Characterization of the Glyoxylate Pathway in *Pseudomonas aeruginosa*

by

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Abstract

Pseudomonas aeruginosa infections are the leading cause of lung dysfunction and mortality in Cystic Fibrosis (CF) patients. Adaptation of *P. aeruginosa* to become a chronic pathogen of the CF lung includes acquisition of mutations that facilitate the prolonged survival of the bacterium in that environment. Thus in order to understand the adaptation strategies of the bacterium, it is imperative to study and characterize the actual CF isolates in addition to the non-CF isolates of *P. aeruginosa*. To identify chronic infection mechanisms of *P. aeruginosa* CF isolates, the Silo-Suh lab isolated transposon insertion mutants of a typical CF isolate strain, FRD1, that were decreased in virulence in an alfalfa seedling model of infection. One of the mutants contained a transposon insertion in *aceA* which encodes for isocitrate lyase, one of two enzymes involved in the glyoxylate pathway. The focus of my research was to characterize the expression of *aceA* and activity of isocitrate lyase to determine whether preferential utilization of the glyoxylate pathway is a part of the adaptation strategy for *P. aeruginosa* in the CF lung. I determined that the expression of the *aceA* gene is deregulated in the CF isolate FRD1 compared to the non-CF isolate PAO1. Moreover, deregulation of *aceA* appears to be common to other CF isolates of *P. aeruginosa*, suggesting this phenotype is important for adaptation of *P. aeruginosa* in the lung. In an effort to elucidate the molecular mechanism of *aceA* deregulation in FRD1, I discovered that RpoN, an alternative sigma factor, negatively regulates *aceA* expression in PAO1. This is a unique role for this

sigma factor. The exact mechanism by which *aceA* expression is deregulated by RpoN in FRD1 is unclear since this sigma factor appears to be active in FRD1. Thus it is likely that during adaptation, *P. aeruginosa* acquires a knockout mutation in a RpoN regulated gene whose role is to repress *aceA* expression in non-CF isolates. Finally, I determined that *glcB*, encoding for malate synthase, the second key enzyme of the glyoxylate pathway, is also required for virulence of *P. aeruginosa* on alfalfa and its expression is deregulated in FRD1 compared to PAO1. In addition, expression of a *glcB* is negatively regulated by RpoN in PAO1. This is the first study to systematically characterize expression of the glyoxylate pathway in *P. aeruginosa* and my data demonstrate the importance of this pathway for chronic infection isolates.

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Chapter 1

Literature Review

Introduction

Pseudomonas aeruginosa is the leading cause of mortality for patients with the genetic disorder Cystic Fibrosis (CF). During infection of the CF lung, *P. aeruginosa* adapts to the lung environment to cause a persistent, chronic infection. This infection is very difficult, if not impossible to treat mainly due to the high intrinsic antibiotic resistance of *P. aeruginosa*. The mechanisms utilized by this bacterium to cause chronic infection are not completely understood and a better understanding of these mechanisms is needed to develop better approaches to treat chronic infections. Therefore, the long-term goal of this research is to identify molecular mechanisms of *P. aeruginosa* adaptation to the CF lung to cause chronic infections. The specific goal of my study is to characterize regulation of the glyoxylate pathway in order to enhance understanding of its role during chronic infection of *P. aeruginosa*. The significant findings of my study are threefold. First, I characterized regulation of the glyoxylate pathway genes in response to carbon source utilization. Second, I determined that regulation of the genes encoding for enzymes of the glyoxylate pathway are altered in some CF isolates of *P. aeruginosa*. Last, I determined that the alternative sigma factor, RpoN, negatively regulates expression of the genes encoding for the key enzymes of the glyoxylate pathway. These findings are the first to implicate RpoN in regulation of this pathway in *P. aeruginosa.*

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative, rod shaped bacterium that has a ubiquitous distribution in nature with predilection for soil and water. *P. aeruginosa* can survive and grow in various environmental niches and has very simple nutritional requirements. *P. aeruginosa's* nutritional versatility allows it to catabolize a diverse group of organic compounds, including branched chain amino acids, fatty acids, dodecan, hexadecane, aliphatic alcohols, including ethanol and propanol, and even vegetable oil (Stanier *et al*., 1966, Zhang, *et al*., 2005). *P. aeruginosa*'s ability to degrade crude oil and pesticides, gasoline, and diesel (Zhang *et al.,* 2005, Fulekar *et al*., 2009, Wongsa *et al.,* 2004), has prompted its use for bioremediation.

P. aeruginosa is found in association with a variety of eukaryotic organisms including many plants and animals in natural environments (Filiatrault *et al*., 2006). In fact, it is a known plant pathogen, causing infections in various plant species (Elrod & Braun, 1942). This bacterium is also an important opportunistic human pathogen. *P. aeruginosa* causes a variety of localized and systemic infections including urinary tract infections, corneal infections, otitis media, dermatitis, and respiratory infections. As an opportunistic pathogen, *P. aeruginosa* infects burn victims, immunocompromised individuals, or those with metabolic disorders and, more notably, CF patients. *Pseudomonas* produces a variety of virulence determinants including adhesins, toxins, and a Type III Secretion System (TTSS) used to deliver toxins and other extracellular proteins into the cytoplasm of host cells (Morihara 1964, Yahr *et al.,* 1997, Yahr *et al.,* 1996). Some of the extracellular cytotoxins involved in virulence include exoenzyme S and exotoxin A.

Exoenzyme S has been shown to play a role in alveolar epithelial injury and dissemination of bacteria within the host (Kudoh *et al.,* 1994, Bjorn *et al.,* 1979), while exotoxin A, ribosylates host elongation factor-2 to inhibit protein synthesis (Iglewski *et al.,* 1975, Iglewski *et al.,* 1977). Another virulence determinant produced by *P. aeruginosa* is elastase. In the lung, elastase damages tight junction-associated proteins thereby increasing alveolar epithelial permeability (Azghani, 1996). Pyoverdin is one of the siderophores produced by *P. aeruginosa* that is essential for virulence, acting as a powerful iron chelator (Meyer *et al*., 1996). Hemolysins produced by *P. aeruginosa* are also important virulence determinants including phospholipase C, which degrades lung surfactant and is essential for virulence on a variety of host organisms (Ostroff *et al*., 1989, Wiener-Kronish *et al*., 1993). *P. aeruginosa* also synthesizes hydrogen cyanide which is detected in the sputum of CF patients infected with *P. aeruginosa*. Although the role of cyanide in infection is not yet completely understood, its presence is associated with decreased pulmonary function (Ryall *et al.,* 2008, Sanderson *et al.,* 2008). Pyocyanin is a phenazine antibiotic that causes oxidative damage to neighboring cells that can result in cell death (Hassan & Fridovich, 1980). Other factors that contribute to infection include pili (Saiman *et al*., 1990, Drake & Montie, 1988) and flagella which play a role in initiating infection by binding the bacterium to respiratory epithelial cells and mediating chemotaxis and motility during infections. A majority of *P. aeruginosa* isolated from the lungs of CF patients produces an exopolysaccharide, alginate which appears to play a role in protecting the bacterium from the host's immune system by protecting it from phagocytosis (Cabral *et al.,* 1987, Krieg *et al.,* 1988). Alginate also plays a role in protecting the bacterium from several antibiotics (Hoilby, 1977) and from

reactive oxygen species (Simpson *et al*., 1989) by preventing their penetration and contributes to biofilm architecture (*Lam et al.,* 1980). Overproduction of alginate results in a mucoid phenotype for most of the CF isolates. Many of the virulence factors produced by *P. aeruginosa* are regulated by two cell-cell communication systems, the *lasRI* system and the *rhlRI* system (Gambello *et al*., 1991, Gambello *et al*., 1993, Toder *et al.,* 1991, Passador *et al.,* 1993, Ochsner & Reiser, 1995). *P. aeruginosa* is resistant to a variety of antibiotics and easily develops a high-level of multidrug resistance (Poole 2001), both of which are largely due to the presence of mutlidrug efflux pumps (Poole & Skirumar, 2001, Poole *et al*.,1993). Constitutive expression of several efflux pumps results in intrinsic multidrug resistance (Poole & Skirumar, 2001) and contributes to the difficulty of treating *P. aeruginosa* infections.

***Pseudomonas aeruginosa* infection models**

Animal models of infections have been invaluable for identification of acute virulence determinants of *P. aeruginosa*. And although *P. aeruginosa* causes many acute infections including pneumonia, urinary tract infections, otitis media, bacterial keratitis, folliculitis associated with hot tubs or swimming pools, *P. aeruginosa* also causes serious chronic infections. Animal models used to study these acute infections caused by *P. aeruginosa* include the burn mouse model, the neonatal mouse model of acute pulmonary infection, the rat corneal infection model, the malnourished mouse model and the mouse model of mucosal colonization (Tan *et al.,* 1999, Tang *et al.,* 1996, Szliter *et al.,* 2006, Boucher *et al.,* 1997, Yu *et al.,* 2000, Pier *et al.,* 1992). Unfortunately, because chronic isolates of *P. aeruginosa* do not produce copious amounts of acute virulence factors, the

animal models that have been highly successful for studying acute infection isolates have not been useful for elucidating the mechanisms of virulence of chronic isolates. Therefore, a lack of a suitable animal model system has hampered studying the virulence mechanisms of chronic isolates. In addition, many chronic infections do not respond well to treatments that are useful against acute infections (Carmeli *et al.,* 1999, Ehrlich *et al.,* 2005, Honer zu Bentrup & Russell, 2001). For example, antibiotics used to treat acute infections only reduce or slow down the destruction caused by *P. aeruginosa* but do not eliminate the bacterium from the lung (Petersen *et al.,* 1981). Furthermore, emergence of antibiotic-resistant strains during chronic infections (Carmeli *et al.,* 1999) limit the possibility that antibiotic therapy will ever be successful against *P. aeruginosa* biofilms (Ehrlich *et al.,* 2005). Vaccinations have also been of limited effectiveness against chronic infections caused by *P. aeruginosa* in CF patients. One study demonstrated that regular vaccination reduced the frequency of chronic infections caused by *P. aeruginosa* and yet 32% of the vaccinated population still became chronically infected over a 10 yr period (while 72% of non-vaccinated group were chronically infected) (Lang *et al.,* 2004). However, in a review of vaccine trials, Johansen *et al.* (2008), concluded that vaccines against *P. aeruginosa* in CF were not very effective or reliable (Johansen & Gotzsche, 2008). The lack of effective vaccines was also recently reviewed by Doring & Pier (2008). The phenotypic changes accompanying *P. aeruginosa* during chronic infection may partially account for the ineffectiveness of vaccines. Unlike many other bacterial infections, complete eradication of *P aeruginosa* from chronically infected CF patients is difficult to achieve. Thus, given ineffectiveness of the current regimen, it is imperative to better understand the mechanisms of chronic

persistence and virulence of *P. aeruginosa* in order to develop more effective therapeutic approaches.

Cystic fibrosis

Cystic fibrosis is a recessively inherited autosomal disease affecting more than 30,000 people in the United States (www.cff.org). It is the result of mutation(s) in a single gene located on the long arm of chromosome 7 which encodes for the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation within the CFTR is a three base pair deletion resulting in loss of a phenylalanine at position 508 (Kerem *et al.,* 1989). Mutations in this gene result in the loss of a functional CFTR protein at the proper cellular location that results in disrupting ion channels in epithelial cell types such as sweat glands, salivary glands, airway epithelium, intestinal epithelium, bile ducts, etc. (Welsh & Smith, 1993, www.cff.org). This defect in ion channels results in reduced chloride ion secretion (Chmiel *et al.,* 2003). Other than chloride ion channels, the amiloride-sensitive epithelial sodium channel (ENaC) within the airway epithelium may also be affected in CF patients (Chmiel *et al.,* 2003). This channel is necessary for proper reabsorption of sodium and water from airway surface liquid, maintaining the necessary depth of airway surface fluid. When the CFTR is disabled, there is an increase in ENaC activity resulting in increased reabsorption of water and salt. Thickening of mucus leads to clogged ducts of the pancreas and in males the sex ducts can become clogged with sticky dehydrated mucus (Govan & Deretic, 1996). The glands will continue to secrete mucus causing the clogged ducts to swell and form cysts (Govan & Deretic, 1996). One of the most important organs affected in CF patients is the lung;

however, CF patients may also experience inflammation at sites other than the lung such as the digestive tract, resulting in inflammatory bowel disease and pancreatitis (Chmiel *et al.,* 2003). In the lung, the thickening of mucus hinders mucociliary clearance, and contributes to bronchiopulmonary infections with various bacteria including *Staphylococcus aureus*, *Heamophilus influenzae*, and *P. aeruginosa* (Govan & Deretic, 1996). There is excessive airway inflammation in CF patients, even in the absence of pathogens (Khan *et al.,* 1995). However, once infection ensues, the inflammatory defense mechanisms of the lung become active which is major cause of tissue damage and impaired lung function (Hoiby, 1977). Cytokines are released which attract neutrophils to the lung, damaging lung tissue and impairing mucocilliary clearance of bacteria even further (Chmiel *et al.,* 2003). As a consequence of the inflammatory response to the infection, many cells die and release DNA which contributes to the viscosity of sputum (Hoiby, 1977) and making it even more difficult to clear the pathogen.

Environment within the CF lung and CF microbiology

P. aeruginosa within the CF lung lives in a complex environment that is altered dramatically due to the complexity of the CF disease and the other organisms that infect the CF lung. Meyer *et al.* (2000) determined that the secretions within the lower respiratory tract of CF patients are different than from non-CF patients. For example, activated neutrophils are abundant in the CF lower respiratory airway which may cause significant alterations of the phospholipid and protein components of airway surfactant. Some of the phospholipids present include phosphatidylcholine,

phosphatidylethanolamine, phosphatidyl-inositol, and sphingomyelin (Meyer, *et al.,* 2000). The fatty acid profiles of bronchoalveolar lavage fluid from the lower respiratory tract were also altered in CF patients when compared to normal volunteers; arachidonic acid was found at high concentrations in both CF and non-CF individuals, but palmitic acid and oleic acid was elevated only in the CF patients (Meyer, *et al.,* 2000). In addition to fatty acids, the amino acid content within CF sputum is altered compared to non-CF sputum (Barth & Pitt, 1996). Amino acids such as leucine, isoleucine, phenylalanine, tyrosine, alanine, serine and methionine, and valine were present in high concentrations in CF sputum compared to non-CF sputum (Barth & Pitt, 1996).

In addition to altered concentrations and profiles of fatty acids and amino acids, CF sputum contains bacterial cells, DNA, and other compounds derived from host, bacteria, and viruses (Hoiby, 1977). Upon examining the diversity of bacterial communities within the sputa of CF patients, many bacterial species were identified, including: *Prevotella oris, Leptotrichia*-like sp. (oral-associated microbes), *Abiotrophia defectiva*, (found in many body sites), *Fusobacterium gonidoformans*, *Bacteroides fragilis*, *Citrobacter murliniae*, *Sarcina ventriculi* (typically associated with the gut), *Lautropia mirabilis*, *S. aureus*, *B. cepacia*, *H. influenzae*, and *Stenotrophomonas maltophilia* and of course *P. aeruginosa* (reported as CF pathogens), indicating the diverse communities of bacteria present in the CF lung (Rogers *et al.,* 2003). However, *P. aeruginosa* is the most prevalent (Gilligan, 1991) and, during the course of infection, the bacterium undergoes changes to adapt to the lung environment. This results in an infection that resists clearing

by both the host immune system and therapeutic agents. The ultimate effect is a chronic infection within the CF lung.

Chronic infection and chronic infection mechanisms

Acute pathogens cause infections in their hosts for a short period of time. Often, the host immune system and standard treatment such as antibiotic therapy are able to clear the infection and eliminate the pathogens. However, there are times when a small number of pathogens continue to survive and persist to cause a chronic, life-long association within the host (Young *et al.,* 2002). For the persistent stage to ensue, the pathogen must first evade clearance from the host immune system and/or manipulate the host's response in order to stimulate colonization. For example, *Helicobacter pylori* can cause inflammation at infection sites within the gastric mucosa surface but the inflammatory response does not lead to clearance of the bacterium for reasons that are unknown (Rhen *et al.,* 2003). Evidence suggests that inflammation may be necessary for initial or prolonged colonization by *H. pylori* (Rhen *et al.,* 2003) because the inflammation results in an increase of specific compounds recognized and bound by the pathogen. Consequently, *H. pylori* promotes its own colonization by inducing inflammation and increasing its adhesion targets in the host (Rhen *et al.,* 2003).

Another obstacle that must be overcome to cause a persistent infection is to ensure that there is a balance between increased bacterial replication and ongoing immune clearance (Rhen *et al.,* 2003). For example, *Mycobacterium tuberculosis* divides very slowly as if in stationary phase during chronic infection. This creates only small amounts of new

antigens which is likely to guarantee the immune system will tolerate the bacteria that are present (Young *et al.,* 2002).

Not only must pathogens avoid attack and clearance from the host immune system, but they must also be able to adapt to the host environment. During prolonged colonization, *H. pylori* undergoes genetic rearrangements to create multiple 'genetic lines' of *H. pylori* in order to better adapt to the host environment (Rhen *et al.,* 2003). This bacterial plurality is common among chronic pathogens. Bacterial plurality results when phenotypic and genotypic diversity exists within a bacterial population (Ehrlich *et al.,* 2005). This diversity, which can arise from altered gene expression or horizontal gene transfer, allows the bacterial population to have strains that may be better equipped to survive in the current environment (Ehrlich *et al.,* 2005). The presence of multiple phenotypes provides a greater chance for survival of a progeny from challenges from the host (Ehrlich *et al.,* 2005). For pathogenic bacteria causing a chronic infection, having multiple strains, and having the ability for horizontal gene transfer, provides a mechanism for gene assortment and increased chance for survival (Ehrlich *et al.,* 2005).

There is also evidence that bacteria utilize an alternate set of genes to persist in the host than those utilized for initial and acute infections. The *pnp* gene product in *Salmonella enterica* sv. Typhimurium, which plays a role in the degradation of RNA, was found to be mutated in strains capable of causing persistent infection (Clements *et al.,* 2002). This mutation results in an increase of a subset of virulence genes found on *Salmonella* pathogenicity islands suggesting they are required for persistent infection. Disruption of

the *pnp* gene could be a strategy for the establishment of persistent infection by *S. enterica* sv. Typhimurium (Clements *et al.,* 2002). In an attempt to better understand the mechanisms utilized by *Brucella abortus* to cause chronic infections, genes were identified that were necessary for this bacterium to cause persistent infection (Hong *et al*., 2000). Remarkably, the identified genes were not required for initial infection (Hong *et al.,* 2000). This data further indicates that chronic pathogens utilize an alternate set of genes that are distinct from those necessary for initial infection to establish a persistent infection. Some genes required for persistence of *B. abortus* are homologous to genes necessary for persistence of other chronic infecting pathogens, indicating that mechanisms for establishment of chronic infections are conserved among pathogenic organisms (Hong *et al.,* 2000, Rhen *et al.*, 2003).

For *M. tuberculosis* to establish a chronic infection, it first must overcome an initial attack from the host's immune system. Its characteristic cell wall of mycolic acids contributes to the survival of the bacterium from the human immune system so that a small number of bacteria survive and continue to persist (Barry, 2001, Young *et al.,* 2002). *M. tuberculosis* is ingested by phagocytes and alters the environment within the phagosome to prevent the normal phagosome maturation process (Honer zu Bentrup & Russell, 2001). This in turn protects the bacterium from a highly degradative environment. In the case where *M. tuberculosis* is ingested by an activated macrophage, a granuloma forms around the macrophage and although this environment protects the host, the bacterium is also protected from other host immune factors (Honer zu Bentrup & Russell, 2001). Other pathogens also seek an intracellular location for persistence as

indicated by the fact that *H. pylori* can enter host cells, and *Brucella*, and *Salmonella,* like *Mycobacterium*, can survive and replicate in macrophages in mice (Rhen *et al.,* 2003).

