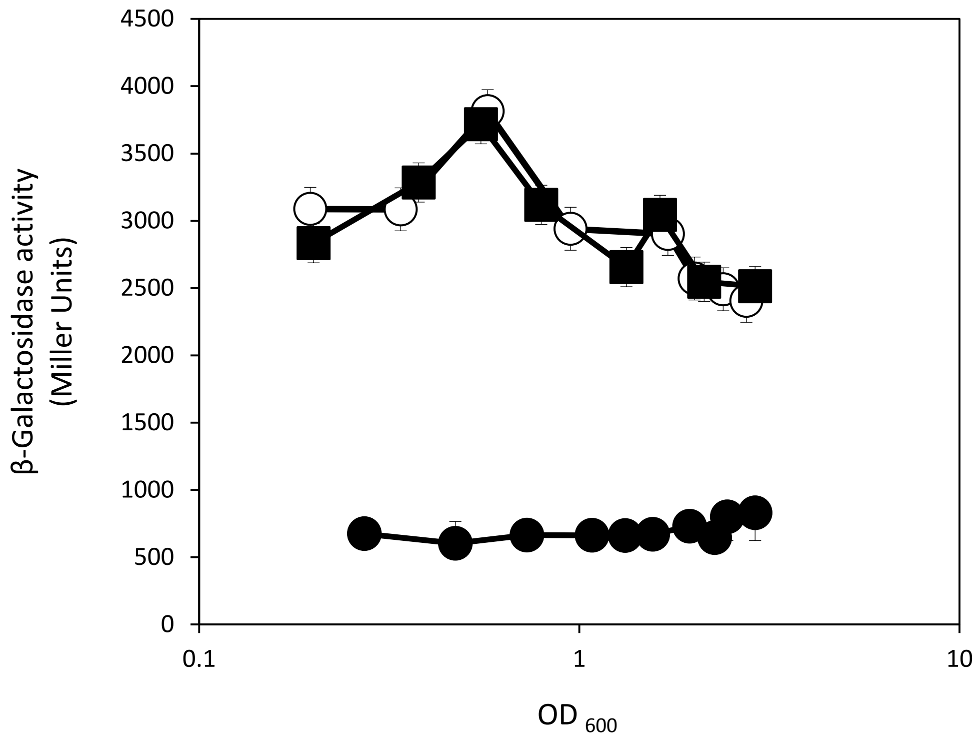

Figure 5.2. ***glcB*::*lacZ* expression in FRD1 and PAO1.** β-Galactosidase assays were conducted using cultures grown in L-broth. Activity is expressed in Miller units and is an average of 3 experiments conducted in duplicate. FRD1 ○ FRD1 *aceA* ■ PAO1 ●

Effect of carbon sources on *glcB* expression

MS encoded by *aceB* has been shown to be regulated by acetate, fatty acids, glyoxylate, and glycolate (Pellicer *et. al.* 1999; Lorca *et. al.* 2007), while MS encoded by *glcB* has been shown to be induced by arabinose, glycolate, glyoxylate, or acetate (Pellicer *e.t al.* 1999; Garcı´a-de los Santos *et. al.* 2002). To more accurately determine the effect of carbon sources on *glcB* transcription in *P. aeruginosa*, expression studies were carried out on FRD1 and PAO1 carrying a *glcB*::*lacZ* transcriptional fusion and grown in the presence of different carbon compounds. Several compounds known to induce *aceA* also induced *glcB* expression in the PAO1 background, including leucine, heptanoic and palmitic acid (Figure 5.3). In contrast, *glcB* expression was highly expressed in FRD1 in most of the compounds tested, suggesting that expression of this gene is largely deregulated in FRD1 (Figure 5.3). *P. aeruginosa* failed to grow appreciably in minimal medium with either glyoxylate or glycolate as the sole carbon source, preventing evaluation of these compounds as sole inducers of *glcB* expression (data not shown).

