Characterization of the Role of *dadA* in *Pseudomonas aeruginosa* Virulence Factor Production

by

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Abstract

Chronic *Pseudomonas aeruginosa* infections remain the leading cause of lung dysfunction and mortality in Cystic Fibrosis (CF) patients. Many other bacteria reside within the CF lung, but *P. aeruginosa* utilizes novel strategies that allow it to colonize the CF lung as the predominant bacterial pathogen. We determined previously that FRD1, a CF *P. aeruginosa* isolate, requires *dadA* for optimal hydrogen cyanide (HCN) production, while PAO1, an acute isolate of *P. aeruginosa*, does not. In order to better understand the increased significance of *dadA* in FRD1 physiology, we characterized the contribution of the *dad* operon to virulence factor production by FRD1 and PAO1. The *dad* operon contains two genes, *dadA* and *dadX*, which encode for enzymes required for the catabolism of alanine. *dadA* encodes for a putative D-amino acid dehydrogenase, while *dadX* encodes for an alanine racemase. In this study, we determined that *dadA* is required for optimal production of alginate, pyocyanin, pyoverdine, rhamnolipid, and biofilm formation by FRD1, as well as optimal virulence of FRD1 in an alfalfa seedling model of infection, while *dadX* is not. In contrast, *dadA* is required only for optimal rhamnolipid production by PAO1. In an attempt to explain the *dadA* phenotype, L- and D-alanine levels were quantitated, but the results were inconclusive. Taken together, the results indicate that *dadA* plays a pleiotropic role in the production of important virulence factors by CF isolates of *P. aeruginosa*.

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Introduction

Chronic *Pseudomonas aeruginosa* infections remain the leading cause of lung dysfunction and mortality in Cystic Fibrosis (CF) patients. Many other bacteria reside within the CF lung, but *P. aeruginosa* utilizes novel strategies that allow it to colonize the lung as the predominant bacterial pathogen. An important aspect of chronic colonization by *P. aeruginosa* is the acquisition of new phenotypes, which are the result of genetic changes promoted by the oxidative stress encountered within the hostile environment of the CF lung. Some of these phenotypes likely facilitate persistence of *P. aeruginosa* within the CF lung. Upregulation of the *dad* operon in some CF-adapted isolates of *P. aeruginosa* during chronic infection suggests this operon plays an important role in persistence of *P. aeruginosa* in the CF lung [Son *et. al.*, 2007].

Microbial pathogens utilize different strategies to maintain chronic infections within hosts, compared to those required to establish acute infections [Costerton *et. al*., 2003; Hong, *et. al*., 2000; Young *et. al*., 2002]. Consequently, treatments that are successful against acute infections are often ineffective at treating chronic infections. Therefore, a better understanding of the strategies utilized by persistent pathogens, such as *P. aeruginosa*, is critical for the development of improved therapies for treating these

infections. We will investigate the role of the *dad* operon in *P. aeruginosa* to gain insight into its role during chronic infection.

Pseudomonas aeruginosa

P. aeruginosa is a member of the class ɣ-Proteobacteria, within the family Pseudomonadaceae, and it is a ubiquitous, motile, gram-negative, rod-shaped bacterium [Nester *et. al.*, 2007]. It is a facultative anaerobe and opportunistic pathogen, whose lifestyle is afforded by the ability to metabolize a wide array of carbon and nitrogen sources [Nester *et. al.*, 2007]. *P. aeruginosa* is ubiquitously distributed across many different environmental niches. It can be found in soil, plants, skin, soft tissue, and even growing on plastic. *P. aeruginosa* is found in many different environments, because it can catabolize many different carbon sources for survival in nutrient-poor conditions. Such carbon sources include sugars, amino acids, fatty acids, and compounds containing aromatic rings [Nester *et. al.*, 2007].

P. aeruginosa's ability to metabolize various substrates for energy likely plays a role in its success as the most common gram-negative causative agent of nosocomial infections. Many hospital patients become infected by this pathogen in a variety of ways. Patients with open wounds and burns can easily acquire cutaneous infections by *P. aeruginosa*. Also within hospitals, most urinary tract infections are caused by *P. aeruginosa,* which can survive on the plastic tips of catheters. *P. aeruginosa* can even cause severe corneal infections, which are sometimes severe enough to cause permanent blindness. These and other infections caused by *P. aeruginosa* are particularly problematic, not only because of

the pathogen's metabolic versatility and ubiquitous nature, but also because this opportunistic pathogen is inherently resistant to most antibiotics available today [Salyers *et. al.*, 2002]. *P. aeruginosa* easily develops a high level of multidrug resistance as well, due to the presence of multiple and highly effective multidrug efflux pumps [Poole *et. al.*, 2001].