Many *M. tuberculosis* genes associated with the persistent infection encode for normal housekeeping functions or play a role in starvation metabolism. This includes isocitrate lyase which is required for persistence in mice (Mckinney *et al.,* 2000). *M. tuberculosis* isocitrate lyase mutants function similarly to wild-type strains during the initial phase of infection but are defective for persistence (McKinney *et al.,* 2000). This suggests that lipids or acetate are a major source of nutrients during this stage of infection. Other pathogens *like Candida albicans* and *S. enterica* sv. Typhimurium depend upon isocitrate lyase during persistent infection but can dispense with the enzyme during acute infection (Lorenz & Fink, 2001, Fang *et al*., 2005). This indicates that persistence is correlated with the ability of the organism to switch its diet within the host (Vereecke *et al.,* 2002). Other proteins have been identified that are required for persistent infection in *M. tuberculosis* including those that are necessary for anaerobic growth and nitrate respiration (Honer zu Bentrup & Russell*,* 2001). Genes that provide a protective advantage against reactive nitrous intermediates within activated macrophages are also induced in *M. tuberculosis* along with the genes involved in fatty acid metabolism (Honer zu Bentrup & Russell*,* 2001). Many genes encoding enzymes of fatty acid degradation, including isocitrate lyase, were upregulated in vivo indicating that *M. tuberculosis* may use host lipids in vivo (Honer zu Bentrup & Russell*,* 2001). In another study almost 200 genes were identified that were specifically required for growth of *M. tuberculosis* in vivo and over half of the genes identified had no annotated function (Sassetti & Rubin,

2003). In summary, the molecular mechanisms of bacterial adaptation to persist within a host are complex and poorly understood at present.

Chronic *P. aeruginosa* infections are the leading cause of mortality of CF patients. Although the mechanisms for establishing infections in CF patients are not completely understood, isolates recovered from the lung produce significantly decreased quantities of virulence determinants that are necessary for establishing acute infection (Dacheux *et al.,* 2001, Hancock *et al.,* 1983, Luzar *et al.,* 1985, Ramsey & Wozniak, 2005, Woods, 1986). Yet *P. aeruginosa* possesses additional characteristics that are associated with chronic infections. Most notably *P. aeruginosa* isolated from chronic infections overproduce alginate which is believed to facilitate formation and persistence of the bacterium within biofilms in the lung (Hoiby, 1977, Govan & Deretic, 1996). Both alginate and biofilm formation confer a protective advantage against neutrophils and antibiotics (Hoiby, 1977). *P. aeruginosa* within the biofilms in the CF lung are composed of genetically diverse population derived from plurality that is similar to what has been observed with other chronic pathogens (Costerton, *et al*., 1994, Ehrlich *et al*., 2005). It is believed that bacterial plurality allows a subset of the population to survive in a challenging environment and facilitates maintenance of a persistent infection (Ehrlich *et al*., 2005). In addition, *P. aeruginosa* adapts to a hypoxic or anaerobic environment during chronic infection in the lung and, similar to *M. tuberculosis*, grows at a slower rate (Boucher, 2004). The observed bacterial plurality is explained by isolates of hypermutable strains of *P. aeruginosa* from the CF lung. Presumably, the hypermutability assures the bacterial plurality and enhances the chance of containing a mutant(s) that can adapt and survive

better in this environment (Oliver *et al.,* 2000, Hogardt *et al.,* 2007). Altered gene expression is common among CF isolates compared to acute isolates. This indicates that alternate mechanisms are being utilized for the pathogen to persist and survive in the lung (Palmer *et al.,* 2005, Silo-Suh *et al.,* 2005, Son *et al*., 2007). Therefore, *P. aeruginosa* isolated from the CF lung possesses many characteristics analogous to other chronic infecting pathogens, including mechanism to evade host clearance, a hypermutable phenotype, bacterial plurality, and altered gene expression. Although some common mechanisms are beginning to emerge from various analyses, there is still more to understand about the adaptation mechanisms of bacterial pathogens to cause chronic infections.

Adaptation of *Pseudomonas* to the cystic fibrosis lung

The CF lung becomes colonized by a variety of bacteria, including pathogens. Before the widespread use of antibiotics, CF patients typically died from *S. aureus* infections very early in life (Govan & Deretic, 1996). Antibiotic treatment facilitated the control of both *S. aureus* and *H. influenzae* infections (Govan & Deretic, 1996). However, due to the inherent resistance of *P. aeruginosa* to many antibiotics, infections by this bacterium are still difficult to eradicate (Poole, 2001).

A comparison of *P. aeruginosa* isolates recovered from the CF lung to environmental and acute clinical isolates indicates that *P. aeruginosa* undergoes adaptation within the CF lung (Woods *et al.,* 1986, Mahenthiralingam & Speert, 1995). Likewise, a comparison of sequential isolates recovered from patients over a period of several years indicates

adaptation of *P. aeruginosa* to the CF lung (Smith *et al.,* 2006). In most cases the CF lung is originally colonized with a single *P. aeruginosa* strain which gives rise to population of derivatives with various mutations that presumably facilitates the survival and persistence within the CF lung (Struelens *et al.,* 1993). *P. aeruginosa* produces a variety of standard virulence determinants that are required for initiating acute infections, but isolates that are recovered from the lungs of CF patients typically do not produce many of these virulence determinants at the level of non-CF isolates (Dacheux *et al.,* 2001, Hancock *et al.,* 1983, Luzar *et al.,* 1985, Ramsey & Wozniak, 2005, Woods, 1986). These phenomena are not restricted only to chronic isolates from CF patients but also from *P. aeruginosa* isolated from chronic obstructive pulmonary disease (COPD) patients (Martinez-Solano *et al.,* 2008). Similar to CF infections, COPD patients appear to be infected with one *P. aeruginosa* clone that gives rise to plurality via hypermutability as it adapts to the lung (Martinez-Solano *et al.,* 2008). Although genetically diverse, the traits that are selected for during chronic infections appear to be conserved and include hypermutability, increased antibiotic resistance, and decreased virulence determinant production (Martinez-Solano *et al.,* 2008). Thus, these traits appear to be important for establishment and maintenance of chronic infections.

Severely decreased and/or lack of production of standard virulence determinants by chronic isolates suggests that *P. aeruginosa* utilizes an alternative set of virulence determinants specific for chronic infections (Silo-Suh *et al.,* 2002). Alternatively, the virulence determinants utilized for acute infections may inhibit establishment and maintenance of the bacterium for chronic infections. For example, most CF isolates of *P.*

aeruginosa have defects in lipopolysacharide O-side chains (Hancock *et al.,* 1983) and in TTSS (Dacheux *et al.,* 2001). CF isolates of *P. aeruginosa* also tend to lack flagella and pili and are non-motile (Luzar *et al.,* 1985, Mahenthiralingam *et al.,* 1994, Saiman *et al.,* 1990). In a burn mouse model, non-motile mutants of *P. aeruginosa* were unable to cause systemic infection and showed a dramatic decrease in virulence compared to motile strains, indicating that motility is important virulence factor in invasive infections (Drake & Montie, 1988). However, as an advantage to CF isolates causing a chronic, persistent infection, Mahenthiralingam *et al.,* showed that non-motile CF isolates deficient in flagella formation were resistant to ingestion by macrophages (1994). Also, Dixon, *et al*. (1992), demonstrated that exotoxin A acts as a super antigen and elastase is also very antigenic (Dixon, *et al*., 1992, Azghani *et al*., 2000). Therefore there may be selective pressure for the loss or decreased production of acute virulence determinants during establishment of chronic infections. Decreased production of acute virulence determinants may provide a protective advantage for persistence of *P. aeruginosa,* possibly by allowing *P. aeruginosa* to evade the host immune system (Smith *et al.,* 2006).

In addition, *P. aeruginosa* characteristically becomes mucoid within the CF lung due to over production of the exopolysaccharide alginate which is considered to be one of the most important chronic virulence determinants for these isolates in this environment (Ramsey & Wozniak*,* 2005). This transformation of initial non-mucoid infecting isolates to the mucoid phenotype marks the transition to a more persistent infection and poor prognosis for the patient (Hoiby, 1977).

Overproduction of alginate by *P. aeruginosa* is associated with mutations that occur in the *muc*-locus of the bacterial genome (Govan & Deretic, 1996). Mutations in *mucA* and *mucB* lead to deregulation of AlgT, an alternative sigma factor that induces expression of the alginate biosynthetic, *algD*, operon (Govan & Deretic, 1996). The most common mutation in CF isolates resulting in the mucoid phenotype has been mapped to *mucA*, which encodes for an anti-sigma factor of AlgT (Martin *et al*., 1993, Boucher *et al*., 1997). Alginate appears to render the bacterium more resistant to phagocytosis, which may provide a survival advantage to the bacterium in the lung (Cabral *et al.,* 1987, Krieg *et al.* 1988). Alginate may also protect the bacterium by scavenging free radicals released by activated macrophages (Simpson *et al.,* 1989).

The nutrient-limited environment within the lung may contribute to the conversion to the mucoid phenotype of *P. aeruginosa* (Terry *et al.,* 1992). Phosphatidylcholine, the major component of lung surfactant, can supply the necessary carbon, nitrogen, and phosphate for *P. aeruginosa.* In 1992, Terry *et al.* demonstrated that growth on this compound was sufficient to convert PAO1 to a mucoid phenotype. The investigators were able to link a decrease in energy metabolism to conversion to the mucoid phenotype and show that the mucoid strains may be better suited than non-mucoid strains of *P. aeruginosa* in environments that are limited in nutrients (Terry *et al.,* 1992).

Alginate is an important component of biofilms formed by mucoid *P. aeruginosa* (*Lam et al.,* 1980). *P. aeruginosa* forms biofilms that are comprised of mushroom-shaped microcolonies of bacteria encased by an extracellular polysaccharide matrix, separated by

fluid-filled channels that likely deliver nutrients and remove metabolic products (Costerton, 1994, O'Toole *et al.,* 2000). Therefore, a biofilm consists of a large number of different microniches. The bacterial adaptation to different microniches may promote genetic diversity within the *P. aeruginosa* population (Ehrlich *et al.,* 2005). The multiple bacterial phenotypes present in the biofilm further aid in colonization (Ehrlich *et al.,* 2005).

P. aeruginosa forms biofilms in most environments that allow for growth (Nickel *et al.,* 1985, Miller & Ahearn, 1987) including CF lung tissues (Govan & Deretic, 1996). Flagella and type IV fimbriae appear to be important for the early stages of biofilm formation (O'Toole & Kolter, 1998a) and lipopolysaccharide (LPS) plays a role in attachment of *P. aeruginosa* to surfaces (Makin & Beveridge, 1996). Once attached to a surface, *P. aeruginosa* uses twitching motility to move along the surface (O'Toole & Kolter, 1998a). Attachment induces changes in gene expression of *algC* which is required for alginate biosynthesis (Davies *et al.,* 1993, O'Toole *et al*., 2000, Lam, *et al*., 1980). Expression of AlgT, the sigma factor that induces alginate production, is accompanied by down regulation of flagellar biosynthetic genes (Garrett *et al.,* 1999) to favor biofilm formation (Davies *et al.,* 1993, *Lam et al.,* 1980). The biofilm mode of growth protects *P. aeruginosa* from the body's defense mechanisms (Jensen *et al.,* 1990). In addition, the slower growth of *P. aeruginosa* in biofilms, or the biofilm barrier, prevents access of antibiotics to the bacterium (Hoilby, 1977). A population of the cells within biofilms survives in an anaerobic environment due to the oxygen gradient present from the top to the bottom of the biofilm (Hoiby, 1977). And although the water filled

channels contain dissolved oxygen (Hoiby, 1977), *P. aeruginosa* via anaerobic nitrate respiration in these anaerobic zones (Boucher *et al*., 2000). This evidence suggests that *P. aeruginosa* is growing anaerobically in the lung (Worlitzsch *et al.,* 2002). Concentrations of nitrate commonly found in the CF sputum can support anaerobic growth of *P. aeruginosa* (Palmer *et al.,* 2007) and *P. aeruginosa* is able to form more robust biofilms under anaerobic conditions compared to aerobic conditions (Yoon *et al.,* 2002). The anaerobic environment in biofilms also promotes alginate production (Worlitzsch *et al.,* 2002) and studies show that *P. aeruginosa* is able to maintain a mucoid phenotype more effectively in an anaerobic or oxygen-limited environment as oppose to a strictly aerobic conditions (Hassett, 1996). However, anaerobic growth via nitrate respiration results in much slower growth rate and this can influence cell physiology and the outcome of the infection (Boucher *et al*., 2000, Brown *et al.,* 1990).

The changes within *P. aeruginosa* promoted by the CF lung environment also affect gene expression (Brown *et al.,* 1990, Palmer *et al.,* 2005). The gene encoding for glucose-6-phosphate dehydrogenase, *zwf*, was found to be upregulated in the CF isolate FRD1 compared to the PAO1 strain, a wound isolate (Silo-Suh *et al.,* 2005). In another study, the genes responsible for transport and metabolism of glucose were repressed in *P. aeruginosa* grown on CF sputum (Palmer *et al.,* 2005). These studies suggest that adaptation to the lung environment may result in altered metabolic activities (Silo-Suh *et al.,* 2005). Over 140 genes were identified as being differentially regulated within *P. aeruginosa* when grown on CF sputum compared to glucose grown bacterium (Palmer *et al.,* 2005). One gene that showed reduced expression was *fliC*, which is likely to

contribute to the loss of motility seen in CF isolates (Palmer *et al.,* 2005). Other genes were induced when grown in CF sputum including those that lyse of *S. aureus* (the initial colonizer of the CF lung). This may contribute to microbial competition that results in the prevalence of *P. aeruginosa* within the CF lung (Palmer *et al.,* 2005). In addition, genes that are involved in branched chain and aromatic amino acid catabolism were highly up-regulated in *P. aeruginosa* grown in CF sputum. In a separate study examining gene expression profiles of *P. aeruginosa* taken directly from the sputum sample, genes involved in fatty acid and lipid metabolism and amino acid degradation were induced. Equally important, many of the same genes were also constitutively expressed in *P. aeruginosa* isolated from the sputum samples (Son *et al.,* 2007). These results suggest that the carbon sources utilized by *P. aeruginosa* in the CF lung are likely lipids and amino acids (Son *et al.,* 2007). Son and his group specifically suggest phosphatidylcholine, the most common lipid in the lung, as being metabolized in vivo (Son *et al.,* 2007). In summary, these studies suggest that one of the most important adaptations of *P. aeruginosa* in the CF lung is in the alteration of cellular metabolism to preferentially utilize the available nutrients in the environment (Meyer *et al.,* 2000, Barth & Pitt, 1996).

The chemical environment and nutrient availability within the CF lung appears to promote changes in *P. aeruginosa* and selects for genetic variants that can persist. In addition, the hostile environment induced by deteriorating lung tissue and human intervention imposes a selective pressure on *P. aeruginosa*. Theses activities, select for *P. aeruginosa* that can survive and cause chronic CF infections (Oliver *et al*., 2000).

Isolation of a large number of hypermutable strains from CF patients indicates that there is an increase in the genetic diversity of the bacterial population which enhances the chance presence of mutants that can better survive in the CF environment (Oliver *et al.,* 2000, Hogardt *et al.,* 2007).

Previous study to identify chronic virulence determinants in FRD1

Various animal models of infection have traditionally been used to study virulence determinants from bacterial pathogens. Unfortunately, screening a large number of mutants in mammalian models tends to be costly and impractical. Thus, in order to genetically screen a large number of mutants to identify virulence determinants of *P. aeruginosa*, alternative models of infection were developed based on insects, nematodes and plants. *P. aeruginosa* virulence genes identified using alternative models were demonstrated to be necessary for virulence in a mouse model of infection (Tan *et al*., 1999, and Rahme *et al*., 1997). This suggested that *P. aeruginosa* utilizes some of the same virulence mechanisms to infect vertebrate and non-vertebrate hosts. In order to study *P. aeruginosa* isolates recovered from the CF lung which typically do not cause infections in animal models, the Silo-Suh lab developed an alfalfa seedlings model of infection to identify virulence determinants from chronic isolates (Silo-Suh *et al.,* 2002). The alfalfa seedlings model of infection is very sensitive and can be utilized to asses virulence of CF isolates that cannot establish infection in other models. Using this assay, Silo-Suh *et al.* (2002) demonstrated that CF isolates utilize alternative strategies to infect alfalfa than non-CF isolates. Some of these alternative infection mechanisms are likely to be important for chronic colonization within the CF lung. In order to identify chronic

virulence genes that are required to cause disease in alfalfa seedlings, a transposon insertion mutant library constructed in a CF isolate, FRD1, was screened for reduced virulence (Lindsey *et al*., 2008). From approximately 2,000 FRD1 transposon insertion mutants screened for virulence in the alfalfa assay, 18 mutants decreased in virulence were isolated. One of the mutants contained a transposon insertion in *aceA* which encodes for isocitrate lyase. As demonstrated in Table 1.1, the *aceA::Tn* mutant was severely decreased in infection of alfalfa. As previously demonstrated by Diaz-Perez *et al.* (2007), the *aceA* mutant was unable to grow in a minimal medium with acetate as a sole carbon source.

Strain	Description	% Infection
FRD1	wild-type	100%
FRD1*aceA*	Tn insertion within PA2634-probable isocitrate lyase	12%

Table 1.1 Isocitrate lyase is required for optimal virulence of FRD1 on alfalfa seedlings. Values represent the average of three experiments where 50 seedlings were tested in each experiment and 100% indicates all seedlings showed disease symptoms.
Data from Lindsey *et al.* (2008)

Isocitrate lyase (ICL) is a key enzyme of the glyoxylate pathway (Figure 1.1). that is responsible for replenishing intermediates of the TCA cycle for some bacteria growing on C2 compounds. To prevent loss of carbon from the TCA cycle as carbon dioxide, isocitrate lyase, along with a second enzyme, malate synthase, is activated to funnel isocitrate into the glyoxylate pathway (Cozzone, 1998). Because isocitrate lyase has a lower affinity for isocitrate than isocitrate dehydrogenase, the first reaction of the glyoxylate pathway occurs only in the presence of high isocitrate concentrations (Holms, 1987), which is achieved when the bacterium is growing on acetate or fatty acids as a sole carbon source. Isocitrate lyase cleaves isocitrate to glyoxylate and succinate and malate synthase condenses acetyl-CoA with glyoxylate to produce malate that can reenter the TCA cycle. For each turn of the cycle, two moles of acetyl CoA are used to generate one molecule of malate (Cozzone, 1998) with no loss of carbon. In the TCA cycle, malate is converted to oxaloacetate, which through gluconeogenesis, can give rise to glucose-6-phosphate and then glucose, with a total of four moles of acetyl-CoA giving rise to one mole of glucose (Cozzone, 1998)

Compounds that induce or require the glyoxylate pathway for catabolism are present within the CF lung including fatty acids and certain amino acids (Meyer *et al.,* 2000, Barth & Pitt, 1996). Furthermore, genes necessary for the transport and catabolism of these nutrients are upregulated in *P. aeruginosa* grown in CF sputum (Son *et al*., 2007, Palmer *et al*., 2005, Barth & Pitt, 1996). Also, compounds like acetate and other short chain fatty acids that can enter the glyoxylate pathway, may be produced by other metabolically active bacteria within the CF lung as fermentation products (Rogers *et al*.,

2005). Taken together, it is likely that *P. aeruginosa* in the lung environment utilizes this pathway to catabolize the carbon sources present in the CF lung.

The role of the glyoxylate pathway in virulence is well documented. Other pathogens, such as *Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium, require the glyoxylate pathway to establish persistent infections in animals (Elias *et al*., 2005, Fang *et al.,* 2005). In addition, clinical isolates of the opportunistic pathogen *C. albicans* show increased glyoxylate cycle enzymatic levels (Lattif *et al*., 2006). Both enzymes of the glyoxylate pathway appear to be important for virulence in animal model systems used to study other chronic pathogens (Munoz-Elias & McKinney, 2005, Fang *et al*., 2005, Lattif *et al.,* 2006, Hancock *et al*., 1983).