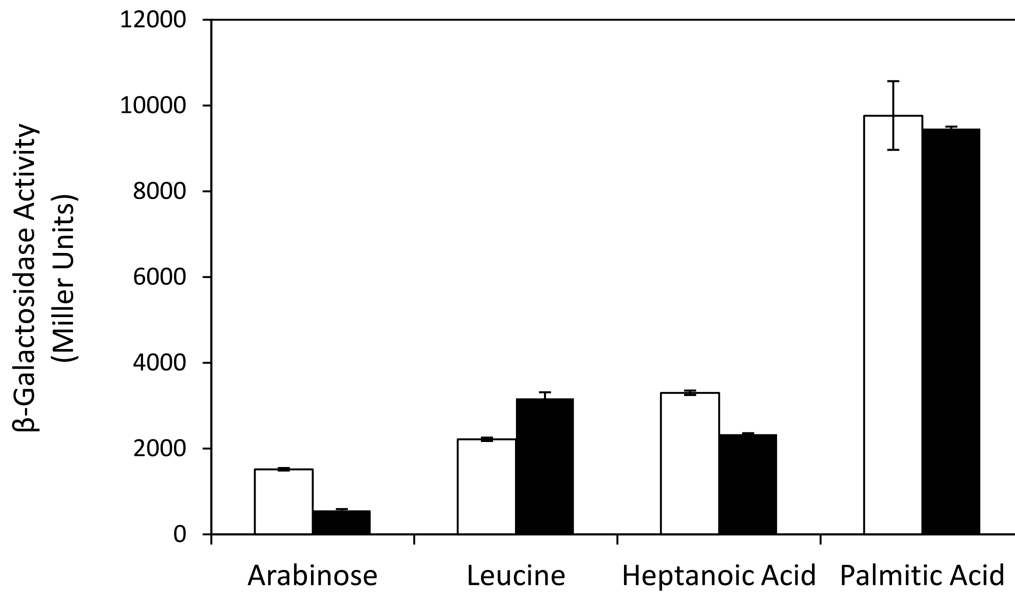

Figure 5.3. Effect of various carbon sources on *glcB*::*lacZ* expression in FRD1 and PAO1. β-Galactosidase assays were conducted using cultures grown in L-broth. Activity is expressed in Miller units and is an average of 3 experiments conducted in duplicate. FRD1 ☐ PAO1 ■

Malate synthase is required for hydrogen cyanide but not alginate production by *Pseudomonas aeruginosa*.

We observed that disruption of *glcB* in *P. aeruginosa* led to reduced production of hydrogen cyanide but not alginate (Figures 5.4 and 5.5). Glyoxylate formed by ICL appears to be converted to glycine, which is the preferred substrate for hydrogen cyanide synthase (Castric 1977; Hagins *et. al.* 2009). While loss of MS activity would likely lead to increased glyoxylate, high glyoxylate concentrations inhibit HCN production, possibly by competing with glycine for binding to HCN synthase (Hagins et al. 2009). Therefore, the reduced HCN production by the *P. aeruginosa glcB* mutants compared with the parental strains likely results from increased cellular glyoxylate concentrations. Consistent with this hypothesis, the double *aceA glcB* mutant in the PAO1 background show higher HCN production than the single *glcB* mutant. This suggests that disruption of *aceA* alleviates the build-up of cellular glyoxylate concentrations formed in the absence of MS activity. Higher HCN production is not observed in the FRD1 double mutant background compared with FRD1. However, this was not an unexpected result because disruption of *aceA* reduces HCN in FRD1 but does not affect HCN production by PAO1 (Hagins *et. al.* 2009). Complementation of the FRD1 and PAO1 *glcB* mutants with a wild-type copy of the gene from FRD1 under the control of a regulatable promoter restored HCN activity in both backgrounds.