Upon initial invasion of a CF patient, *P. aeruginosa* causes an acute infection of the respiratory tract, which transitions into a chronic infection. The bacterium undergoes several morphological changes during the infection process, which include, but are not limited to, conversion to the mucoid phenotype, loss of motility, increased biofilm capabilities, and decreased production of various virulence factors [Driscoll *et. al.*, 2007]. *P. aeruginosa*'s diverse metabolic capabilities facilitate persistence of the bacterium within very unfavorable environmental niches, including the CF lung. In this environment, *P. aeruginosa* utilizes the glyoxylate shunt to generate energy for cellular needs, such as rotation of the flagellum and synthesis of macromolecules. The glyoxylate shunt is utilized in catabolism of C2 carbon sources to avoid losing carbon in the form of CO_2 during the conversions of isocitrate to α-ketoglutarate and α-ketoglutarate to succinyl-CoA (Figure 1.1). More importantly, the glyoxylate shunt is upregulated in *P. aeruginosa* growing in the CF lung, and in some CF isolates, this upregulation becomes permanent via unknown mutations [Son *et. al.*, 2007; Lindsey *et. al.*, 2008; Hagins *et. al.*, 2011]. The combined upregulation of the glyoxylate pathway, in addition to the newly-characterized *dad* operon, leads to increased production of hydrogen cyanide (HCN) in many CF isolates of *P. aeruginosa* [Hagins *et. al.*, 2009; Carterson *et. al.*, 2004]. This is

consistent with the observation that high HCN levels are present in the CF lung and correlate with increased pulmonary damage and dysfunction [Ryall *et. al.*, 2008; Sanderson *et. al.*, 2008].