Known regulators of the glyoxylate pathway in other organisms

Many enzymes are feed-back regulated by concentrations of their end-products or compounds from downstream reactions acting as allosteric inhibitors. The first enzyme of the glyoxylate pathway, isocitrate lyase was thought at one time to be allosterically inhibited by phosphoenolpyruvate (Ashworth & Kornberg, 1963). It appears, however that phosphoenolpyruvate behaves more like an analog of succinate and no correlation appears to exist between intracellular levels of other TCA cycle intermediates, such as oxaloacetate, pyruvate, citrate, or isocitrate and activity of isocitrate lyase and malate synthase (Williams *et al*., 1971, Lakshmi & Helling, 1978). The flux of carbon through the glyoxylate cycle is largely controlled by the phosphorylation state of isocitrate dehydrogenase (IDH), the enzyme in the TCA cycle at the branch-point of the TCA cycle

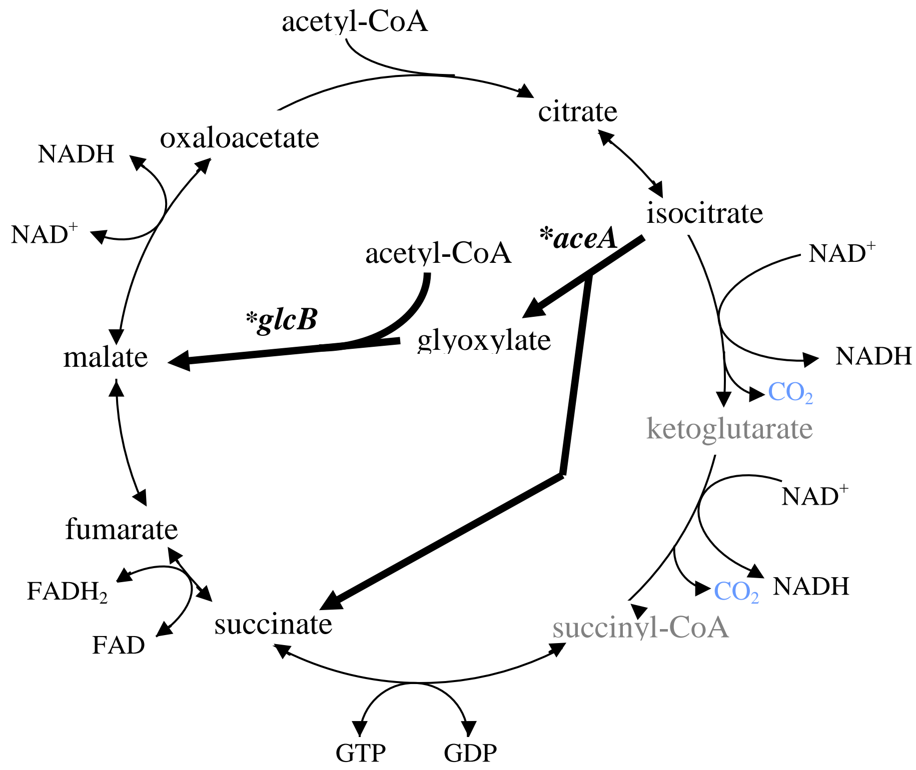

Figure 1.1 The Glyoxylate Pathway
*genes encoding key enzymes of the glyoxylate pathway

and the glyoxylate pathway (Cozzone, 1998). The protein in *E. coli* responsible for the phosphorylation of ICD is encoded for by *aceK,* isocitrate dehydrogenase kinase/phosphatase (Borthwick *et al*., 19984). Unlike in *P. aeruginosa*, this gene in *E. coli*, along with the genes encoding malate synthase (*aceB*), and isocitrate lyase (*aceA*), are encoded as a single operon, *aceBAK,* (Maloy & Nunn, 1982, LaPorte *et al*., 1985, Chung *et al*., 1988). Isocitrate dehydrogenase competes with isocitrate lyase for isocitrate (LaPorte *et al*., 1984) and is in the inactive form when it is phosphorylated at a serine residue by ICD kinase/phosphatase (Borthwick *et al*., 1984). In its phosphorylated state, ICD can no longer bind isocitrate. Therefore the flow of carbon into the glyoxylate pathway is favored with isocitrate lyase having access to isocitrate (Bennett & Holms, 1975, Nimmo & Nimmo, 1984, Laport *et al*., 1984, Cozzone, 1998). In *E. coli* when glucose is limited but acetate is pressent, ICD is maintained in a partially inactivated state (Bennett & Holms, 1975) due to reversible phosphorylation (LaPorte & Koshland, 1982).

Another mechanism by which the glyoxylate pathway is regulated involves the activity of two proteins in *E. coli* designated IclR and FadR (Maloy & Nunn, 1982, Gui, *et al*., 1996). IclR is a repressor of isocitrate lyase and malate synthase that acts at the transcriptional level by binding the *aceA* promoter to prevent the binding of RNA polymerase (Maloy & Nunn, 1982, Cortay *et al*., 1991). Mutations in *fadR* result in increased expression of the *ace* operon in *E. coli* (Maloy & Nunn, 1982). However, there does not appear to be a FadR binding site within the *ace* promoter region (Gui *et al*., 1996). Gui *et al* determined that FadR activates *iclR* transcription by binding immediately upstream of the *iclR* promoter (1996). Their studies indicate that FadR prevents an

increase in *iclR* expression during growth on fatty acids but activates transcription of this gene during growth on other carbon sources (Gui *et al*., 1996).

Cozzone noted that there are three different factors, FruR, IHF, and UP that stimulate *aceBAK* expression, all of which induce conformational changes in DNA (1998). This indicated that the function of this operon is greatly dependent on the structural organization of the DNA helix. FruR, a repressor of the fructose regulon, also plays a role in regulating the glyoxylate pathway by stimulating the synthesis of isocitrate lyase and malate synthase (Chin *et al.*, 1989). FruR activates other genes by binding to specific sites upstream of the promoter region to induce a bend in the DNA helix that is thought to play a role in promoter activation (Cozzone, 1998). IHF, encoded for by *himA* and *himD*, stimulates expression of *aceBAK* even in the absence of any other activator (Cozzone, 1998). Since IHF is a DNA binding protein that promotes bending, this supports the importance of localized DNA structure for *ace* operon expression. Finally, expression of the *aceBAK* operon is controlled by the UP element that strengthens RNA polymerase binding to a promoter by interacting with the carboxy-terminus of the α-subunit (Negre *et al*., 1997, Ross *et al*., 1993, Cozzone, 1998).

Summary

In several pathogenic bacteria that cause chronic infections, the glyoxylate pathway appears to be important for establishment and maintenance of persistent infections. A transposon insertion in *aceA* caused a severe decrease in the ability of a *P. aeruginosa* CF isolate, FRD1, to infect alfalfa seedlings. This suggested that *aceA* was important for *P.*

aeruginosa to cause chronic infections. The goals of my study were threefold. First, determine and characterize the role of *aceA* on virulence and persistence of *P. aeruginosa*. Second, determine if other genes involved in fatty acid utilization, including *glcB* which encodes for the second key enzyme of the glyoxylate pathway, are also required for virulence of *P. aeruginosa*. Finally, characterize the effect of various carbon sources on *aceA* expression and ICL activity. The results of my study should enhance our understanding of the role of the glyoxylate pathway in *P. aeruginosa* physiology during establishment and maintenance of chronic infections

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Chapter 2

Initial Characterization of *aceA* and isocitrate lyase from *Pseudomonas aeruginosa*

Note: This chapter contains results that have been published as Lindsey, T. L., Hagins, J. M., Sokol, P. A. & Silo-Suh, L. (2008). Virulence determinants from a Cystic Fibrosis isolates of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiology* **154**, 1616-1627. Remaining data will be published separately.

Abstract

Pseudomonas aeruginosa causes chronic pulmonary infections in cystic fibrosis (CF) patients. The metabolic pathways utilized by *P. aeruginosa* during these infections, which can persist for decades, are poorly understood. Several lines of evidence suggest that the glyoxylate pathway, which utilizes acetate or fatty acids to replenish intermediates of the TCA cycle, is an important metabolic pathway for *P. aeruginosa* growing within the CF lung. In a previous study, we determined that *P. aeruginosa* is dependent upon *aceA* to cause disease on alfalfa seedlings. In this study, I demonstrate that *aceA* encodes for isocitrate lyase (ICL) activity in *P. aeruginosa*, which is one of two major enzymes of the glyoxylate pathway. More importantly, *aceA* is required for *P. aeruginosa* strain PAO1, an acute isolate of *P. aeruginosa*, to cause disease in rat lungs. Expression of *aceA* in PAO1 responds to carbon sources that utilize the glyoxylate

pathway for catabolism. In contrast, expression of *aceA* from FRD1, a CF isolate, is constitutively upregulated. Moreover, deregulation of *aceA* is common to other chronic isolates of *P. aeruginosa,* suggesting that high ICL activity facilitates adaptation of *P. aeruginosa* to the CF lung. Complementation of FRD1 with a PAO1 genomic clone library identified *rpoN* to negatively regulate *aceA*. However, *rpoN* appears to be active and does not seem to be altered in FRD1. This is supported by complementation of a PAO1*rpoN* mutant with a wild-type copy of *rpoN* from either PAO1 or FRD1 in *cis*. Thus, regulation of the glyoxylate pathway by RpoN is likely indirect and constitutes a unique regulatory role for this sigma factor in bacterial metabolism.

Introduction

Bronchopulmonary infections caused by *Pseudomonas aeruginosa* are the leading cause of mortality for CF patients. These infections resist eradication by antibiotic therapy and the host immune system and indicate a need for novel therapeutic strategies. The ability of *P. aeruginosa* to maintain decade long infections within the CF lung is attributed in part to virulence mechanisms that evolve as *P. aeruginosa* adapts to this environment (Lindsey *et al.*, 2008; Nguyen & Singh, 2006). Although *P. aeruginosa* is nutritionally versatile, within human niches it must adapt to the availability of host-derived nutrients. Within the lungs of CF patients, these nutrients are likely represented in sputum.

The composition of CF sputum is complex. It contains host and bacterial cells as well as various host and bacterial derived compounds (Hoiby, 1998). Transcriptome studies indicated that PAO1, a wound isolate of *P. aeruginosa*, primarily used amino acids as a

carbon source while a CF isolate used amino acids and lipids when grown in CF sputum (Palmer *et al.*, 2005; Son *et al.*, 2007). The different carbon source preference by these isolates suggests that *P. aeruginosa* alters its metabolic pathways during chronic infection of the CF lung. This is supported by the observation that regulatory control of several central metabolic enzymes is altered in FRD1 compared to PAO1 (Lindsey *et al.*, 2008; Silo-Suh *et al.*, 2005).

In a previous study, we used the alfalfa seedling infection model to identify virulence determinants from *P. aeruginosa* isolates already adapted to the CF lung. Our analysis revealed that the *aceA* gene, which encodes for isocitrate lyase, is critical for infection of alfalfa seedlings (Lindsey *et al.*, 2008). In this study, I determined that *aceA* is also required for optimal virulence of *P. aeruginosa* in a rat lung chronic infection model. Isocitrate lyase is one of the two key enzymes of the glyoxylate pathway which is utilized by some bacteria when growing on fatty acids or acetate as the sole carbon source. In this study I demonstrate that *aceA* expression is upregulated in the CF isolate FRD1 but not in the acute isolate PAO1 when grown in L-broth, a peptide rich medium. Therefore, *aceA* gene expression responds to different environmental cues in FRD1 compared to PAO1. Alternatively, *aceA* may be constitutively expressed in FRD1. In addition, I compared the regulation of *aceA* expression and ICL activity in PAO1 and FRD1 in response to various carbon sources and determined that RpoN negatively regulates *aceA* in a non-CF isolate of *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). Additional carbon sources were used at the following concentrations: Palmitic acid at 2.5 mM, Heptanoic acid at 5 mM, Valeric acid at 10 mM. All amino acids were used at 1% (w/v) except histidine and tryptophan at 0.5% (w/v), and tyrosine at 0.1% (w/v). Ethanol was used at 0.5%. The remaining carbon sources were used at 20 mM unless indicated. For the catabolite repression studies, 10 mM succinate and 0.1% (v/w) tyrosine were used.

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 (Ap^r) ml^{-1} for *E. coli*; 180 µg carbenicillin (Cb^r) ml^{-1} for *P. aeruginosa*; 20 µg gentamicin (Gm^r) ml^{-1} for *E. coli* and 200 µg for *P. aeruginosa*; 20 µg tetracycline (Tet^r) ml^{-1} for *E. coli*; 100 µg ml^{-1} for *P. aeruginosa,* and 50 µg kanamycin ml^{-1} for *E. coli*, and 700 µg kanamycin ml^{-1} for *P. aeruginosa*. When indicated, glutamine was used at 1mM concentration. 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 (Suh *et al.*, 1999). 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).

Complementing the *aceA* mutant: To complement the isocitrate lyase mutants, the *aceA* gene was amplified from FRD1 using *Pfu*. The PCR fragment was digested with *Eco*RI and the resulting fragment, containing 50 bp upstream of the coding sequence, was cloned into an *Eco*RI and *Sma*I site of pLS1155, containing an inducible promoter (Silo-Suh *et al*., 2005). The m*oriT* was inserted at the *Hind*III site on the plasmid and the plasmid was transformed into *E. coil*. The plasmid (pJH122) was transferred to both the FRD1*aceA* and PAO1*aceA* via conjugation. The complementing colonies were selected for on PIA media containing kanamycin. The colonies were then PCR verified. The

complemented *FRD1aceA* and PAO1*aceA* mutants were designated FRD1*aceA*+ (JH168) and PAO1*aceA*+ (JH166) respectively.

Construction of *aceA* transcriptional fusions. The *aceA::lacZ* transcriptional fusion was constructed using the *aceA* gene fragment obtained from FRD1 via PCR using *Pfu*. The fragment, which included 430 bp upstream from the coding sequence, was digested with *Eco*RV and cloned into the *Sma*I site of pSS223 (Suh *et al.,* 2004). The plasmid (pJH132), containing the 5' coding sequence for *aceA* in the proper orientation, was verified by PCR and restriction digests and conjugated into FRD1 and PAO1.

Identification of *aceA::lacZ* regulator. To identify regulators of *aceA* expression in FRD1, a PAO1 library was introduced into JH139 (FRD1 *aceA::lacZ*) via triparental mating (Goldberg & Ohman, 1984). Approximately 1,000 transconjugants were tested for alterations in *aceA::lacZ* expression via β-galactosidase activity. Plasmids were recovered from three transconjugants showing reduced *aceA* expression compared to JH139. The plasmids were reintroduced into JH139 and verified for restoration of *aceA::lacZ* regulation that is observed in non-CF isolates. In order to identify the complementing gene, the plasmids were mutagenized in vitro using EZ-Tn5 (Epicentre, Madison, WI) and the pool of mutants was transformed into *E. coli* DH10B. The pool of *E. coli* transformants was re-conjugated into JH139 and then screened for loss of complementing activity that restored the deregulation of *aceA::lacZ* in FRD1 background. Two transposon insertion plasmid clones were recovered that had lost the

ability to regulate the *aceA*::*lacZ* fusion in FRD1. Both constructs contained transposon insertions in *rpoN*.

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 (Lindsey *et al*., 2008): *P. aeruginosa* cells were harvested from stationary cultures and washed with saline. The cells were resuspended in TE Buffer pH 6.9 and broken open via ultrasonificaiton with (Fisher Scientific, Model 100 Sonic dismembrator) under the following conditions: (9 rounds, 10 seconds each time, keeping cultures on ice). 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 ΔA_{324} was determined using only the linear part of the reaction.

Rat chronic lung infection. *P. aeruginosa* strains were tested for their ability to cause respiratory infections in the agar bead model in rats as described by Cash *et al*. (Cash *et al*., 1979). The rat lung infections were performed by Dr. Pam Sokol of the University of Calgary. For each *P. aeruginosa* strain tested, eight male Sprague-Dawley rats weighing 200-220 g (Charles River Breeding Laboratories, Willmington, MA) were tracheostomized under anesthesia and inoculated with ~10^4 c.f.u. bacteria embedded in

agar beads. On day 14 postinfection, the animals were euthanized and the lungs were aseptically removed. For each set of strains tested, lungs from four animals were homogenized in PBS (Polytron homogenizer, Brinkmann Instruments, Inc., Westbury, NY) and serial dilutions were plated onto trypticase soy agar to determine bacterial counts. The remaining lungs were fixed in 10% formalin and examined for quantitative histopathological changes as previously described (Bernier *et al.*, 2003). The lung sections were scanned using an Epson 1650 scanner. Areas of inflammation, characterized by cellular infiltration were identified and digitized with Scion Image software and reported as the percentage of the total area of the lung section that was covered by inflammatory exudates.

Strain or Plasmid	Genotype, relevant characteristics	Source
Strains		
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)
PAO1*crc* (LS1441)	PAO1Δ*crc101*	Silo-Suh *et al.* (2002)
PAO1*iclR* (LS1662)	PAO1*iclR101::aacCI*	Dr. Laura Silo-Suh
PAO1*retS* (LS1540)	PAO1*retS101::aacCI*	Dr. Laura Silo-Suh
FRD1*aceA* (LS1539)	FRD1*aceA101*::*aaC*I	Lindsey *et al*. (2008)
PAO1*aceA* (LS1545)	*PAO1aceA101::aaCI*	Lindsey *et al*. (2008)
FRD1*aceA*+ (JH168)	FRD1 *aceA* complemented for *aceA*	Lindsey *et al*. (2008)
PAO1*aceA*+ (JH166)	PAO1 *aceA* complemented for *aceA*	Lindsey *et al*. (2008)
FRD1*rpoN* (LS1775)	FRD1*rpoN101::*ΩTc	Dr. Laura Silo-Suh
FRD1*rpoN*+*F*(LS1790)	FRD1*rpoN* complemented with FRD1 *rpoN*	This study
FRD1*rpoN*+*P*(JH271)	FRD1*rpoN* complemented with PAO1 *rpoN*	This study
PAO1*rpoN* (LS1773)	PAO1*rpoN101::* ΩTc	Dr. Laura Silo-Suh
PAO1*rpoN*+*F*(LS1788)	PAO1*rpoN* complemented with FRD1 *rpoN*	This study
PAO1*rpoN*+*P*(JH273)	PAO1*rpoN* complemented with PAO1 *rpoN*	This study
JH139	FRD1 carrying *aceA::lacZ* fusion	Lindsey *et al*. (2008)
LS1638	PAO1 carrying *aceA::lacZ* fusion	Lindsey *et al*. (2008)
P3, P6, P13, P18-P19, P22, P24-P27	Clinical isolates	This study
ENV2, ENV10, ENV46, ENV54	Environmental isolates	Mahenthiralingam *et al.* (1994)
C1-C4	Environmental isolates	Ferguson *et al.* (2001)

PAM57-15, PA2192	CF isolates	van Heeckeren & Schluchter (2002)
P70, P77, P79, P81	CF isolates	This study
DO5	CF isolate	Silo-Suh *et al.* (2005)
DO11, DO108, DO110	CF isolates	This study
JH202	FRD1 complemented for *aceA* activity with PAO1 genomic DNA	This study
JH228	JH202 with Tn insertion that eliminates *aceA* complementing activity	This study
Plasmids		
pLS1653	*iclR*101 in pBluescript K(+)	Dr. Laura Silo-Suh
pLS1537	*retS*101 in pBluescript K(+)	Dr. Laura Silo-Suh
pLS574	*rpoN*101 in pBluescript K(+)	Dr. Laura Silo-Suh
pLS1536	*aceA*101 in pBluescript K(+)	Lindsey *et al.* (2008)
pSS213	plasmid with regulatable promoter	Suh *et al.* (2004)
pLS1155	plasmid with regulatable promoter	Silo-Suh *et al.* (20050
pLS214	pUC19 with m*oriT* at *Hind*III	Suh *et al.* (2004)
pLS1785	*rpoN* complementing plasmid with FRD1 *rpoN*	This study
pJH264	*rpoN* complementing plasmid with PAO1 *rpoN*	This study
pJH132	*aceA::lacZ* transcriptional fusion in pSS223	Lindsey *et al.* (2008)
pJH122	*aceA* complementing plasmid	Lindsey *et al.* (2008)
pJH204	plasmid complemented for *aceA* activity with PAO1 genomic DNA	This study
pJH241	plasmid with Tn insertion that eliminates *aceA* complementing activity	This study

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.

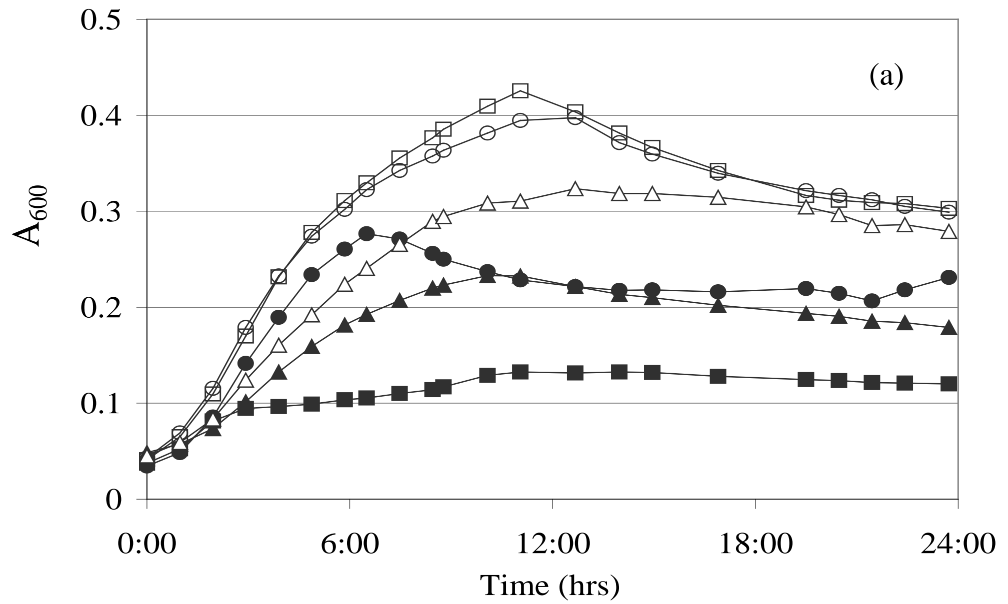
Results

FRD1*aceA* and PAO1*aceA* mutants are defective for growth on acetate

To demonstrate that *aceA* encodes for the isocitrate lyase in *P. aeruginosa*, I determined the ability of FRD1 *aceA* and PAO1 *aceA* mutants to grow in NCE minimal medium with either acetate or succinate as a sole carbon source. As shown in Figure 2.1, the *aceA* mutation abolished the ability of *P. aeruginosa* to grow on acetate as a sole carbon source. However, the *aceA* mutation had no effect on the ability of *P. aeruginosa* to grow on succinate. This demonstrated that the *aceA* mutation only affected acetate utilization and that *aceA* likely encoded for isocitrate lyase. Complementation of the *aceA* mutation with a wild-type copy in *trans* partially restored the ability of FRD1 *aceA* and PAO1 *aceA* mutants to grow on acetate.