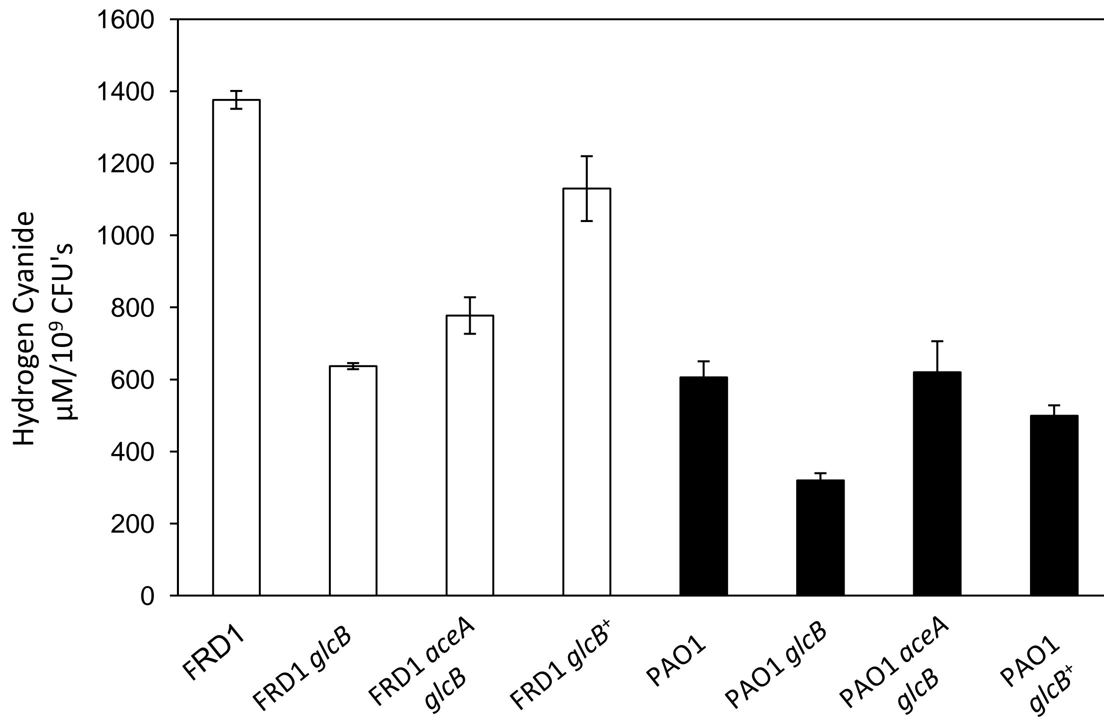

Figure 5.4. Malate synthase is required for hydrogen cyanide production by *Pseudomonas aeruginosa*. Cyanide concentrations were normalized to the colony-forming units (CFUs) of bacteria recovered from Pseudomonas Isolation Agar plates. Values represent the mean ± standard error of 2 independent experiments conducted in duplicate.

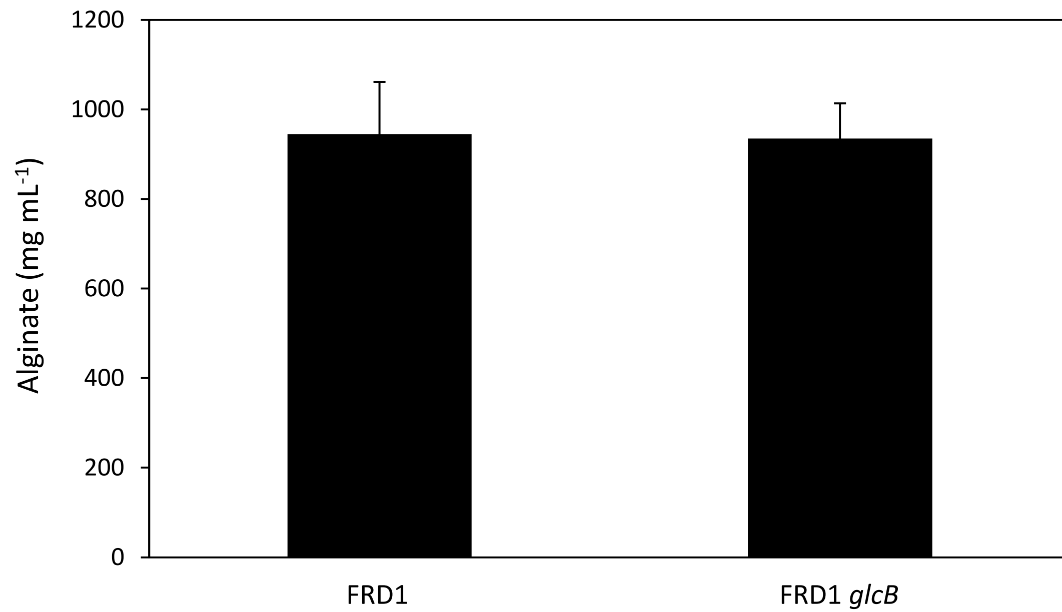

Figure 5.5. Malate synthase is not required for alginate production by FRD1. Alginate was assayed from cultures grown in L-broth and values represent the mean ± standard error of 3 experiments.