Isocitrate lyase is required for chronic lung infection in rats.

aceA is required for persistence of other bacterial pathogens in their respective animal model systems of infection (Fang *et al.*, 2005; McKinney *et al.*, 2000). To determine whether *aceA* is required for virulence and persistence of *P. aeruginosa*, a PAO1 *aceA* mutant was tested in the rat chronic lung infection model. We had to test the PAO1 *aceA* mutant because our previous data demonstrated that FRD1 was unable to establish an infection in this model system (Silo-Suh *et al.*, 2002). As shown in Table 2.2, the PAO1 *aceA* mutant was significantly reduced for virulence on alfalfa seedlings. More importantly, disruption of *aceA* in PAO1 led to significantly reduced histopathology in rat lungs. These data indicate that isocitrate lyase is required for optimal virulence of *P. aeruginosa* in mammals.



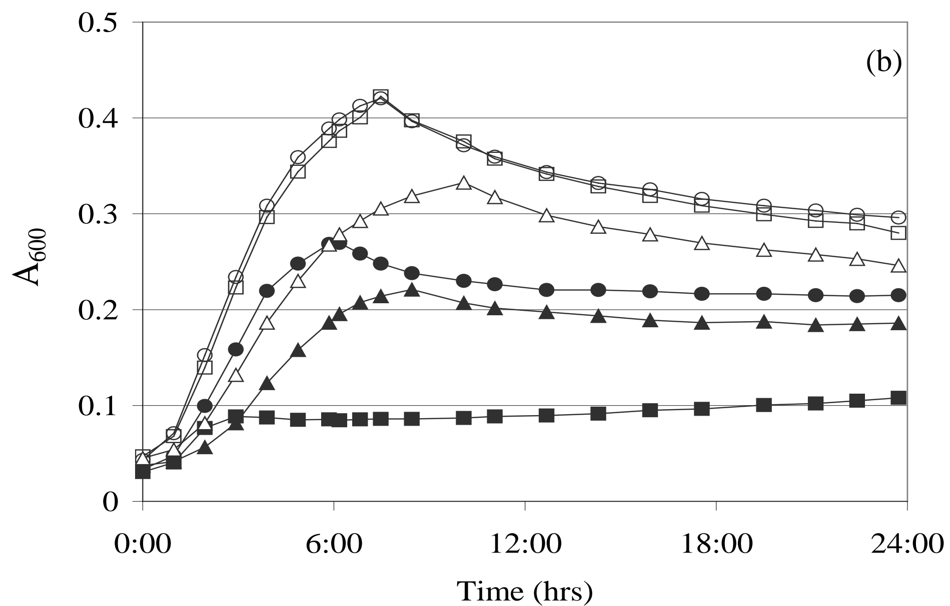


Figure 2.1 *aceA* is required for optimal growth of both FRD1 and PAO1 on acetate as a sole carbon source. (a) FRD1 and derivatives. (b) PAO1 and derivatives. Cultures were grown in minimal media with 20 mM succinate and 20 mM acetate as a sole carbon source for 24 hours. ●, wild-type grown in acetate;○, wild-type grown in succinate; ■, *aceA* mutant grown in acetate; □, *aceA* mutant grown in succinate; ▲ *aceA* complemented strain grown in acetate; Δ, *aceA* complemented strain grown in succinate.

Strain	% Seedling Infection *	Rat lung Infection: Mean ± SD bacteria (c.f.u./ml/lung) †	Mean ± SD % Inflammation ‡
PAO1	77 ± 17.5	$4.54 \times 10^5 \pm 5.96 \times 10^5$	44.6 ± 7.84 (38.2-55.7)
PAO1*aceA*	26 ± 12.1	$5.12 \times 10^4 \pm 3.34 \times 10^4$	11.8 ± 2.27 (9.2-14.6)

Table 2.2 Isocitrate lyase is required for *P. aeruginosa* virulence on alfalfa and in the rat lung model.
* Values represent the % of alfalfa seedlings with maceration symptoms (± standard deviation). The data are representative of two experiments. Significantly different than PAO1 ($p < 0.001$)
†‡ Values are mean ± SD for 4 animals at 14 days post infection. Numbers in parentheses equal the range. † Number of bacteria recovered from rats lungs. ‡ Significantly different than PAO1 ($p<0.001$, T test).

The enzyme encoded by *aceA* is responsible for isocitrate lyase activity that is upregulated in FRD1.

In order to address whether *aceA* functions similarly in FRD1 and PAO1, I tested cell free extracts of these strains for ICL activity. The *aceA* mutants of FRD1 and PAO1 and their respective *aceA* complemented strains were also tested for ICL activity. As demonstrated in Figure 2.2, FRD1 cell free extracts showed increased ICL activity (over 5-fold) compared to PAO1 extracts. The low ICL activity observed for PAO1 grown in L-broth is consistent with published results that *aceA* is specifically induced by carbon sources that utilize the glyoxylate pathway such as fatty acids and acetate (Diaz-Perez *et al.*, 2007; Honer Zu Bentrup *et al.*, 1999). As expected, the *aceA* mutants of each parental strain were deficient for ICL activity. Genetic complementation via integration of pJH122 that carries a wild-type copy of *aceA* derived from FRD1 restored the ICL defective phenotype in the *aceA* mutants of both FRD1 and PAO1 (Figure 2.2).

To address whether the enhanced ICL activity in FRD1 was caused by increased gene expression at the transcriptional level, an *aceA::lacZ* fusion was constructed and introduced into both wild-type *P. aeruginosa* isolates (FRD1 and PAO1). As illustrated in Figure 2.3, the relative expression of β-galactosidase from the *aceA::lacZ* fusion paralleled the activity of ICL under the same growth conditions in both FRD1 and PAO1. These data indicated that the increased ICL activity in FRD1 was due to increased expression of *aceA* at the transcriptional level (Fig. 2.3). The high expression of *aceA* in FRD1 compared to PAO1 is consistent with Son *et al*.'s data which demonstrated that in CF isolates, *aceA* expression was higher than in non-CF isolates (Son *et al*., 2007).

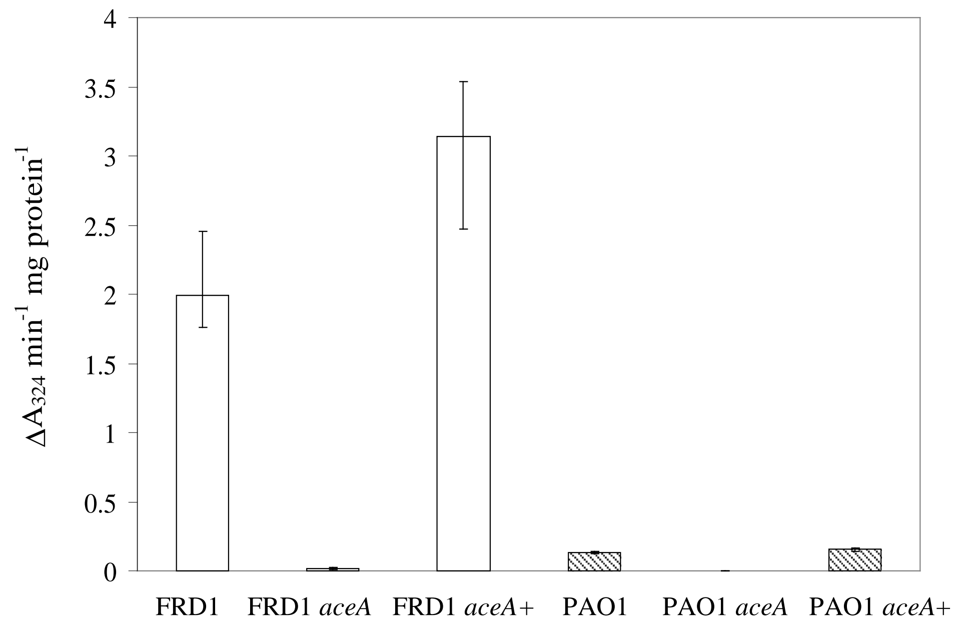


Figure 2.2 Activity of ICL is altered in FRD1. The data represents the average of four experiments.

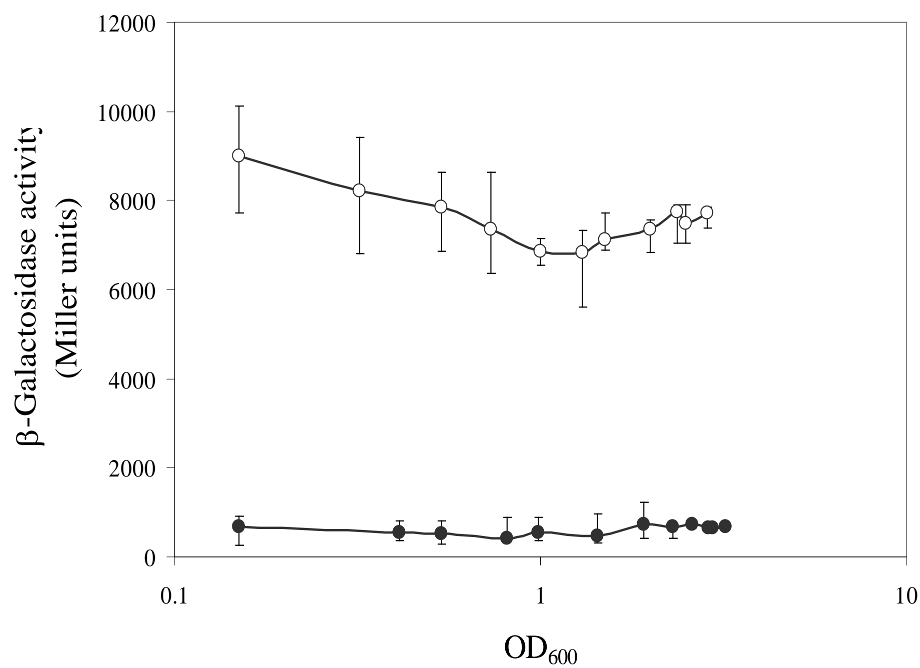


Figure 2.3 Expression of *aceA* is altered in FRD1. The data represent the average of four experiments. ○ FRD1, ● PAO1.

High ICL activity is common to CF *P. aeruginosa*

When grown in L-broth, *aceA* expression and ICL activity are upregulated in the *P. aeruginosa* CF isolate FRD1 compared to the wound isolate PAO1. The low ICL activity observed for PAO1 under these conditions is consistent with published reports that *aceA* is specifically induced by carbon sources that utilize the glyoxylate pathway (Diaz-Perez *et al.*, 2007; Honer Zu Bentrup *et al.*, 1999). Thus, our data suggest that *aceA* expression is deregulated in FRD1 and which results in high ICL activity. The abnormally high ICL activity of FRD1 may be a consequence of *P. aeruginosa* adaptation to the CF lung environment. Thus, to address whether the putative deregulation of *aceA* is common adaptive strategy for chronic isolates, I tested other acute clinical isolates, CF isolates and environmental isolates of *P. aeruginosa* for ICL activity. As demonstrated in Figure 2.4, all ten acute isolates had similar low ICL activity as PAO1. Similarly, all eight environmental isolates had low ICL activity. In contrast, three out of ten CF isolates possessed high ICL activity similar to FRD1. While not all of the CF isolates showed deregulated ICL activity, this phenotype appears to be more common in CF isolates versus other sources of *P. aeruginosa*. Unfortunately, lack of patient history for all of the *P. aeruginosa* CF isolates tested here prevented generation of a correlation between infection duration and emergence of the deregulated ICL phenotype.

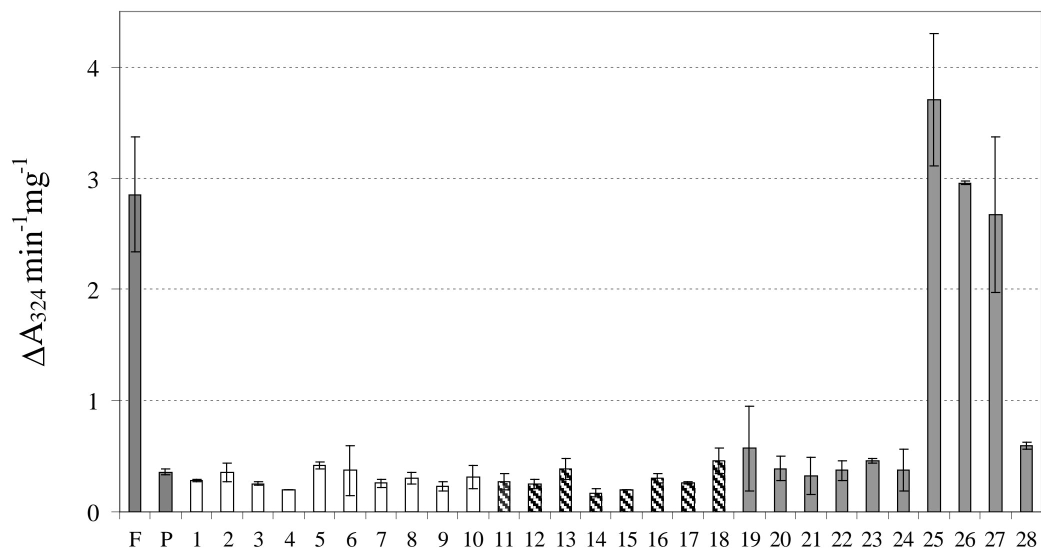


Figure 2.4 Survey of ICL activity in *P. aeruginosa* isolates. ICL activity was assayed from overnight cultures of *P. aeruginosa* grown in L-broth and normalized to protein concentrations. Values represent the average of 2 experiments conducted in duplicate (± standard deviation). Clinical Isolates ; Environmental Isolates ; CF 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, C4; 19, PAM57-15; 20, PA2192; 21, P70; 22, P77; 23, P79; 24, P81; 25, DO11; 26, DO5; 27, DO108; 28, DO110.

Effect of carbon sources on *aceA* expression and ICL activity

In various bacteria, *aceA* expression is regulated by the presence of certain carbon sources in the growth medium that yield acetyl-CoA during catabolism. Both ICL activity and *aceA* expression are upregulated in FRD1 compared to PAO1 when grown in L-broth. This was an unexpected result because a peptide rich medium had not previously been shown to induce *aceA* expression. To more accurately determine the effect of various carbon sources on *aceA* transcription, expression studies were preformed on FRD1 and PAO1 carrying an *aceA::lacZ* transcriptional fusion.

The analysis was initiated by testing the effect of several TCA cycle intermediates and compounds known to induce *aceA* in *P. aeruginosa* (Diaz-Perez *et al.*, 2007; Kretzschmar *et al.*, 2008). Consistent with published reports, expression of *aceA* in PAO1 was activated only when known inducers, incluing ethanol, acetate and various fatty acids, were exogenously provided as the sole carbon source (Figure 2.5a). In contrast, expression of *aceA* was relatively high in FRD1 in response to all of the carbon sources tested. These data supported my hypothesis that expression of *aceA* is deregulated in FRD1. However, variations in *aceA* expression levels in response to the various carbon sources suggest some regulatory controls are still active in FRD1.

ICL enzymatic activity closely correlated with *aceA* promoter activity in PAO1 for most of the tested carbon sources with the notable acceptation of palmitic and hepatonic acid (Fig. 2.5b). For these two fatty acids, the correlation is reversed for unknown reasons. Although ICL activity was consistently high in FRD1 compared to PAO1 in response to

growth in the given carbon sources, ICL activity did not strictly mirror *aceA* expression in this background. However, ICL activity in FRD1 was highest following growth on carbon sources that strongly induced *aceA*::*lacZ* in PAO1. Taken together, the results suggest that ICL activity in both backgrounds is affected by posttranscriptional regulation.

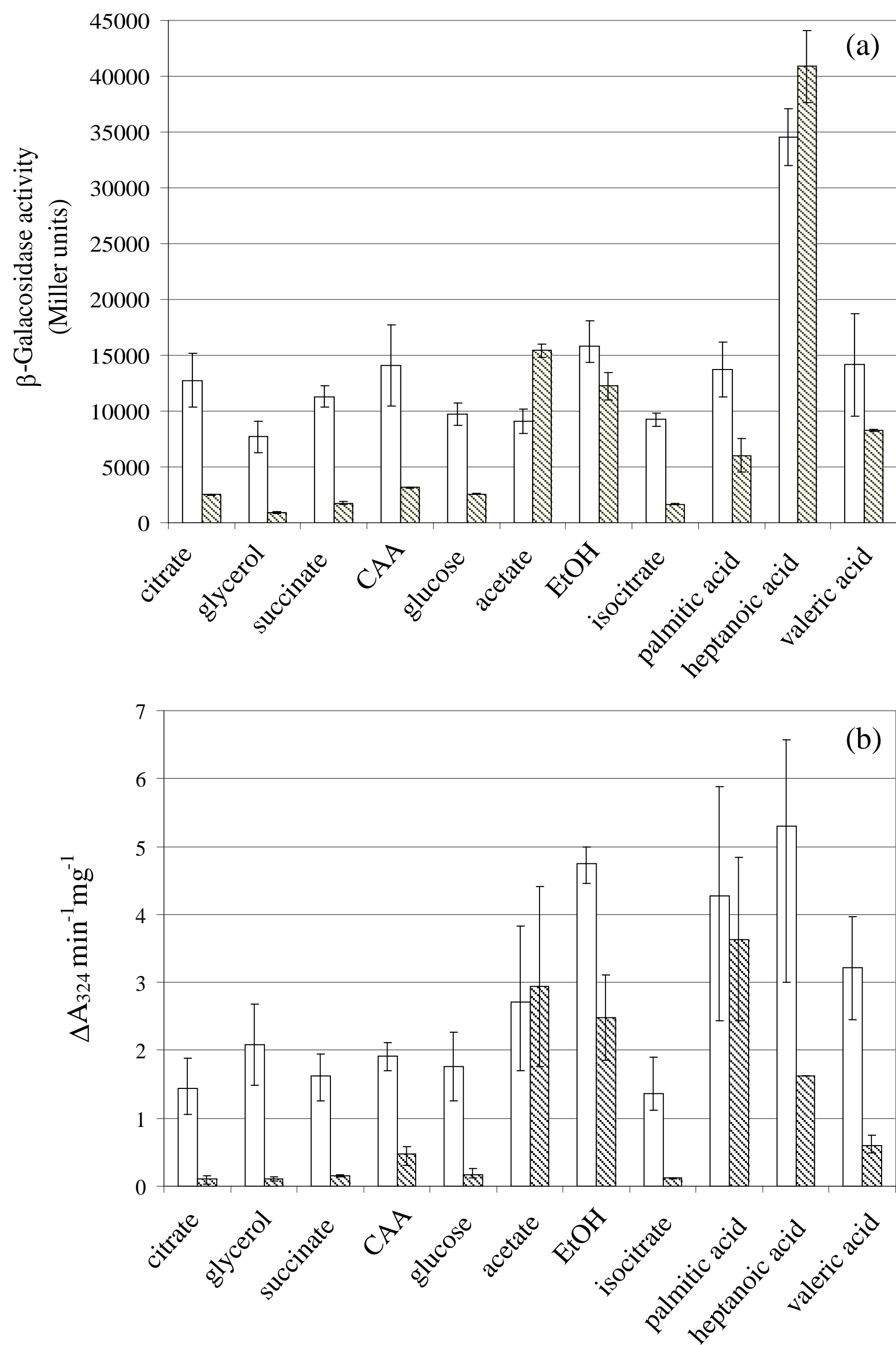


Figure 2.5 Effect of various carbon sources on *aceA::lacZ* expression and ICL activity. *P. aeruginosa* cultures were grown overnight in NCE medium supplemented with the given carbon sources. (**a**) β-galactosidase activity is presented in Miller units and **(b)** ICL activity was normalized to protein concentrations. Values presented are the mean + SD of two experiments conducted in duplicate. FRD1 ☐; PAO1 ▧.

Isocitrate lyase is induced by ketogenic amino acids in PAO1.

Current reports suggest that *P. aeruginosa* utilizes amino acids and some fatty acids as carbon sources while growing in the CF lung (Palmer *et al.*, 2005; Son *et al.*, 2007). Although the glyoxylate pathway is known to be induced in various bacteria by growth on fatty acids, much less is known about the role of the glyoxylate pathway during catabolism of amino acids. Some ketogenic amino acids yield acetyl-CoA during catabolism and would require the glyoxylate pathway for catabolism. As expected several of the ketogenic amino acids induced *aceA* expression in PAO1 including leucine, isoleucine, and tyrosine (Figure 2.6). For unknown reasons, tyrosine induced *aceA* more strongly than the other tested amino acids although expression was highly variable. PAO1 cultures grown on succinate plus tyrosine, showed ICL activity similar to growth on succinate alone (2500 Miller units) (Figure 2.7). The results confirm earlier reports that ICL expression is subjected to catabolite repression by TCA intermediates (Diaz-Perez *et al.*, 2007).

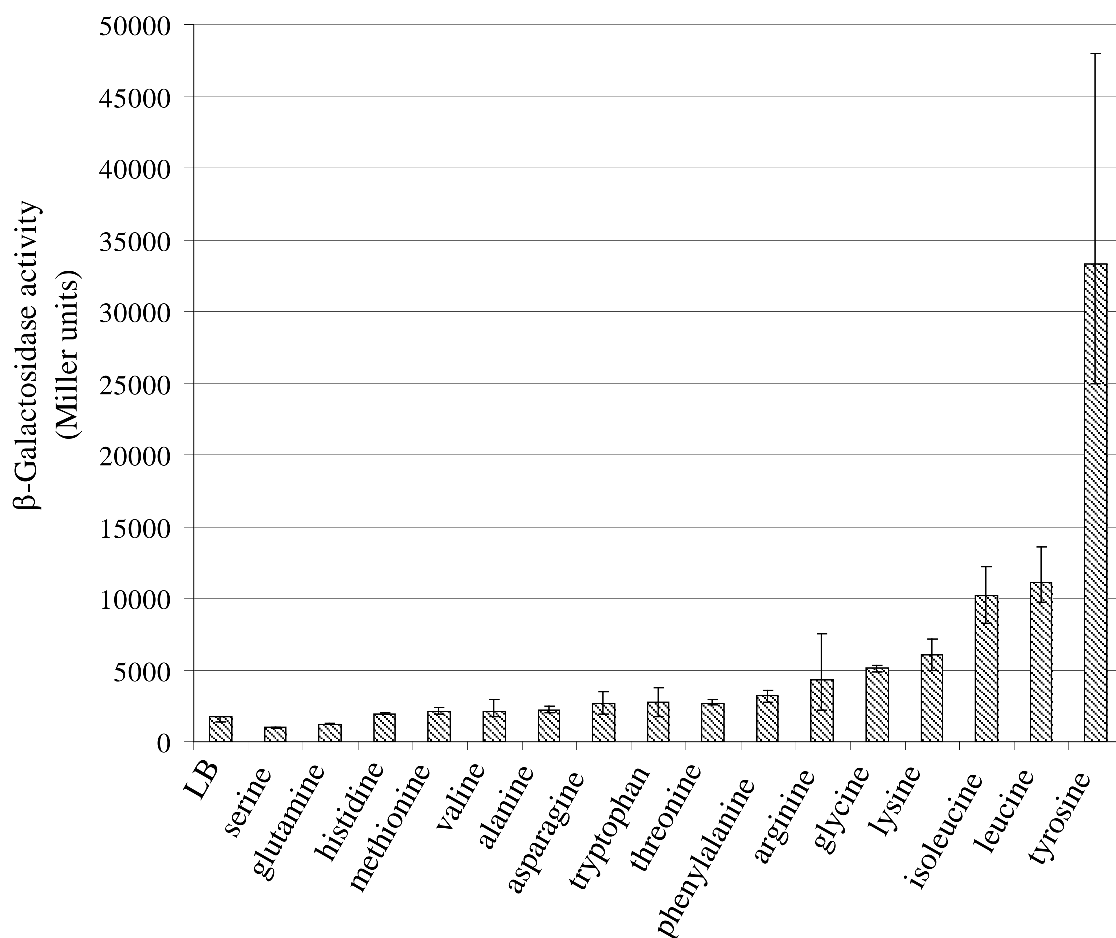


Figure 2.6 Effect of various amino acids as carbon sources on *aceA::lacZ* in PAO1. *P. aeruginosa* cultures were grown overnight in NCE supplemented with the given amino acids as carbon sources. β-galactosidase activity is presented in Miller units. Values presented are the mean $\pm$ SD of two experiments conducted in duplicate.

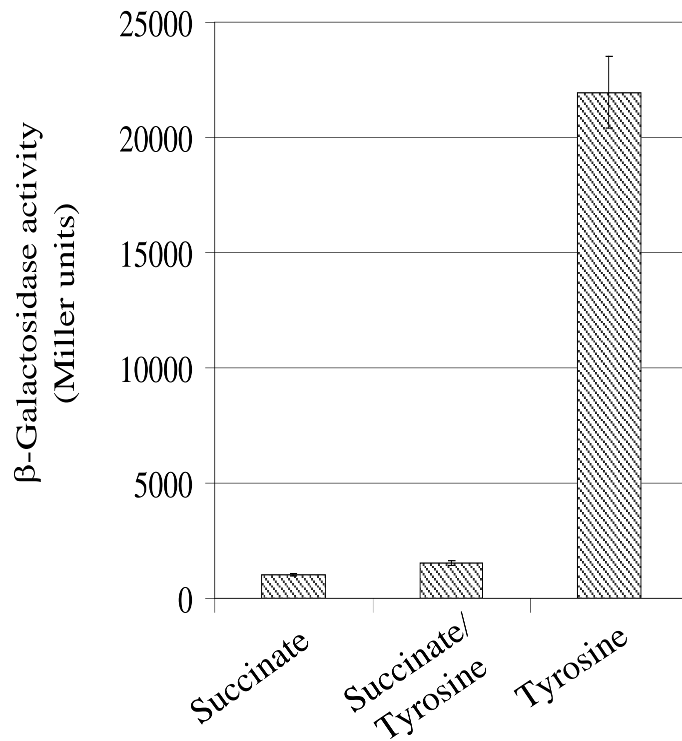


Figure 2.7 Catabolite repression of *P. aeruginosa aceA* by succinate. *P. aeruginosa* cultures were grown NCE supplemented 10 mM succinate, 0.1% (v/w) tyrosine, or both succinate and tyrosine. Cultures were taken during early log phase. β-galactosidase activity is presented in Miller units. Values presented are the mean + SD of two experiments conducted in duplicate.

Isocitrate lyase regulation

In an attempt to identify the mechanism of deregulation of *aceA* in FRD1, we measured *aceA* expression in *P. aeruginosa* derivatives disrupted for known regulators of ICL, carbon catabolism or chronic virulence. Because the catabolite repression control protein, Crc, functions post-transcriptionally to regulate carbon catabolism in Pseudomonads, we examined the effect of a *crc* knockout on *aceA* expression in PAO1. Crc is involved in repression of multiple carbon catabolic pathways in *P. aeruginosa* when preferred substrates are present (Collier *et al.*, 1996) but does not appear to control expression of *aceA* in PAO1 (Figure 2.8). Disruption of an ORF (PA0236) predicted to encode for IclR, a repressor for *aceA* in various bacteria (Gui *et al.*, 1996), also did not affect *aceA* expression in PAO1. A second repressor for *aceA* expression in other bacteria is FadR which is thought to interact with the promoter region of *iclR*, although it may also regulate *aceA* expression independently (Maloy & Nunn, 1982, Gui, *et al*., 1996). Disruption of the ORF predicted to encode for FadR however, did not affect *aceA* expression in PAO1 (data not shown). Finally, *algT* and *retS* have been characterized as encoding for regulators of chronic virulence in *P. aeruginosa*. AlgT becomes active in *P. aeruginosa* during colonization of the CF lung via acquired mutations and is responsible for overproduction of the exopolysaccharide alginate (Xie *et al.*, 1996). In order to examine this mutation in FRD1, cultures had to be grown at 32°C because a mutation in AlgT renders FRD1 temperature sensitive. In contrast, RetS down-regulates genes in PAO1 predicted to be involved in chronic virulence (Goodman *et al.*, 2004). As shown in Figure 2.8, AlgT is not responsible for the high *aceA* activity in the FRD1 background and RetS appears to positively regulate *aceA* in PAO1. Therefore, a predictive approach

to identify regulators of *aceA* transcription in *P. aeruginosa* failed to identify the reason for the deregulation of *aceA*/ICL in FRD1.

Based on the available data, it appeared that *aceA* expression is deregulated in the CF isolate while it is still regulated tightly in the non-CF isolate. Thus, to identify the mechanism(s) by which *aceA* expression is deregulated in FRD1, a PAO1 library was introduced into FRD1 carrying the *aceA::lacZ* fusion and the resulting transconjugants were screened to isolate clones with a decrease in β-galactosidase activity. From this analysis, I isolated two PAO1 genomic clones that restored *aceA* regulation in FRD1 (Figure 2.9). Since the genomic DNA carried in these plasmids was approximately 20 kb in size, I performed a transposon mutagenesis to identify the gene(s) responsible for restoring the regulation of *aceA* expression in FRD1. Two plasmids were mutagenized in vitro with EZ::TN and transformed into *E. coli*. The resulting EZ::TN library was subsequently conjugated into FRD1 carrying the *aceA::lacZ* fusion and transconjugants were again screened for β-galactosidase activity (Fig 2.9). Plasmids carrying EZ::TN insertions that have lost the restoration of aceA expression in FRD1 were further characterized to identify the mutated gene. Through transposon mapping and DNA sequencing, I determined that the transposon had inserted in *rpoN* in both plasmids. *rpoN* encodes for an alternative sigma factor, RpoN or Sigma54. To verify whether sigma-54 regulates *aceA* in *P. aeruginosa*, we disrupted *rpoN* in both FRD1 and PAO1 and then measured ICL activity. As demonstrated in Figure 2.10a, the PAO1 *rpoN* mutant had increased ICL activity compared to the parent, while the FRD1 *rpoN* mutant had a similar ICL activity to FRD1 (Figure 2.10a). Unexpectedly FRD1*rpoN* showed no decrease in

ICL activity following addition of a copy of *rpoN* from either FRD1 or PAO1. This further supports that ICL activity is likely subject to some post-translational controls. Low ICL activity was restored to the PAO1*rpoN* mutant following complementation with a wild-type copy of *rpoN* from either FRD1 or PAO1 *in cis*. Although RpoN appears to be unaltered in FRD1, increased RpoN concentrations may account for the negative impact on *aceA* expression in FRD1 (Figure 2.9). Transcription of the *aceA::lacZ* fusion in the PAO1*rpoN* mutant increased steadily over a growth cycle in cultures grown in L-broth compared to PAO1 (Figure 2.10b). These results are consistent with a regulatory effect of sigma-54 on ICL activity by affecting *aceA* transcription in PAO1.

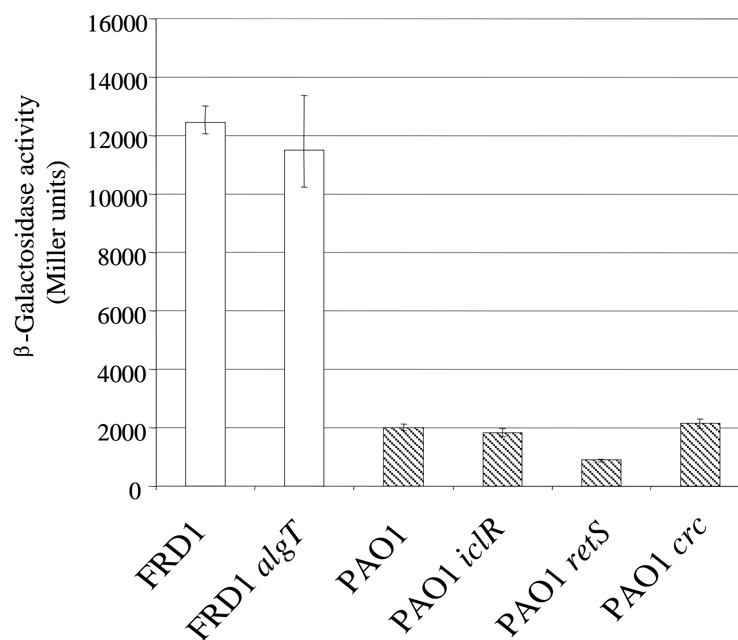


Figure 2.8 Effect of various mutations on *aceA* expression in *P. aeruginosa.* β-galactosidase activity is presented in Miller units and assayed from overnight cultures of *P. aeruginosa* grown in L-broth. Values represent the average of 2 experiments conducted in duplicate. FRD1 and FRD1*algT* were grown at 32°C while PAO1 and derivatives were assayed using cultures grown at 37°C.

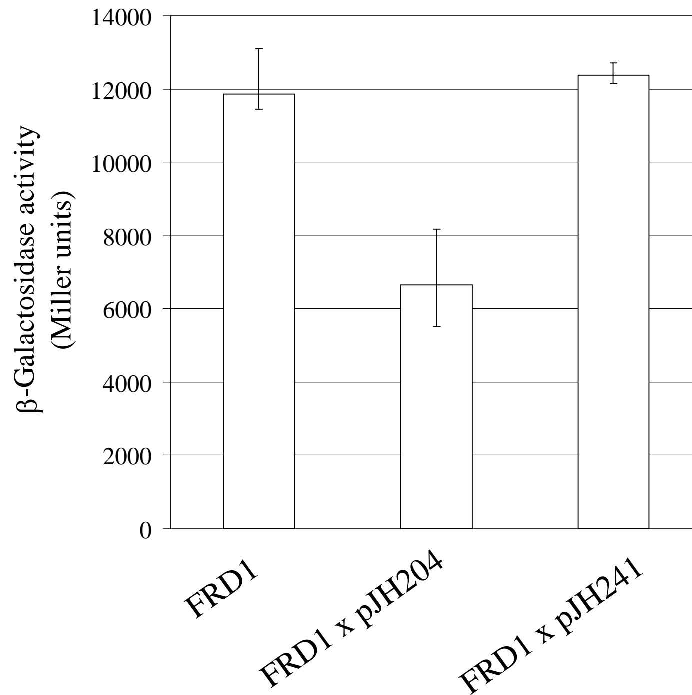


Figure 2.9 Complementation of FRD1 for *aceA* expression. β-galactosidase activity is presented in Miller units. Values represent an average of two experiments done in duplicate. Lane 1 shows *aceA::lacZ* expression in FRD1. Lane 2 represents a FRD1 *aceA*::*lacZ* complemented for restoration of regulation of *aceA* expression with a PAO1 clone from the genomic library. Lane 3 represents loss of complementing activity following transposon mutagenesis of the complementing vector.

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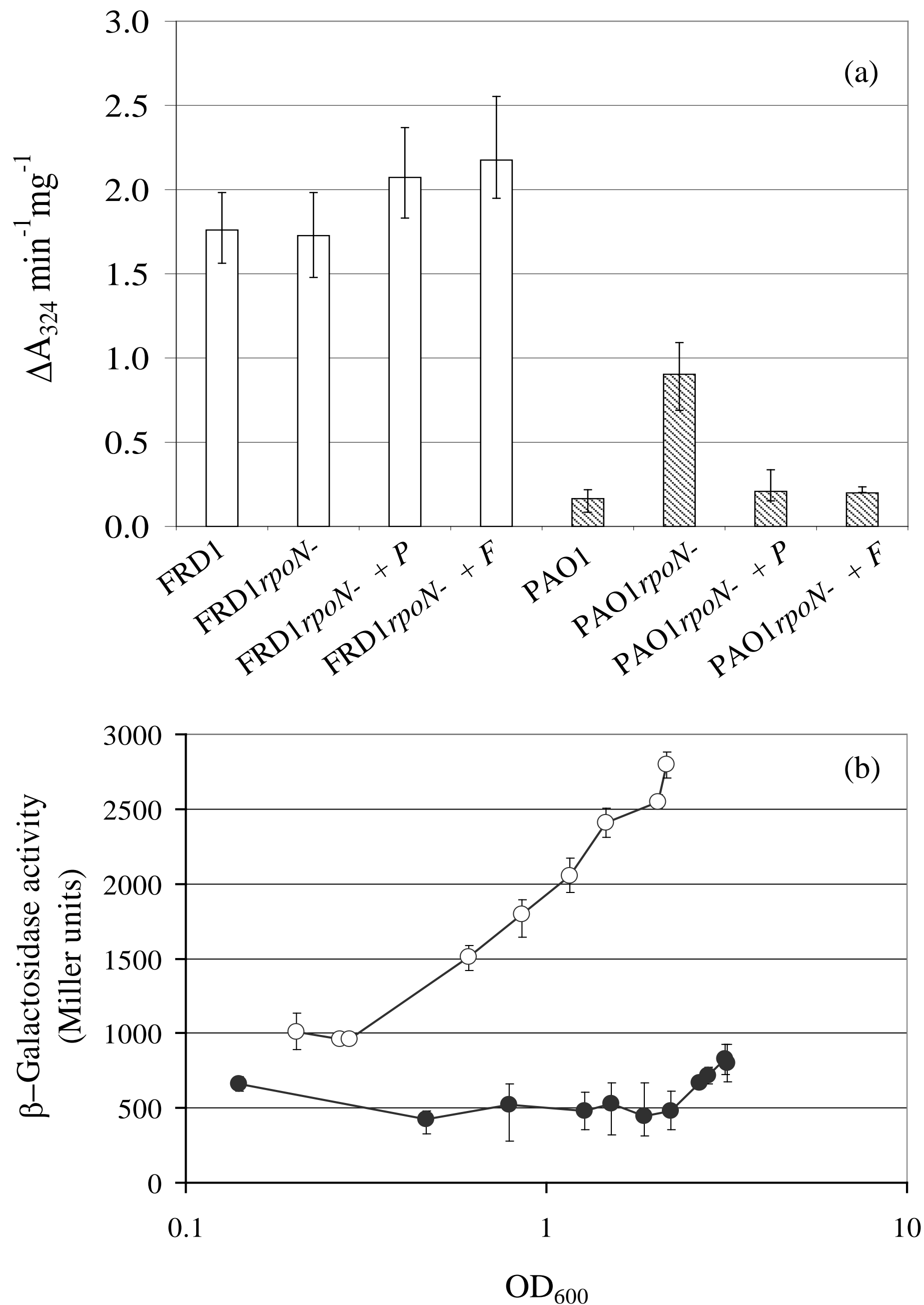

Figure 2.10 Effect of *rpoN* mutation on ICL activity and *aceA* expression. (a) ICL activity was assayed from overnight cultures of *P. aeruginosa* grown in L-broth amended with 5 mM glutamine. Complements are designated as "+ P" for those containing a wild-type copy of *rpoN* from PAO1, and "+ F"for those containing a wild-type copy of *rpoN* from FRD1. Values represent the average of 2 experiments conducted in duplicate. **(b)** β-galactosidase activity is presented in Miller units. ●, PAO1; ○, PAO1*rpoN*.

Discussion

In order to develop effective alternative strategies to combat chronic infections, it is essential to gain a better understanding of the survival and adaptive mechanisms of the pathogens. One potentiallly viable alternative strategy is to disrupt the nutrient acquisition and catabolism by the pathogen. In order to achieve this goal, it is essential to first understand the metabolic needs of chronic pathogens. In this study, we characterized regulatory control of the gene encoding for isocitrate lyase in *P. aeruginosa*. ICL is the first enzyme of the glyoxylate pathway that allows growth of some bacteria on C_2 compounds. Such compounds appear to be present in the CF lung as indicated by induction of *aceA* expression when *P. aeruginosa* is grown in CF sputum (Son *et al.*, 2007). Phosphatidylcholine (PC) is the major lipid in lung surfactant and can be degraded via lipases to produce a variety of long-chain fatty acids including palmitic acid. In addition, amino acids also appear to be major carbons sources for *P. aeruginosa* in the CF lung, including ketogenic species (Barth & Pitt, 1996; Palmer *et al.*, 2005; Son *et al.*, 2007). Long chain fatty acids and ketogenic amino acids yield acetyl-CoA during degradation and therefore would require the glyoxylate pathway for catabolism. Taken together, the glyoxylate pathway might be necessary for *P. aeruginosa* to utilize a variety of potential nutrients present in CF lungs. At present it is unclear whether catabolite repression is active in this environment to influence utilization of compounds requiring the glyoxylate pathway.