3. Unpublished Results

Isocitrate Lyase Regulation

Deregulation of the glyoxylate pathway in FRD1 suggests the loss of a negative regulator as FRD1 adapted to the CF lung. In an attempt to identify the mechanism responsible for the deregulation of the glyoxylate pathway in FRD1, we tested the effect of several mutations in transcriptional regulators on *aceA* expression and ICL activity in PAO1. In *E. coli*, IclR regulates the gloxylate pathway in conjunction with FadR (Gui *et. al.* 1996). Therefore, we tested the effect of mutations in three *iclR* homologs on *aceA*::*lacZ* expression in PAO1. Disruption of three *iclR* homologs resulted in a significant reduction in *aceA*::*lacZ* expression in PAO1, which suggests that these genes may play a role in activating the glyoxylate pathway (Figure 5.6). Similar results were seen in a mutation in the response regulator *erdR* (PA4341). ErdR regulates the *acsA* gene, which is required for acetate activation in *P. aeruginosa* (Kretzschmar *et. al.* 2010). Due to the induction of *glcB* expression on leucine, we also tested the effect of a mutation in *lrp* on ICL activity. Lrp (Leucine-responsive Regulatory Protein) proteins are global transcriptional regulator involved in cellular metabolism and respond to exogenous amino acids (Brinkman *et. al.* 2003). As shown in figure 5.7, disruption of *lrp* resulted in an increase in ICL activity in PAO1, which suggests a possible role in the negative regulation of the glyoxylate pathway. However, sequence analysis of the *lrp* gene from FRD1 revealed only 3 silent mutations, therefore, it is unlikely that this regulator is responsible for deregulation of the glyoxylate pathway in FRD1 (data not shown).

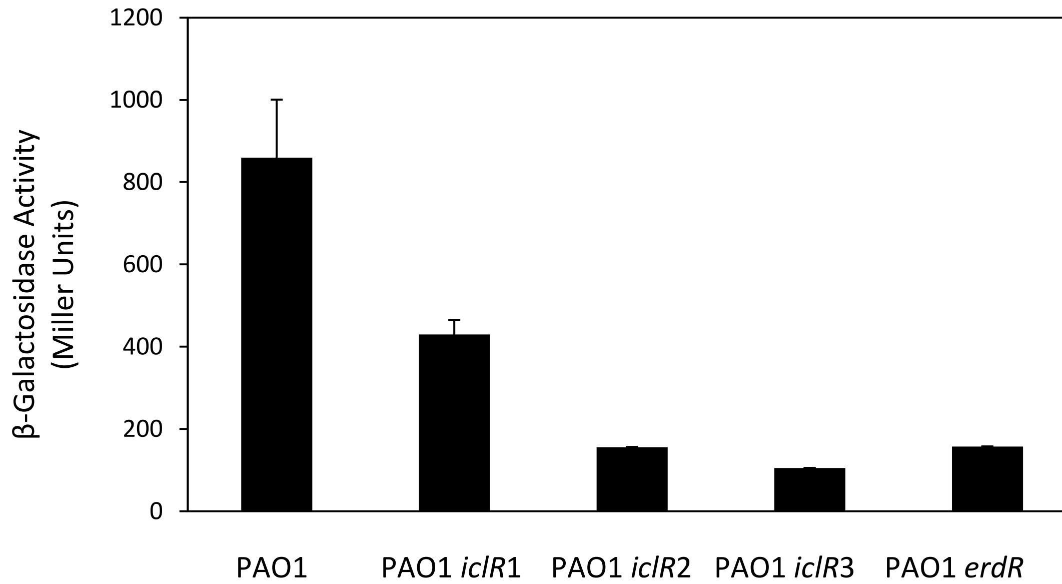

Figure 5.6. Effect of various mutations on *aceA*::*lacZ* expression in *P. aeruginosa*. β-Galactosidase activity was assayed from overnight cultures of *P. aeruginosa* grown in LB. Values represent the mean (±standard error) of two experiments conducted in duplicate.