As shown in this study, some *P. aeruginosa* derivatives adapted to the CF lung, including FRD1, constitutively express *aceA*. This suggests that even in the absence of C_2

compounds ICL activity is beneficial to the bacterium in this environment, possibly for virulence determinant production. Alternatively, the FRD1*aceA* mutant grew slightly better on succinate than the parental strain, indicating that upregulation of *aceA* may provide a growth advantage on some carbon sources.

We previously showed that alginate production is reduced in the absence of ICL in FRD1 (Lindsey et al., 2008). This may reflect the need for the generation of oxaloacetate (OAA) via glyoxylate pathway (via an end product of the glyoxylate shunt, malate) to produce fructose-6-phosphate, a precursor for the exopolysaccharide, via gluconeogenesis. Alginate plays a protective role for *P. aeruginosa* during chronic virulence by limiting the diffusion of oxidative radicals, antibiotics, opsonizing antibodies and phagocytes (Hatch & Schiller, 1998; Oliver & Weir, 1985; Pedersen *et al.*, 1990; Simpson *et al.*, 1989).

One of the products of isocitrate lyase activity is glyoxylate which is condensed with acetyl-CoA by malate synthase to produce malate that can enter back into the TCA cycle. Glyoxylate can also be converted to glycine by a D-amino acid oxidase which can then be used to produce HCN which is detected in the sputum of CF patients and is associated with decreased pulmonary function (Ryall *et al.*, 2008, Sanderson *et al*., 2008). Interestingly, mucoid *P. aeruginosa* isolates have been shown to produce increased levels of HCN compared to PAO1. In addition, CF isolates such as FRD1 was suggested to utilize an alternate mechanism for increased HCN production (Carterson *et al.*, 2004). Given our data which indicated higher production of glyoxylate in CF isolates, it

appeared that there was a correlation between the preference of CF isolates for glyoxylate pathway and increased HCN production. Furthermore, we determined that the FRD1 *aceA* mutant produced a decreased amount hydrogen cyanide and that this could be restored by the addition of glyoxylate or glycine to the growth medium. In contrast, there was no significant effect on hydrogen cyanide production in the PAO1 *aceA* mutant (Hagins *et al*., 2009). Thus, we believe that glyoxylate from isocitrate lyase is converted to glycine which can then be used for HCN synthesis in FRD1. In support of this hypothesis, we recently identified PA5304, *dadA*, predicted to encode for D-amino acid oxidase which has homology to known enzymes of other organisms that catalyze the conversion of glyoxylate to glycine (Hagins *et al*., 2009). The results from the study by Hagins *et al.,* (2009) suggest the high ICL activity in FRD1 contributes to high levels of glycine via conversion of glyoxylate. DadA was demonstrated to play a role in this process and represents one possible mechanism utilized by FRD1 for increased HCN production in the lung. These data indicate that central metabolism is not only necessary for utilization of carbon sources present during infection but it is also important for the production of virulence determinants by *P. aeruginosa* (Hagins *et al*., 2009).

The correlation between ICL enzymatic activity and *aceA* transcription in PAO1 has been noted previously (Kretzschmar *et al.*, 2008). However, we show here that ICL activity is also regulated post-transcriptionally. While heptanoic acid induces *aceA::lacZ* activity more strongly than palmitic aid, the resulting ICL activity is higher following growth of PAO1 on palmitic acid compared to heptanoic acid. In the FRD1 background, ICL activity is clearly upregulated following growth on C_2 carbon sources for which a

corresponding increase in *ace::lacZ* is lacking. ICL activity in other bacteria is allosterically affected by various metabolites (Hoyt *et al.*, 1991; Kumar & Bhakuni, 2008; MacKintosh & Nimmo, 1988; Reinscheid *et al.*, 1994). However, such information is lacking for *P. aeruginosa* ICL.

While a predictive approach failed to identify the mechanisms of deregulation of *aceA* in FRD1, it did identify RetS as a potential positive regulator of *aceA* in PAO1. The alternative approach to complement the low *aceA* activity in FRD1 with a PAO1 genomic library identified RpoN to negatively regulate *aceA*. However, RpoN appears to be functional in FRD1, indicating that complementing activity of *rpoN* on *aceA* expression in FRD1 may be due to copy effect or increased *rpoN* expression. These data also support the idea that ICL activity is subject to some post-translational controls. No other complementing clones were identified from the PAO1 genomic library using this approach. This suggests either the library is incomplete, the genetic screen was faulty, multiple mutations are required to cause deregulation of *aceA* in FRD, or that multiple copies of the affected regulator renders the cell unviable. We are continuing to identify regulators of *aceA* using alternative approaches.

RpoN is widely distributed among bacteria and is involved in regulation of a variety of functions including nitrogen assimilation, motility, virulence and carbon source utilization. In other Pseudomonads, disruption of *rpoN* affects growth on a several C_4-dicarboxylic acids such as succinate and fumarate (Hendrickson *et al.*, 2000; Kohler *et al.*, 1989) indicating a role in central metabolism. However, this is the first report of

RpoN affecting transcription of *aceA* in any bacterium to our knowledge. In *P. aeruginosa*, RpoN plays a role in alginate biosynthesis (Kimbara & Chakrabarty, 1989), flagellin and pilin production (Totten *et al.*, 1990) and production of several virulence determinants (Heurlier *et al.*, 2003). Indeed, RpoN is required for PA14 virulence on various hosts (Hendrickson *et al.*, 2001). However, loss of RpoN may facilitate chronic colonization as suggested by persistence of *P. aeruginosa* within the CF lung lacking RpoN (Smith *et al.*, 2006). Benefits acquired by loss of RpoN include resistance to several antibiotics (Smith *et al.*, 2006; Viducic *et al.*, 2007), increased production of elastase and rhamnolipid (Heurlier *et al.*, 2003), and resistance to phage infection (Webb *et al.*, 2003). Our data indicate that RpoN negatively regulates *aceA* gene expression that is deregulated in CF isolates presumably due to adaptation. Thus, it appears that selective activity of RpoN maybe advantageous for adapting to the CF environment. Since RpoN is reminiscent of eukaryotic transcriptional factors and it needs to act with specific activator proteins to induce transcription from target genes, it is possible that CF isolates may have acquired a mutation in an activator that is involved in repression of *aceA*. Further study is required to decipher the mechanism by which RpoN regulates *aceA* and whether it plays a role in chronic infection.

Induction of the glyoxylate pathway during chronic infection is common to several bacterial pathogens, including *P. aeruginosa* (Fang *et al.*, 2005; McKinney *et al.*, 2000; Son *et al.*, 2007). However, the role of this pathway in chronic infection is poorly understood. Our studies suggest that the glyoxylate pathway may be required for catabolism of certain nutrients by *P. aeruginosa* within the CF lung and it is required for

optimal production of several virulence determinants. Bacterial metabolic pathways active during infection are attractive targets for therapeutic intervention if proven to be essential and non-redundant. A better understanding of the glyoxylate pathway and other metabolic pathways that are active in chronic isolates of *P. aeruginosa* is required before such therapeutic approaches can be identified.

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Chapter 3

Initial characterization of *Pseudomonas aeruginosa glcB* that encodes for malate synthase

Abstract

Pseudomonas aeruginosa causes chronic infections in patients with cystic fibrosis (CF). However, the molecular mechanisms utilized by this pathogen to survive and persist in the CF lung are not completely understood. Our previous study demonstrated that *aceA,* which encodes for isocitrate lyase, is required by CF and non-CF isolates of *P. aeruginosa* to cause infections in the alfalfa seedling model. In addition, *aceA* is also required by PAO1, a wound isolate of *P. aeruginosa*, to cause disease in a rat chronic lung infection model. Isocitrate lyase is one of two enzymes that catalyze the glyoxylate pathway which allows some bacteria to grow on acetate and fatty acids as the sole carbon source. In an attempt to determine if other genes involved in acetate metabolism play a role in pathogenesis of CF isolates, I mutagenized *P. aeruginosa* to identify genes required for growth on acetate. From this analysis, *glcB*, predicted to encode for malate synthase, the second key enzyme of the glyoxylate pathway, was identified to be required for growth of *P. aeruginosa* on acetate. Moreover, *glcB* was also required for *P. aeruginosa* infection of alfalfa seedlings. In this study, I demonstrate that *glcB* does encode for malate synthase activity. In addition, I characterized expression of *glcB* in

response to various carbon sources. Expression of *glcB* appears to be regulated similarly to *aceA,* although it is not in the same operon as *aceA*. Likewise, similar to expression of *aceA*, *glcB* expression is deregulated in the CF isolate FRD1 compared to a non-CF isolate, PAO1.

Introduction

High fatty acid concentrations have been reported to be present within the lung (Meyer *et al*., 2000). Thus, it is not surprising that genes involved in fatty acid metabolism are upregulated in *P. aeruginosa* isolates growing *in vivo* (Son *et al*., 2007). These data suggest that lipids and lung surfactant are the carbon sources utilized by *P. aeruginosa* within the CF lung to grow and persist (Son *et al*. 2007). To grow on compounds such as acetate or fatty acids, *P. aeruginosa* requires a functioning glyoxylate pathway (Kretzxchmar *et al*., 2002). Data from several studies show that the glyoxylate pathway is important for various chronic pathogens (McKinney *et al.,* 2000, Lorenz & Fink, 2001, Fang *et al*., 2005). The first enzyme of the glyoxylate pathway is isocitrate lyase, which cleaves isocitrate to glyoxylate and succinate. Malate synthase is the second enzyme of the glyoxylate pathway and combines acetyl-CoA with glyoxylate to produce malate, which can enter back into the TCA cycle. In *E. coli*, glyoxylate can be generated via the glyoxylate pathway, by isocitrate lyase or via an alternative pathway from glycolate degradation (Ornston & Ornston, 1969). Glyoxylate can then be utilized via one of two enzymes to form malate in *E. coli*. These enzymes are malate synthase A (MSA), encoded by *aceB*, and malate synthase G (MSG), encoded by *glcB* (Vanderwinkel & De Vlieghere, 1968). In *E. coli* the *aceB* gene is located in an operon with *aceA*, which

encodes for isocitrate lyase, and *aceK*, which encodes for isocitrate dehydrogenase kinase/phosphatase (Chung *et al*., 1988). The *aceBAK* operon in *E. coli* is induced by acetate (Maloy & Nunn, 1982, LaPorte *et al*., 1985). The second *E. coli* malate synthase encoded by *glcB*, is found in a separate operon, *glcDEFGB* and is induced by glycolate (Molina *et al*., 1994). MSG is also induced when acetate is present in the environment (Pellicer *et al.*, 1999). This induction is mediated at the transcriptional level by the *glcC* gene product, which acts as an activator (Pellicer *et al.*, 1999). The gene encoding malate synthase in *P. aeruginosa* and *M. tuberculosis* is not located in an operon with *aceA* (Smith *et al*., 2003). In *P. aeruginosa* the gene encoding for MS has been designated *glcB* because of high homology (75%) to the *E. coli glcB* gene (Winsor *et al*., 2009, Stover *et al*., 2000). However, this gene has not been characterized in *P. aeruginosa.* Therefore, the goal of this study was to provide a preliminary characterization of *glcB* in *P. aeruginosa* physiology and virulence.

Materials and Methods

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 3.1. Bacteria were cultured in L-broth (LB) or on L-agar. No-carbon-E-minimal medium (NCE) supplemented with 0.1% (w/v) casamino acids (CAA) was used supplemented with various carbon sources. The carbon sources that were used include: Leucine, and CAA at 1% (w/v), 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, Valeric acid at 10 mM, other carbon sources were used at 20 mM. To select for acetate mutants, 40 mM of succinate and 30 mM of acetate was used and the media was

solidified with 1.5% (w/v) Bacto Agar (Difco; Becton Dickinson). To select for *Pseudomonas* transconjugants and counter select for *E. coli*, a 1:1 mixture of L-agar and Pseudomonas Isolation Agar (PIA) was used. For growth of the *rpoN* mutants, 1 mM glutamine was added to the media. Antibiotic from Sigma-Aldrich (St. Louis, MO) were used at the following concentrations: 100 µg ampicillin ml^{-1} for *E. coli*, 180 µg carbenicillin ml^{-1} for *P. aeruginosa*, 20 µg tetracycline ml^{-1} for *E. coli*; 100 µg ml^{-1} for *P. aeruginosa,* 50 µg kanamycin ml^{-1} for *E. coli*, and 700 µg kanamycin ml^{-1} for *P. aeruginosa*, and 20 µg and 200 µg of gentamicin ml^{-1} for *E. coli* and *P. aeruginosa* respectively. A Shimadzu UV-1601 spectrophotometer was use to record UV-visible absorption spectra. 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 regularly used as a host strain for cloning. Electroporation was used to introduce DNA into *E. coli* while conjugation was used for *P. aeruginosa* as previously described (Suh *et al.*, 1999). Plasmids and DNA fragments excised from agarose gels were purified with QIAprep Spin Miniprep columns and Qiaex II DNA gel extraction kit respectively (Qiagen, Valencia, CA) according to the manufacturer's instructions. Restriction enzymes, ligase, and *Taq* were purchased from New England Biolabs while *Pfu* was purchased from Stratagene (La Jolla, CA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Strain or Plasmid	Genotype, relevant characteristics	Source
Strains		
FRD1	CF isolate, mucoid	Ohman & Chakrabarty (1981)
PAO1	Wound isolate, nonmucoid	Holloway *et al.* (1979)
FRD1*rpoN* (LS1775)	FRD1*rpoN101::*ΩTc	Chapter 2
PAO1*rpoN* (LS1773)	PAO1*rpoN101::* ΩTc	Chapter 2
FRD1*glcB* (JH104)	FRD1*glcB101::aaCI*	This study
PAO1*glcB* (JH105)	PAO1*glcB101::aaCI*	This study
FRD1*glcB*+ (JH148)	FRD1 *glcB* complemented for *glcB*	This study
PAO1*glcB*+ (JH151)	PAO1 *glcB* complemented for *glcB*	This study
FRD1 *glcB::lacZ* (JH133)	FRD1 carrying *glcB::lacZ* fusion	This study
PAO1*glcB::lacZ* (JH135)	PAO1 carrying *glcB::lacZ* fusion	This study
Plasmids		
pLS1155	plasmid with regulatable promoter	Silo-Suh *et al.* (2005)
pJH130	*glcB::lacZ* transcriptional fusion in pSS223	This study
pJH101	*glcB101* in pBluescript K(+)	This study
pJH141	*glcB* complementing plasmid	This study
pAG408	Mini Tn5 containing plasmid	Suarez *et al.* (1997)

Table 3.1 Bacterial strains and plasmids.

Biochemical assays. Malate synthase activity was measured according to the Sigma Aldrich protocol (EC 4.1.3.2), with slight modification: *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 DTNB 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)$^{-1}$ where the ΔA_{412} min^{-1} was determined using the linear rate of the reaction. β-galactosidase assays were preformed as described by Miller (1972). Cultures were grown in L-broth or minimal medium with a specific carbon source.

Transposon mutagenesis of FRD1. *P. aeruginosa* strain FRD1 was mutagenized with Tn*gfp* carried on plasmid pAG408 (Suarez *et al*., 1997). Plasmid pAG408 was introduced into FRD1 via triparental mating (Suh *et al*., 1999) and transconjugants were plated onto PIA agar containing gentamicin to select for FRD1 cells containing the transposon. Transconjugants that grew on NCE agar plates containing 40 mM succinate but failed to grow on NCE agar plates containing 30 mM acetate were isolated and characterized.

Mapping the transposon insertion sites. Genomic DNA was obtained from the transposon insertion mutants using Wizard Genomic (Promega, Madison, WI) and digested with appropriate enzymes. The digested DNA was then cloned into pBluescript

K(+) and electroporated into *E. coli* and plated on L-agar containing gentamicin. The colonies that were gentamicin resistant were verified for the presence of the transposon via PCR. Those that contained the transposon were sent for sequencing to the Auburn University Research and Instrumentation Facility.

Alfalfa seedling infection assay. Alfalfa seedlings were infected according to the model described by Silo-Suh *et al* (2002). The assay was carried out as follows: Bacterial cultures, grown to mid-to-late log phase, were diluted in saline and used to inoculate wounded alfalfa seedlings. After inoculation, the alfalfa seedlings were incubated for 6-7 days after which time they were scored for disease symptoms. Disease symptoms include yellowing of the leaf tissue, water soaked regions, and often a brown "goop" appearance on the leaf and surface. Alfalfa seedlings that exhibited any of the given symptoms were designated as infected.

Construction of the *P. aeruginosa glcB* mutants. Primers specific for *glcB* were used to PCR amplify the sequence from FRD1 using *Pfu*. The PCR fragment was digested with *Eco*RI and *Hind*III resulting in a fragment containing the *glcB* ORF and 380 bp upstream of the coding sequence. The 2.8 kb fragment was cloned into pBluescriptSK (+) at the *Eco*RI/*Hind*III site. The resulting plasmid was digested with *Stu*I and a 1.1 kb fragment was removed from within the *glcB* coding region and replaced with the *aacC1* gene encoding gentamicin resistance (Schweizer, 1993). An origin of transfer (m*oriT)* of RP4 was cloned into the plasmid at the *Hind*III site (Suh *et al*., 2004) to yeild pJH101. This plasmid was introduced into *P. aeruginosa* strains and potential *glcB* mutants were

isolated as gentamicin resistant carbenicillin sensitive colonies that resulted from a double crossover event. Replacement of the wild-type *glcB* gene with the *glcB101::aacCI* allele was verified by PCR analysis and checked for inability to grow on minimal media with acetate as a sole carbon source.

Construction of the *glcB* complemented strains. To complement the *glcB* mutation, the wild-type gene was PCR amplified from FRD1 using *Pfu*. The resulting fragment was digested with *Eco*RI and cloned into the *Eco*RI and *Sma*I site of pLS1155, which contains a regulatable promoter upstream of the multiple cloning site (Silo-Suh *et al*., 2005). The resulting plasmid was digested with *Hind*III and the m*oriT* was inserted to allow for mobilization of the plasmid (pJH141) into *P. aeruginosa*. The plasmid was mobilized into *P. aeruginosa* by triparental mating and potential complemented strains were isolated as kanamycin resistant colonies. Complementation was verified by PCR analysis and the complemented FRD1*glcB* and PAO1*glcB* isolates were designated FRD1*glcB* + (JH148) and PAO1*glcB* + (JH151) respectively.

Construction of *glcB* transcriptional fusions. The *glcB*::*lacZ* transcriptional fusion was constructed using the *glcB* gene fragment obtained from FRD1 via PCR using *Pfu* and an upstream primer that contained an *Eco*RI site. The fragment was digested with *Pvu*II and *Eco*RI and the resulting fragment, which included 380 bp upstream from the coding sequence, was cloned into the *Eco*RI and *Sma*I digested pSS223 (Suh *et al*., 2004). The plasmid, pJH130, containing the promoter region for *glcB*, was verified by PCR and restriction digests and conjugated into FRD1, FRD1 *aceA* mutant and PAO1.

Results

Isolation and characterization of FRD1 mutants unable to utilize acetate as a sole carbon source in the alfalfa seedling model

I screened approximately 22,000 random Tn*gfp* insertion mutants of FRD1 and isolated 7 mutants that were unable to grow with acetate as a sole carbon source. The transposon insertion site was identified for 6 of the 7 mutants and all were tested for their ability to infect alfalfa seedlings. As shown in Table 3.2, 6 of the 7 mutants were reduced for virulence compared to FRD1, indicating that growth on acetate plays an important role in the ability of FRD1 to cause disease. Not surprisingly, a FRD1*aceA* mutant was recovered from the mutagenesis and screening process. More importantly, another identified mutant was severely reduced for virulence on alfalfa and contained a transposon insertion in the gene encoding for putative malate synthase (*glcB*). Therefore, an intact glyoxylate pathway is required for FRD1 virulence on alfalfa. This study supported our hypothesis that the glyoxylate pathway is likely a critical component in chronic infections of *P. aeruginosa.* In order to determine whether the glyoxylate pathway also plays a role in pathogenesis of a non-CF isolate of *P. aeruginosa*, an insertion mutation was generated within the *glcB* gene in the wound isolate PAO1 and tested for virulence on alfalfa. An identical mutation was generated in FRD1 for comparison. As shown in Table 3.3 *glcB* is required for optimal virulence of both CF and non-CF isolates on alfalfa seedlings.

FRD1*glcB* and PAO1*glcB* are defective for growth on acetate

To determine whether disruption of *glcB* led to a growth defect on a defined medium, the mutant strains were compared to the parental strain over a growth period in NCE minimal medium containing acetate or succinate as a sole carbon source. As shown in Figure 3.1, neither mutant was defective for growth on succinate. However, both mutants showed reduced growth on acetate, which was restored via genetic complementation with a wild-type copy of *glcB*. These results verify that *glcB* is required for both FRD1 and PAO1 to grow optimally on acetate. Furthermore the data demonstrate that these genes encode for the enzymes of the glyoxylate pathway in *P. aeruginosa.*

Strains	Description	% Infection[1]
FRD1	wild-type	96%
FRD1 *aceA*	Tn insertion within PA2634-*aceA*, isocitrate lyase	24%
FRD1 *glcB*	Tn insertion within PA0482-probable malate synthase G	17%
FRD1 *acsA*	Tn insertion within PA0887-probable acetyl-CoA synthetase	60%
FRD1 *PA3604*	Tn insertion within PA3604- probable two-component response regulator	59%
FRD1 *PA3271* (*putP*)	Tn insertion within PA3271-probable two-component sensor (*putP*)	91%
FRD1 *epd*	Tn insertion within PA0551-D-erythrose 4-phosphate dehydrogenase	58%
FRD1 *unknown*	Tn insertion unknown	64%

Table 3.2 Infection of alfalfa with FRD1 acetate mutants.
[1]Values represent the % of 50 seedlings with severe symptoms.

Strain[1]	% Infection[2]
FRD1 wild-type	91%
PAO1 wild-type	100%
FRD1 *glcB101*::*aacCI*	13%
PAO1 *glcB101*::*aacCI*	40%

Table 3.3 Malate synthase is required for optimal infection of CF and non-CF isolates on alfalfa seedlings.

[1.] *aacCI*: gentamicin resistant cassette

[2.] Values represent the number of seedlings showing maceration symptoms and is the average of 3 or more experiments with 50 seedlings each.

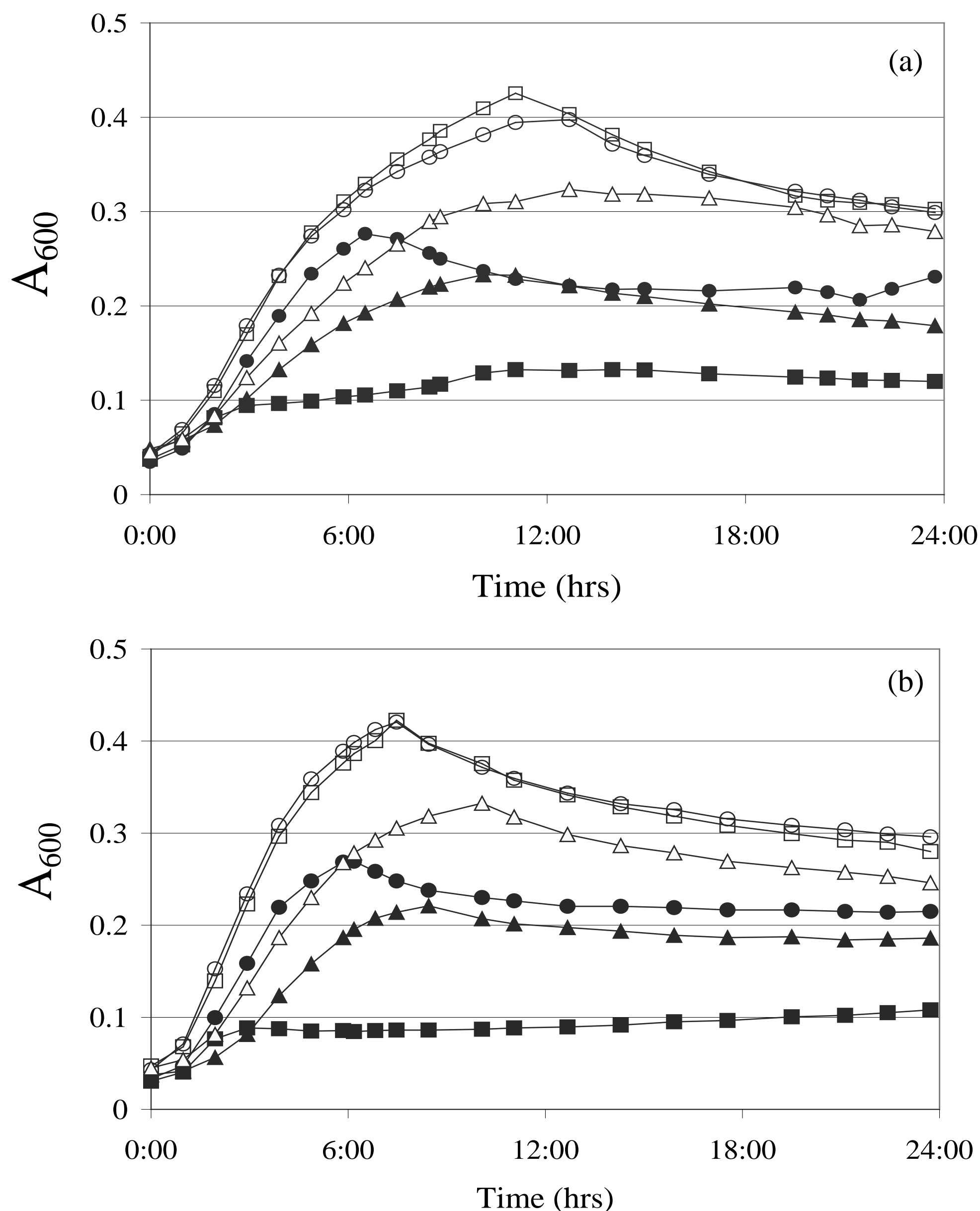

Figure 3.1 *glcB* is required for optimal growth of FRD1 and PAO1 on acetate as a sole carbon source. (a) FRD1 and derivatives. **(b)** PAO1 and derivatives. Cultures were grown in minimal media with succinate and acetate as a sole carbon source for 24 hours. ●, wild-type grown in acetate;○, wild-type grown in succinate; ■, *glcB* mutant grown in acetate; □, *glcB* mutant grown in succinate; ▲ *glcB* complemented strain grown in acetate; Δ, *glcB* mutant grown in succinate.

***glcB* encodes for malate synthase enzymatic activity**

In order to determine if *glcB* encodes for malate synthase activity as predicted by DNA and predicted amino acid sequence homology, the parent, the *glcB* mutant and the complemented strains were tested using a standard malate synthase (MS) assay. As shown in Figure 3.2, cell free extracts prepared from the parental strains FRD1 and PAO1 grown in L-broth exhibit MS activity while disruption of *glcB* led to reduced MS activity. Complementation of the FRD1 and PAO1 *glcB* mutant with a wild-type copy of the gene from FRD1 restored the MS activity. The behavior of *glcB* in PAO1 suggests this gene is induced by substrates that are catabolized by the glyoxylate pathway, such as C_2 carbon sources. Similar to the ICL data, FRD1 cell free extracts showed increased MS activity (approximately 3-fold) when grown on L-broth compared to PAO1 extracts, suggesting regulation of *glcB* is altered in FRD1. The residual MS activity observed in FRD1*glcB* mutant may be due to the presence of another gene encoding for MS activity in *P. aeruginosa*. However, examination of the PAO1 genome did not reveal the presence of such a gene although this does not eliminate the possibility of another gene that encodes for MS in FRD1 genome. Alternatively, the residual MS activity may be an artifact of the assay.

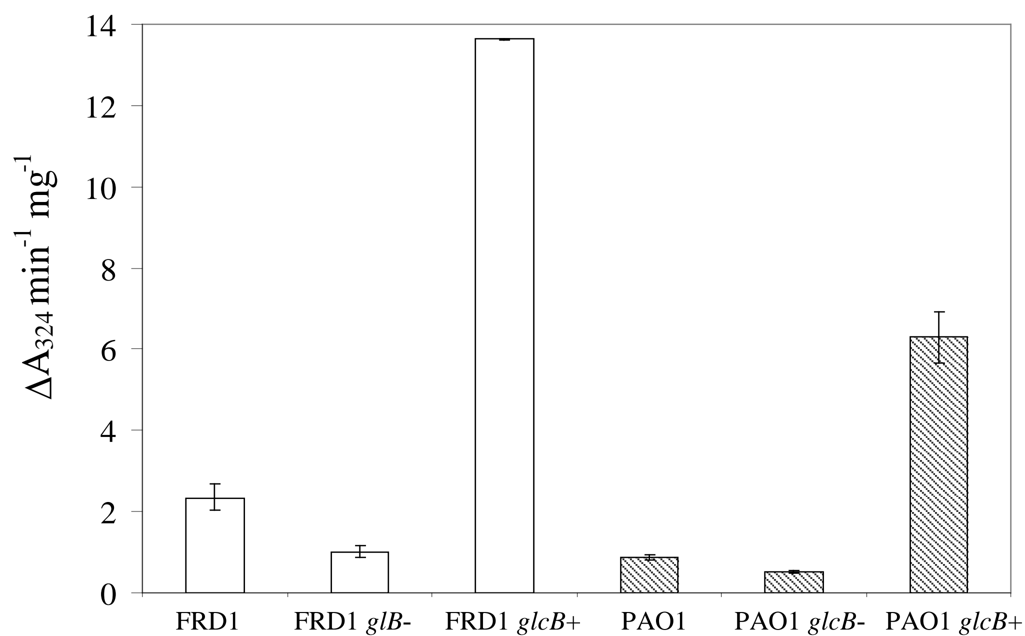

Figure 3.2 Activity of MS is altered in FRD1. The data represents the average of three experiments. Malate synthase activity was measured from cells grown in L-broth overnight. MS activity is expressed as ΔA_{324} min^{-1} mg^{-1}. FRD1 and derivatives ▯; PAO1 and derivatives ▨.

Malate synthase activity correlates with *glcB* expression level

In order to study *P. aeruginosa glcB* expression, a *glcB*::*lacZ* transcriptional fusion was constructed by cloning the promoter region of the gene from FRD1 into a *lacZ* reporter vector. The fusion plasmid was integrated into FRD1 and PAO1 genomes via homologous recombination between the *glcB* promoter sequences and *glcB*::*lacZ* expression was determined. As shown in Figure 3.3, *glcB* is expressed at a significantly higher level in FRD1 than in PAO1 over a growth cycle when grown in L-broth. Peptides, which comprise the L-broth have not been reported to induce *glcB* expression in other bacteria. The expression pattern of *glcB* in FRD1 under these conditions suggests its expression might be deregulated in a similar manner to *aceA* expression in FRD1. Malate synthase activity correlates with *glcB* expression with cells grown in L-broth indicating that enzymatic activity is largely controlled by gene expression at the transcriptional level.

High ICL activity does not contribute to increased *glcB* expression in FRD1

I questioned whether high *glcB* expression in FRD1 might be a consequence of increased ICL enzymatic activity that would result in high glyoxylate concentrations. However the *glcB*::l*acZ* fusion retained high expression in the FRD1*aceA* mutant, indicating that there is an alternative explanation for the high *glcB* expression in FRD1 (Figure 3.4).

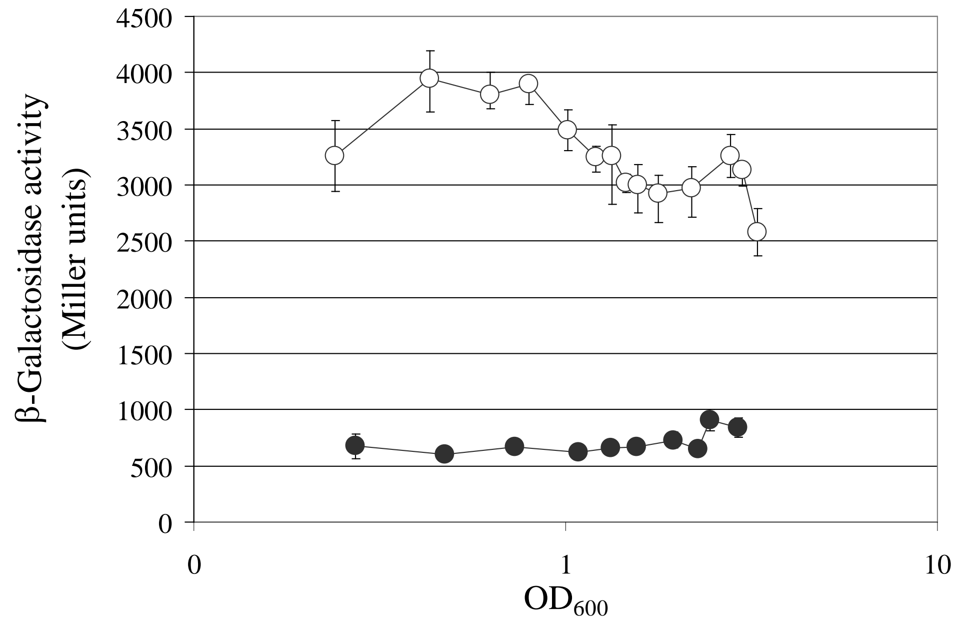

Figure 3.3 Expression of *glcB*. β-galactosidase assays were done using cultures grown in L-broth and samples taken throughout a growth cycle. Activity is expressed in Miller Units and is an average of 3 experiments conducted in duplicate. ○ FRD1; ● PAO1.

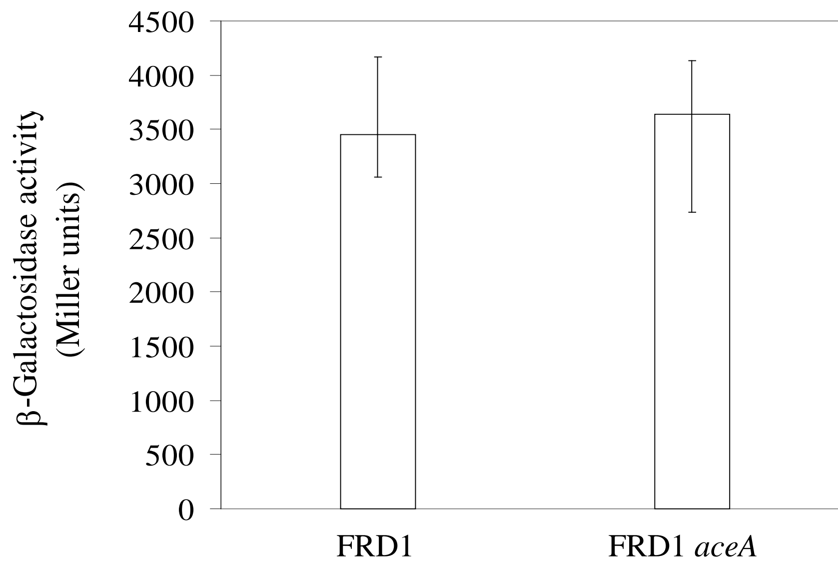

Figure 3.4 Increased expression of *glcB* is not a result of high ICL activity. β-galactosidase assays were performed using cultures from late-exponential growth phase. Activity is expressed in Miller units the data shown are an average of 3 independent experiments done in duplicate.

Effect of carbon sources on *glcB* expression and MS activity

The expression of *glcB* and the activity of MS were characterized in both FRD1 and PAO1 in response to various carbon sources to clarify whether *glcB* is deregulated in FRD1, or induced by peptides. Carbon sources that affected expression of *aceA* were tested along with the TCA cycle intermediates, which are preferred carbon sources for *P. aeruginosa*. Enzymatic assays were conducted in a subset of the same carbon sources to verify the expression studies. As shown Figure 3.5a, expression of *glcB* in FRD1 is higher than in PAO1 in response to almost all of the carbon sources tested. Not surprisingly, some of the same carbon sources that induce expression of *aceA* also induce expression of *glcB* including palmitic acid, heptanoic acid, and the amino acid, leucine. One exception was the valeric acid which did not greatly induce *glcB* expression as it did *aceA* expression in PAO1. Interestingly, acetate induced *glcB* expression in PAO1, while glyoxylate did not. Taken together, my results suggest that *glcB* is subject to regulatory control(s) similar to *aceA* in *P. aeruginosa* and it is deregulated in the CF isolate FRD1. However induction of *glcB* expression in FRD1 by fatty acids suggests some regulatory control remains intact. For most of the carbon sources tested, MS activity correlated with *glcB* expression in both FRD1 and PAO1 (Figure 3.5b).

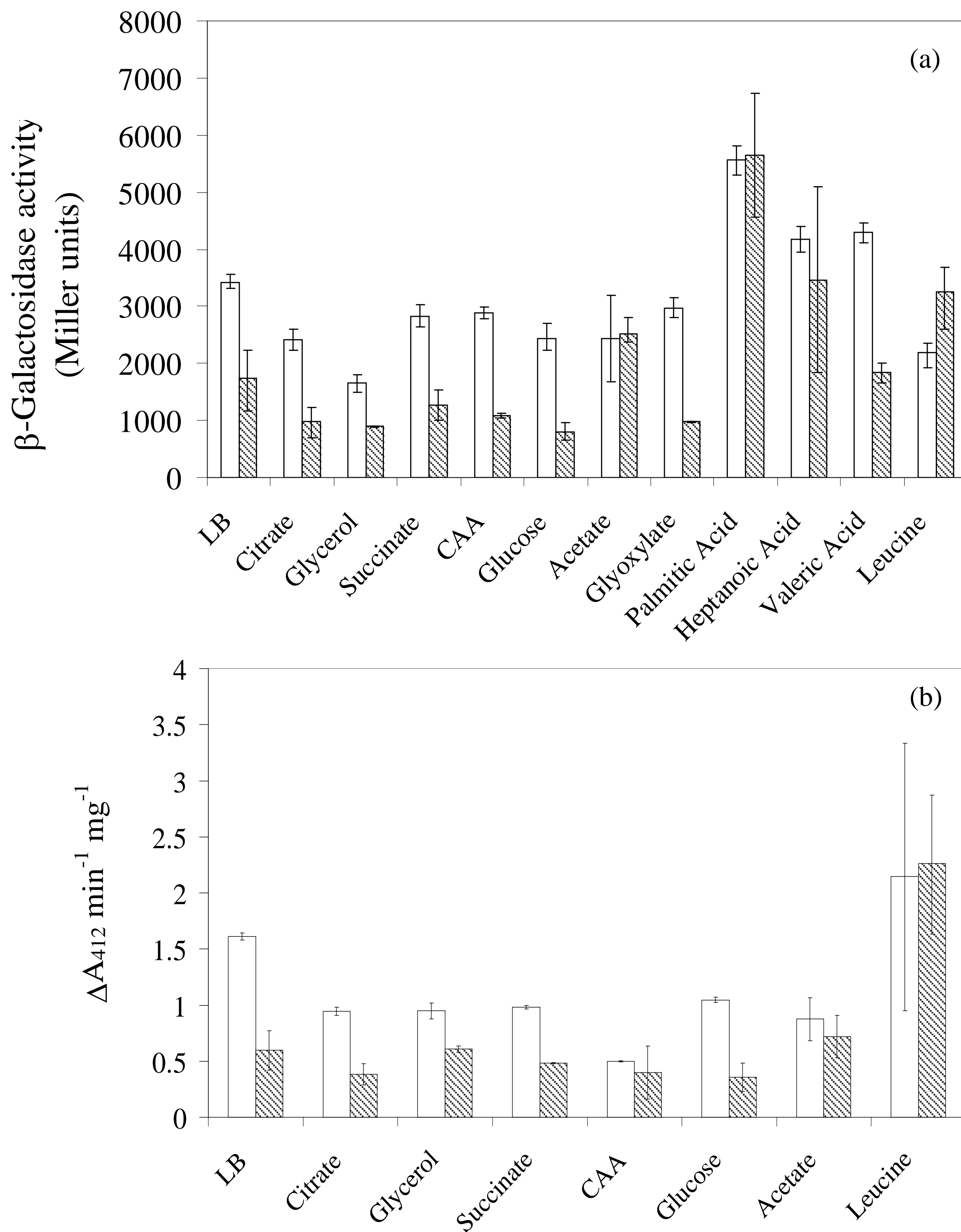

Figure 3.5 Effect of various carbon sources on *glcB::lacZ* expression and MS activity. *P. aeruginosa* cultures were grown overnight in NCE supplemented with the given carbon sources. Values presented are the mean ± SD of two experiments. FRD1 ▯; PAO1 ▨. **(a)** β-galactosidase activity is presented in Miller units. **(b)** MS activity is presented as $\Delta A_{412}min^{-1}mg^{-1}$.