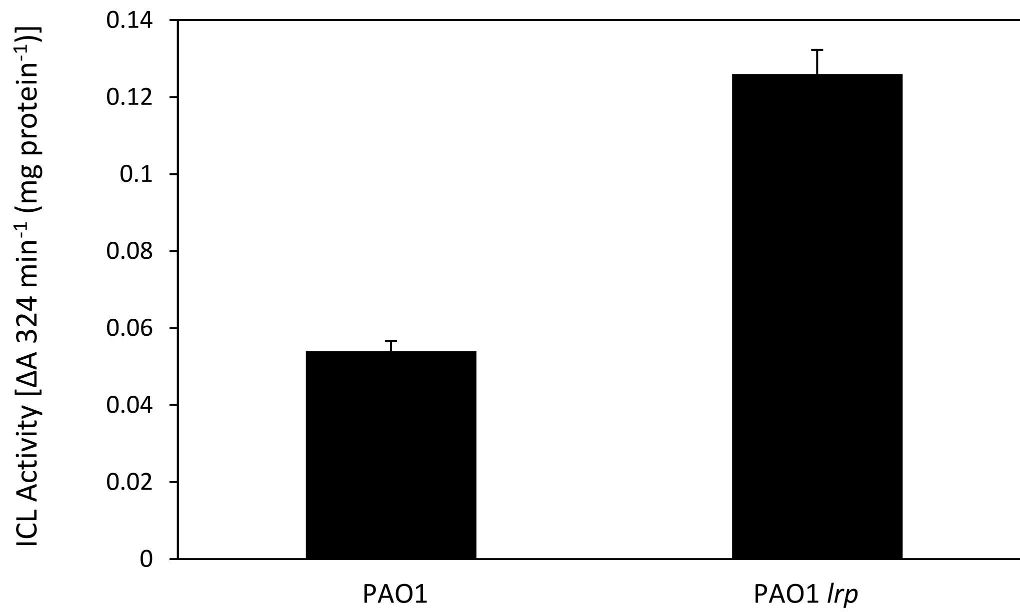

Figure 5.7. Effect of a mutation in the *lrp* gene on ICL activity in PAO1. Malate synthase activity was assayed from overnight cultures of *P. aeruginosa* grown in LB. Values represent the mean (± standard error) of two experiments.

Discussion

Initial characterization of malate synthase revealed that it is deregulated in a chronic isolate of *P. aeruginosa*. In addition, *glcB* expression was induced on carbon sources that require the glyoxylate pathway. Although MS was required for the optimal production of hydrogen cyanide, it was not required for alginate production. In an attempt to identify the mechanism responsible for deregulation of the glyoxylate pathway, we tested the effect of several mutations in *iclR* homologs, *erdR*, and *lrp* on *aceA* expression or ICL activity. All of the *iclR* homologs increased *aceA* expression, which suggests they probably play a role in the activation of the glyoxylate pathway. Similar results were seen in the *erdR* mutant. However, a mutation in *lrp*, which encodes for the leucine-responsive regulatory protein, showed increase ICL activity, which suggests a role in negative regulation, however, LRP appeared to be unaltered in FRD1.

The glyoxylate pathway has been shown to be important for pathogenesis of several microorganisms. We reported that ICL in *P. aeruginosa* is required for alginate production and for virulence in the rat lung and alfalfa models of infection (Lindsey *et. al.* 2008), in addition to optimal production of hydrogen cyanide (Hagins *et. al.* 2009). In two separate studies, *Mycobacterium tuberculosis* and *Rhodococcus equi* strains lacking ICL were demonstrated to be reduced for virulence (McKinney *et. al.* 2000; Wall *et. al.* 2005). In addition, ICL was required for persistence of *M. tuberculosis*. Similarly, ICL is required for persistence in *Burkholderia pseudomallei* during pulmonary melioidosis infection (Schaik *et. al.* 2009). Taken together, these studies demonstrate that the glyoxylate pathway is not only important for growth on certain carbon sources but also for virulence and persistence.