RpoN regulates *glcB* in PAO1

We previously established that the alternative sigma factor RpoN negatively regulates *aceA* gene expression in PAO1. To determine whether RpoN also regulates *glcB*, the *glcB*::*lacZ* transcriptional fusion was moved into both the FRD1 and PAO1 *rpoN* mutants. Characterization of β-galactosidase activity over a growth cycle revealed that like *aceA*, *glcB* appears to be negatively regulated by RpoN in PAO1 (Figure 3.6a). In contrast, expression of *glcB* in FRD1 *rpoN* mutant is comparable to FRD1 wild-type levels. The same conclusion was drawn from measurement of MS activity from the RpoN mutants (Figure 3.6b). These data support the deregulation of *glcB* expression and MS activity in FRD1, presumably as an adaptive mechanism for surviving and persisting in the CF lung.

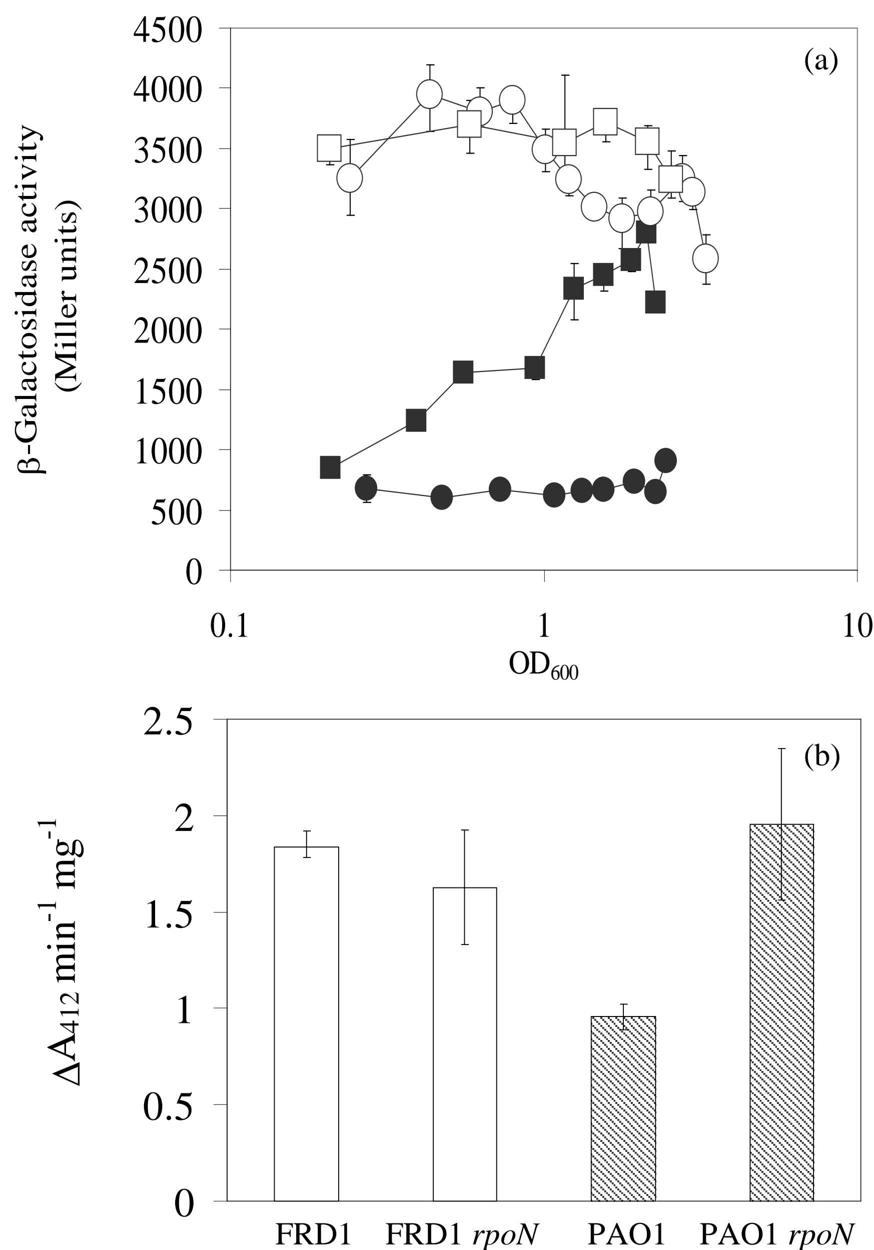

Figure 3.6 RpoN **regulates MS activity at the transcriptional level in PAO1. (a)** β-Galactosidase assays were done from cultures of *P. aeruginosa* grown in L-broth containing glutamine and collected over a growth cycle. Activity is presented in Miller units. Values represent the average of 3 experiments done in duplicate. ○FRD1; □ FRD1*rpoN*-; ●PAO1; ■ PAO1*rpoN*-. **(b)** MS activity is presented as $\Delta A_{412}min^{-1}mg^{-1}$. *P. aeruginosa* cultures were grown overnight in L-broth supplemented with glutamine. Values presented are the average of two experiments. FRD1 and derivatives □; PAO1 and derivatives ▨.

Discussion

Pseudomonas aeruginosa requires the glyoxylate pathway to cause optimal infection in the alfalfa model of infection and isocitrate lyase is required for optimal infection in the rat lung model of infection by PAO1 (Lindsey *et al*., 2008). The requirement of this pathway for infection is not unique to *P. aeruginosa.* Many other human pathogens also require the glyoxylate pathway for causing persistent infections, including *M. tuberculosis, S. typhimurium,* and *C. albicans* (McKinney *et al.,* 2000, Lorenz & Fink, 2001, Fang *et al*., 2005). Our previous studies demonstrate that the first key enzyme of the glyoxylate pathway, isocitrate lyase, is deregulated in the CF isolate, FRD1, compared to PAO1. Furthermore, this phenotype is common to other CF isolates of *P. aeruginosa.* Recent studies indicate that both amino acids and fatty acids are present in the CF lung and are likely important carbon sources for *P. aeruginosa* in that environment (Barth & Pitt, 1996, Palmer *et al*., 2005, Son *et al*., 2007). This study was pursued in order to identify other functions that are involved in C2 and fatty acid metabolism in *P. aeruginosa* CF isolate FRD1. Although I identified a total of six genes that may be involved in the catabolism of acetate, I focused on characterizing *glcB* and its gene product, malate synthase, which is the second enzyme of the glyoxylate pathway. As illustrated in Tables 3.2 and 3.3, *glcB* function is required for *P. aeruginosa* infection of alfalfa seedlings. Along with my previous study on *aceA*, these data clearly demonstrate importance of the glyoxylate pathway in virulence and pathogenesis of the CF isolate, FRD1. This is the first time that *glcB* and its product, malate synthase, has been characterized in CF isolate of *P. aeruginosa*.

To better understand the regulation of the *glcB* in FRD1 and PAO1 in response to carbon sources, a *glcB*::*lacZ* transcriptional fusion was generated. I determined that *glcB* expression and MS activity correlate well with each other and also with *aceA* expression and ICL activity. In PAO1 *glcB* expression behaves similarly to *aceA* in *E. coli*, including its induction by acetate. However, in FRD1, *glcB* expression and MS activity are permanently upregulated during growth in most of the tested carbon sources. The highest induction of *glcB* in both FRD1 and PAO1 was observed with cells grown in palmitic and heptanoic acid. High induction of *aceA* expression in both isolates was also observed for heptanoic acid grown cells but not for palmitic acid. Taken together, the data suggest that *aceA* and *glcB* share some regulatory controls while others are unique to each gene. Furthermore, RpoN negatively regulates expression of *glcB* in PAO1 providing another common regulatory mechanisms for both glyoxylate pathway enzymes. The role of RpoN in *P. aeruginosa* physiology is well documented. RpoN negatively regulates elastase, rhamnolipid and hydrogen cyanide synthesis (Heurlier *et al*., 2003), and *P. aeruginosa rpoN* mutants have a higher survival rate against some antimicrobial compounds (Viducic *et al.,* 2007). Others have suggested that for *P. aeruginosa* to persist, as in chronic infections, there may be selective pressure to lose expression of RpoN-dependent surface ligands (like flagella and pili). Consistent with this idea, *P. aeruginosa* with mutations in *rpoN* have been isolated from the CF lung (Mahenthiralingam & Speert, 1995, Smith *et al*., 2006). Conversely in PA14, another acute isolate, *rpoN* is necessary for the production of certain virulence determinants (Hendrickson *et al*., 2001) and is required for optimal infection of PA14 in the xenograft model of infection, mouse thermal injury model, and infection of *C. elegans* (Cohn *et al*.,

2001, Hendrickson *et al.,* 2001). Therefore there are conflicting data for the function of RpoN in *P. aeruginosa* infections. RpoN is required by some species of Pseudomonads to utilize several amino acids and C4-dicarboxylates as carbon sources (Hendrickson *et al*., 2000) indicating a role in metabolism. The results from my study also implicate RpoN in metabolism, a regulatory role that will likely affect chronic infection during which the pathogen must find a stable source of nutrients.

This study reveals that like *aceA*, *glcB* expression in *P. aeruginosa* is deregulated in the CF isolate FRD1, and that FRD1 and PAO1 utilize different mechanisms to regulate expression of *glcB*. The high expression of both *aceA* and *glcB*, indicate that the glyoxylate pathway is important in FRD1 and likely results from adaptation of FRD1 to the CF lung. Thus therapeutic approaches that target either malate synthase or isocitrate lyase to regulate or control chronic *P. aeruginosa* infections appear attractive. However, a better understanding of the regulation of this pathway in CF isolates is necessary to fully elucidate the molecular mechanisms of bacterial adaptation.

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Chapter 4

Conclusion and Future Directions

Conclusion

In order to develop effective strategies to treat chronic infections, it is essential to gain a better understanding of how the chronic infecting pathogens cause and maintain infection. *Pseudomonas aeruginosa* undergoes adaptation within the cystic fibrosis (CF) lung during chronic infection (Woods *et al*., 1986, Smith *et al*., 2006) and typically loses the ability to synthesize standard virulence determinants that are necessary for initiating acute infections (Hancock, *et al*., 1983, Dacheux, *et al.,* 2001, Luzar *et al*., 1985a, Luzar *et al*., 1985b, Ramsey & Wozniak, 2005, Woods *et al*., 1986). Consequently, chronic infection isolates of *P. aeruginosa* also lose the ability to cause infections in animal models that are typically used to study acute virulence determinants (Silo-Suh *et al*., 2002). This situation prompted development of a highly sensitive alternative model system of infection based on alfalfa seedlings to identify chronic virulence determinants from *P. aeruginosa* isolates adapted to the CF lung. Using the alfalfa seedling model of infection, *aceA* was identified as a virulence determinant for both FRD1, a CF isolate, and for PAO1, an acute isolate. The goal of my project was to initiate characterization of *aceA* in order to better understand its role in *P. aeruginosa* physiology and virulence during chronic infections. Prior to my analysis *aceA* had not been characterized in *P.*

aeruginosa. The first step of this process was to determine whether *aceA* did in fact encode for isocitrate lyase activity as suggested by annotation analysis. Secondarily, I characterized expression of *aceA* in response to carbon source utilization. During this analysis, I determined that regulation of *aceA* is altered in FRD1 most likely as a consequence of adaptation of *P. aeruginosa* to the CF lung. The third goal of my project was to initiate a characterization of *glcB* which was postulated to encode for the second key enzyme of the glyoxylate pathway, malate synthase. I also determined that malate synthase is required for *P. aeruginosa* infection in the alfalfa seedling model. This gene had not been characterized prior to my study. From this study, I determined that expression of *aceA* and *glcB* in PAO1 responds to carbon sources similarly to what has been observed in other bacteria. Likewise, ICL and MS activity correlates strongly with gene expression as seen in other bacteria. However, both *aceA* and *glcB* expressions are permanently upregulated in the CF isolate FRD1 and in other CF isolates. These data suggest that this alteration must benefit *P. aeruginosa* within the CF lung. Currently, we recognize three possible benefits of permanent upregulation of the glyoxylate pathway in *P. aeruginosa* growing in the CF lung, including catabolism of carbon sources present in this environment, optimal production of alginate for protection, and increased production of hydrogen cyanide to out compete other organisms.

A variety of studies indicate that amino acids and fatty acids are likely the growth substrates for *P. aeruginosa* in the CF lung (Meyer *et al*., 2000, Barth & Pitt 1996, Palmer *et al*., 2005, Son *et a.*, 2007). While fatty acids would induce the glyoxylate pathway, we expect the presence of certain amino acids to catabolite repress expression

of both *aceA* and *glcB* even in the presence of fatty acids. This is supported by the lack of *aceA* and *glcB* induction observed by Palmer *et al.* (2005) when PAO1 was added to CF sputum. In contrast, Son *et al*. (2007) observed that CF *P. aeruginosa* induced expression of *aceA* and *glcB* under similar conditions, suggesting some isolates adapted to the CF lung can override catabolite repression of the glyoxylate pathway. Other pathogens also upregulate ICL and MS activity during infection and appear to require this pathway to establish a persistent infection in animals (Fang *et al*., 2005, Munoz-Elias & McKinney, 2005, Lattif *et al*., 2006, Hancock *et al*., 1983). However, utilization of these pathways has not been linked to virulence factor production in other bacterial pathogens as we have shown for *P. aeruginosa*.

High ICL activity likely contributes to high levels of two virulence factors produced by *P. aeruginosa* isolates in the CF lung, alginate and hydrogen cyanide. The FRD1*aceA* mutant produces less alginate than the parental strain (Lindsey *et al.*, 2008), indicating that the glyoxylate pathway supplies downstream intermediates for biosynthesis of alginate. The glyoxylate produced by isocitrate lyase can also be converted to glycine via a process that involves *dada.* The glycine from this reaction is then converted to hydrogen cyanide by hydrogen cyanide synthase (Hagins *et al*., 2009). Central metabolic metabolism pathways are known to impact virulence of other pathogens. However, this is the first indication that these pathways can be altered during chronic infection in order to increase production of certain virulence determinants.

Finally, in an attempt to determine the mechanism for deregulation of *aceA* expression in the CF isolate, FRD1, I identified RpoN as a negative regulator of *aceA* and *glcB* in PAO1. However, RpoN does not appear to play a direct role in deregulation of *aceA* and *glcB* in FRD1. This suggests that the actual regulator of *aceA* and *glcB* may be inactive in FRD1 to result in deregulation of these genes. Alternatively, RpoN regulates *aceA* and *glcB* via different mechanisms in CF and non-CF isolates. Futher study is necessary to determine the mechanisms by which RpoN regulates *aceA* and *glcB*. My data suggest that RpoN may play different roles in acute infections compared to chronic infections.

Future Direction

The goal of my study was to initiate the characterization of they glyoxylate pathway in *P. aeruginosa* virulence and physiology, which had not been previously reported. Much still remains to be elucidated about the regulation of the pathway including transcriptional and post-transcriptional regulation. Specifically, the intermediate between RpoN and *aceA* and *glcB* repressions should be identified and the mechanisms of deregulation of *aceA* in FRD1 and other CF isolates should be determined.

Identification of regulators of *aceA* gene expression in *P. aeruginosa*

Additional studies to identify other regulators of *aceA* in *P. aeruginosa* are needed to understand the mechanism(s) behind the altered regulation of this gene in the CF background. One possible method is to mutagenize PAO1 carrying the *aceA::lacZ* fusion to identify putative mutants with altered β-galactosidase activity. This approach has the

potential to identify both the intermediate between RpoN and *aceA* and the mechanisms of deregulation of *aceA* in FRD1.

Alternatively, FRD1 may have acquired additional DNA in the form of a plasmid or pathogenicity island while in the CF lung that encodes for a transcriptional activator of *aceA* expression. Three *P. aeruginosa* pathogenicity islands have been identified to date (Liang *et al*., 2001, Finnan, *et al*., 2004). PAGI-1 (*P. aeruginosa* genomic island 1) was found in 85% of the clinical isolates tested (Liang *et al*., 2001) and it is possible that additional genes could be present in FRD1 that may account for the phenotype described in this study. The annotation of PAGI-1 identified the presence of two putative transcriptional regulators (Liang *et al*., 2001), one of which shows significant similarity to the RpoN-dependent subfamily of regulatory elements. This particular regulator is closely related to the PrpR of *Salmonella* which is required for growth on propionate by activating the *prpBCDE* operon (Palacios & Escalante-Semerena, 2000). The other putative PAGI-1 regulator shows similarity to regulatory proteins in various *Mycobacterium* and *Streptomyces* species (Liang *et al*., 2001). Further examination will determine whether acquired DNA sequences play a role in regulation of *aceA* and *glcB* in the CF isolates of *P. aeruginosa*. One method is to mutagenize FRD1 carrying the *aceA::lacZ* fusion with a transposon and then screen the putative mutants for alterations in β-galactosidase activity. This method may allow for the identification of a positive regulator or activator present in the additional DNA acquired by FRD1 during infection that is not present in PAO1.

Expression of *aceA* in PAO1 is likely regulated by mechanisms utilized by other bacteria. However, a predictive approach to identify such regulators has thus far been unsuccessful. The PAO1 genome contains several uncharacterized ORFs that have homology to IclR, a known regulator of *aceA* in other bacteria. The uncharacterized ORF with the most homology to IclR from *E. coli* include: PA3508, PA1630, and PA4341. PA4341 is approximately 45% similar to the transcriptional regulator of the *mhp* operon (responsible for the degradation of 3-(3-hydroxyphenyl)-propionic acid) in *E. coli* (Torres *et al*., 2003), but has not been characterized in *P. aeruginosa*. In *Yersinia pestis*, isocitrate lyase is constitutively expressed and this phenotype was found to be a result of a mutation in *iclR* (Sebbane *et al.*, 2004). Therefore I feel that a more extensive search for a *iclR* homologue may be beneficial in understanding the regulation of *aceA*.

A mutation in PA4769, encoding for a probable *fadR*, also had no effect on *aceA* expression in *P. aeruginosa*. However, there are two other genes (PA1627 and PA5356) in *P. aeruginosa* that encode for proteins with homology to the FadR protein sequence of *E. coli*. Although PA5356 has been annotated as a *glcC*, a regulator of malate synthase, it has not been fully characterized in *P. aeruginosa*. It may be beneficial to examine the role of these genes in *aceA* expression.

Alternatively, ICL activity may be regulated by other mechanisms in addition to transcription of *aceA*. For example, the isocitrate dehydrogenase activity is controlled by phosphorylation/dephosphorylation. Thus, ICL could also be regulated via post-translational modification. In addition, ICL may be regulated by the presence or absence

of isocitrate dehydrogenase. The PAO1 genome contains two genes that likely encode for isocitrate dehydrogenase, which are designated as *icd* and *idh*. These genes are located next to each other in the PAO1 genome, but are divergently transcribed. They have not been characterized to date and there is no information on whether both are functional. Low levels of isocitrate dehydrogenase activity in FRD1 would likely funnel isocitrate to the glyoxylate pathway. When this enzyme is phosphorylated by isocitrate dehydrogenase kinase it becomes inactive and can not bind to isocitrate and thereby increases the activity of the glyoxylate pathway (Nimmo & Nimmo, 1984). Sequencing both *icd* and *idh* in FRD1 would confirm the possibility of isocitrate dehydrogenase being mutated or absent in FRD1. Examining isocitrate dehydrogenase activity in FRD1 compared to PAO1 may provide insight as to the mechanism behind high ICL activity in FRD1. The predicted ORF of *aceK*, encoding isocitrate dehydrogenase kinase/phosphatase (*aceK*) is PA1376, and this has also not been characterized in *P. aeruginosa*.

Further characterization of malate synthase

The PAO1*glcB* mutant should be tested in an animal model system of infection and the FRD1*glcB* mutant should be tested for alginate and cyanide productions. These studies would lend support for the role of the glyoxylate pathway in *P. aeruginosa* virulence. One major question left to be answered is whether the complete pathway is required for catabolism of carbon sources, or whether the isocitrate lyase the important part of the pathway because of its role in hydrogen cyanide production. Finally, once the key regulators of *aceA* are identified, they should be tested for regulation of *glcB*. These

genes are not located within the same operon; however, they appear to share some regulatory controls.

Potential Drug Targets

One major goal of this study is to facilitate development of new approaches to control chronic *P. aeruginosa* infections in the CF lung with drugs that target the glyoxylate pathway. Because the glyoxylate pathway is absent in mammals, such drugs have the potential to be highly specific and effective for the bacterial pathogen.

In conclusion this study demonstrates that the glyoxylate pathway is important in nutrient catabolism and virulence determinant production for *P. aeruginosa* that are adapting to the unique environment of the CF lung. Continued study of the metabolic pathways that are active in *P. aeruginosa* during infection will enhance our understanding of the roles these pathways play in virulence and perhaps identify new targets for drug intervention.

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