Several transcriptional regulators of the glyoxylate pathway have been identified in other bacteria. For example, in *E. coli*, the glyoxylate pathway is controlled by two regulators, FadR and IclR (Gui *et. al.* 1996). However, neither appears to regulate the glyoxylate pathway in *P. aeruginosa* (Hagins). In addition, RamB has been shown to regulate the glyoxylate pathway in *M. tuberculosis* and *Corynebacterium glutamicum* (Cramer *et. al.* 2007; Micklinghoff *et. al.* 2009). To date, putative regulators of the glyoxylate pathway have not been identified with the exception of RpoN, an alternative sigma factor (Hagins *et. al*. 2010) in *P. aeruginosa*. Identification of the mechanism(s) responsible for the deregulation of the glyxoylate pathway in the chronic *P.aeruginosa* isolate, FRD1, would give a clearer indication of how *P. aeruginosa* uses this pathway to persist in the lung and perhaps lead to the development of improved therapies.

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Chapter 6

Conclusion and Future Directions

Conclusion

The improved effectiveness and development of novel therapies used to treat chronic bacterial infections is dependent upon the ability to enhance our understanding of the intricate mechanisms used by bacteria to cause infection and persist in the host. *P. aeruginosa* establishes life-long chronic infections in the CF lung by utilizing various strategies for adaptation (Bragonzi *et. al.* 2009). One strategy used by some chronic isolates of *P. aeruginosa*, including FRD1, may include the upregulation of the genes encoding for the glyoxylate pathway enzymes, *aceA* and *glcB,* which encode for isocitrate lyase (ICL) and malate synthase (MS), respectively (Hagins *et. al.* 20010, 2011; Lindsey *et. al.* 2008). In addition, ICL has also been shown to be required for the optimal production of alginate and hydrogen cyanide, which are two virulence determinants commonly found within the CF lung (Hagins *et. al.* 2009; Lindsey *et. al.* 2008). Furthermore, ICL is required for infection in the rat lung and alfalfa models of infection (Lindsey *et. al.* 2008). Previous data suggest deregulation of the glyoxylate pathway may benefit *P. aeruginosa* growing within the CF lung. However, the mechanism(s) responsible for the deregulation of these genes have yet to be elucidated. The initial goal of my project was to determine the mechanism of regulation of the glyoxylate pathway in an acute isolate (PAO1) and CF isolate (FRD1) of *P. aeruginosa*. In an attempt to identify the mechanism of deregulation of the glyoxylate pathway I first tested IclR homologs that are known to regulate the glyoxylate

pathway in other bacteria. In *E. coli, aceA* is negatively regulated by FadR and IclR (Gui *et. al.* 1996). However, none of the IclR homologs were found to be negative regulators of the glyoxylate pathway in *P. aeruginosa*. Eventually, I determined that GlpR, a negative regulator of glycerol metabolism (Schweizer *et. al.* 1996), also negatively regulates the glyoxylate pathway in PAO1. However, GlpR was not responsible for the deregulation of the glyoxylate pathway in FRD1. My second objective was to characterize glycerol metabolism in several *P. aeruginosa* isolates. I discovered that some CF isolates, such as FRD1, are able to utilize glycerol more efficiently as a carbon source than non-CF isolates. Isolates that were able to grow proficiently on glycerol displayed the mucoid phenotype and it was determined that the overproduction of alginate was responsible for enhanced growth on glycerol. Alginate provides micraerophilic growth conditions and it is likely that *P. aeruginosa* metabolizes glycerol better when oxygen is limited. Genes involved in glycerol metabolism have been shown to be upregulated in the CF lung (Son *et. al.* 2007). In addition, glycerol is a likely nutritional source for *P. aeruginosa* growing within the CF lung. The enhanced ability to metabolize glycerol by CF isolates may provide some benefit to *P. aeruginosa.*

The third goal of my project was to characterize the role of GlpR in *P. aerugionsa* virulence. I determined that GlpR is required for optimal virulence in the chronic CF isolate, FRD1, and that loss of *glpR* increases the formation of persister cells in both FRD1 and PAO1. Further analysis is needed to determine if GlpR regulates these virulence factors directly, or by re-routing carbon intermediates. This was the first study to demonstrate the potential connection between fatty acid and glycerol metabolism in *P. aeruginosa*. Moreover, this study showed the importance of glycerol metabolism on virulence in the chronic CF isolate, FRD1. In summary,

these data show that the glyoxylate pathway and *glp* regulon are important factors that may contribute to adaptation and persistence during chronic infection.

Future Directions

The major goal of my project was to characterize the regulation of the glyoxylate pathway in *P. aeruingosa*. Although GlpR appears to regulate the glyoxylate pathway in PAO1, the mechanism responsible for the deregulation of this pathway in FRD1 remains to be elucidated. More studies are needed to determine the cause of high ICL and MS activity in CF adapted isolates. In addition, this study focused largely on the contribution of GlpR to *P. aeruginosa* virulence. The contribution of GlpR regulated genes to *P. aeruginosa* should also be characterized.

Identification of additional regulators of the glyoxylate pathway

It is likely that the glyoxylate pathway is regulated by factors other than GlpR. Predictive approaches have failed to identify other transcriptional regulators and a non-predictive approach identified RpoN as an indirect regulator of the glyoxylate pathway (Hagins *et. al.* 2010). An alternative method for identifying regulators of this pathway is to perform a DNA pull down assay. Biotinylated *aceA* and *glcB* promoters can be complexed with proteins in a *P. aeruginoa* cell free extract and then isolated using a streptavidin column. The proteins can then be eluted from the DNA-protein complex and N-terminal amino acid sequencing conducted to identify the bound proteins.

It is also possible that the glyoxylate pathway is regulated by factors involved in stress response. DNA alterations acquired by *P. aeruginosa* in the CF lung are likley caused by oxidative (Ciofu et. al. 2010), and possibly osmotic stress. Overexpression of ICL has been

demonstrated in *P. fluorescens* in response to aluminum stress (Hamel *et. al.* 2004). Other evidence suggests that in *E. coli* the glyoxylate pathway is additionally regulated by RpoS, the stress response regulator (Dong *et. al.* 2009). In fact, loss of RpoS increases isocitrate lyase and malate synthase activites under stress conditions (Maharjan *et. al.* 2005). Lastly, this study is one of the first to show a relationship between fatty acid and glycerol metabolism. It would be interesting to see if genes involved in fatty acid or acetate metabolism are required for growth on glycerol, and vice versa. Genes involved in glycerol metabolism have been shown to be upregulated during growth on acetate in *Citrobacter* sp. (Kim *et. al.* 2012).

Anaerobic utilization of glycerol by *P. aeruginosa.*

In this study, chronic mucoid CF isolates of *P. aeruginosa* were able to utilize glycerol more efficiently compared to the non-mucoid isolate, PAO1. I determined that this phenotype was due to the overproduction of alginate which may provide microaerophilic growth conditions. Further confirmation of these results would be to examine glycerol utilization by PAO1 under strict anaerobic conditions. Finally, it would be interesting to see if CF adapted isolates show a growth advantage on other carbon sources commonly found within the CF lung compared to PAO1 or other clinical isolates.

In conclusion this study provides novel insight into the regulatory interchange involved in glycerol and fatty acid metabolism in *P. aeruginosa,* and will promote our understanding of how these two networks enable *P. aeruginosa* to establish and maintain chronic infections. Furthermore, it suggests a more important role for nutrient catabolism in *P. aeruginosa* adaptation and virulence in the CF lung. The absence of the glyoxylate enzymes in humans suggests the potential for controlling *P. aeruginosa* CF lung infections by targeting these

enzymes for therapy. Understanding how the glyoxylate pathway is regulated will provide a better understanding of the role it plays in pathogenesis.

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