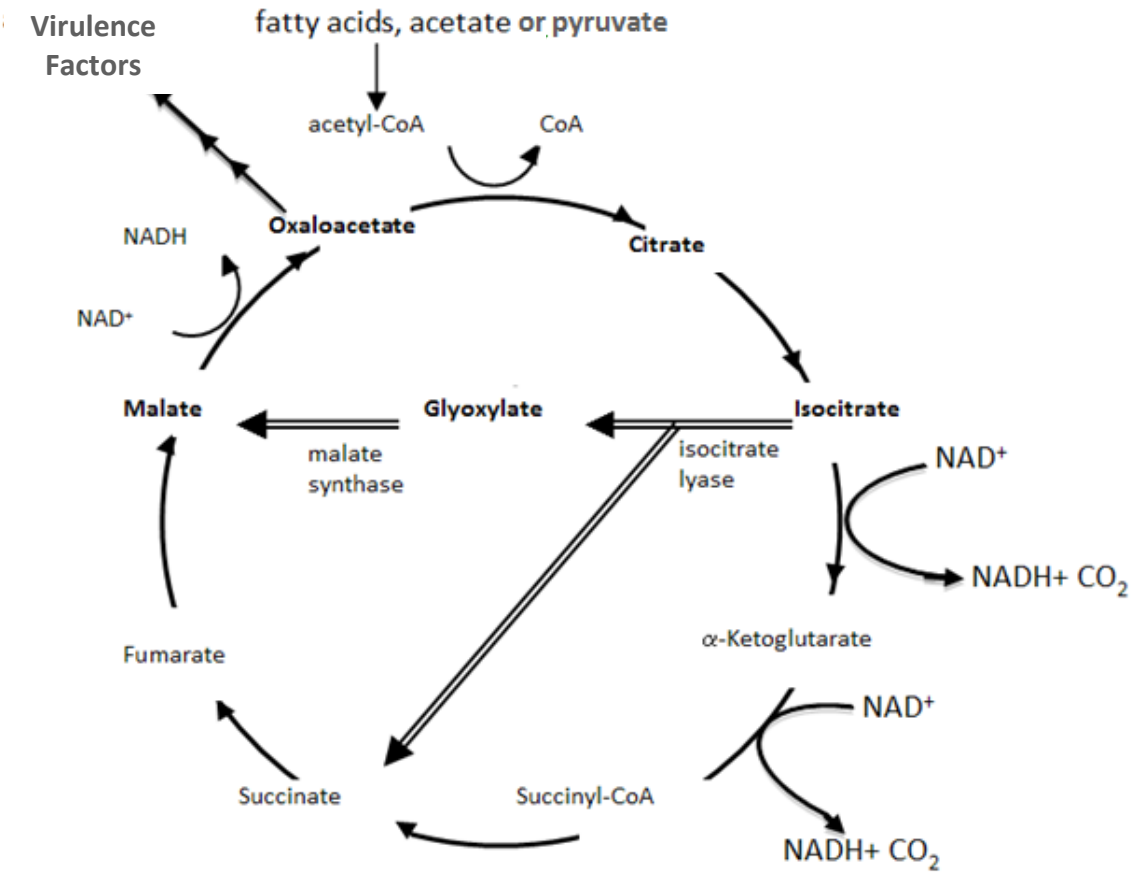

Figure 1.1. The TCA cycle and glyoxylate shunt. This figure is courtesy of Dr. Laura Silo-Suh.

Cystic Fibrosis

CF is an autosomal recessive genetic disorder that affects millions of people worldwide [Boat *et. al.*, 1989]. According to a survey completed in 2010 by the U.S. Department of Energy, Biological, and Environmental Research, in the U.S. alone, one in 2,500 children is born with CF every year, and one in 20 people are asymptomatic carriers. CF is caused

by alterations in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), encoded by a gene located on chromosome seven, which functions in the transport of small molecules [Riordan *et. al.*, 1989]. CFTR normally regulates the function of other ion channels, such as those that transport sodium ions across cell membranes in the lungs and pancreas [Cohn, 2005]. The mutation in CFTR affects many secretory glands throughout the body, including but not limited to, secretory glands located in the lungs, pancreas, liver, gastrointestinal tract, and various reproductive organs, causing a wide variety of symptoms in patients [Riordan *et. al.*, 1989].

Under normal conditions, CFTR also functions as a channel transporting chloride ions in and out of secretory cells. The transport of these ions helps regulate the movement of water molecules in tissues, which is necessary for the cilia lining those tissues to remain hydrated [Cohn, 2005]. When CFTR is defective, those cilia become dehydrated, and therefore, cannot properly function in the removal of mucus out of the respiratory and digestive systems. CF is characterized by an overproduction of sticky, dehydrated mucus that cannot be cleared from the respiratory tract, due to the dysfunctional CFTR protein. This altered mucus allows opportunistic pathogens to invade, colonize, and proliferate within host respiratory systems [Riordan *et. al.*, 1989]. *P. aeruginosa* infections of the CF lung ultimately lead to the early demise of CF patients, following a period of chronic infection. Although *P. aeruginosa* is capable of direct damage of host tissues via various secreted virulence determinates, the majority of lung damage in CF patients is caused by the host's immune system in a desperate attempt to eradicate *P. aeruginosa* [Wieland *et. al.*, 2002].

The CF Lung Environment

The CF lung is dramatically altered in terms of the secretions, fatty acids, and amino acids present. The lower CF respiratory tract contains copious amounts of activated neutrophils, which may significantly alter the phospholipid and protein components of the airway surfactant [Meyer *et. al.*, 2000]. In addition, palmitic and oleic acid are elevated in the bronchoalveolar lavage fluid from the lower respiratory tract of CF patients as compared to non-CF patients [Meyer *et. al.*, 2000]. Amino acids such as valine, tyrosine, serine, methionine, leucine, phenylalanine, and alanine are also elevated in CF sputum compared to non-CF sputum [Barth *et. al.*, 1996]. These alterations in fatty acid and amino acid content are due to the complexity of the CF disorder, as well as the presence of other microbial organisms. CF sputum has been found to harbor *Staphylococcus aureus*, *Burkholderia cepacia*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Lautropia mirabilis*, *Bacteriodes fragilis*, and various other bacteria, fungi, and phage [Rogers *et. al.*, 2003; Armougom *et. al.*, 2009]. *P. aeruginosa* is the most prevalent pathogen, and due to the changes the bacterium undergoes throughout the course of infection, chronic persistence by *P. aeruginosa* ensues throughout the lifespan of the CF patient.

Adaptations of *Pseudomonas aeruginosa* to the CF Lung

P. aeruginosa adopts many different metabolic strategies for persisting in the CF lung environment. The bacterium reduces its metabolism within a biofilm, which reduces the likelihood that it will be attacked by the host's immune system. *P. aeruginosa* also

adopts bacterial plurality, meaning that within a single lung environment, many different genotypes and phenotypes of the bacterium will be present. This strategy ensures the continuation of the bacterial infection, because genetic variability gives the bacterial community additional antimicrobial and/or anti-phagocytic resistance. In addition, *P. aeruginosa*'s nutrient acquisition is extremely diverse. It can catabolize various carbon and nitrogen sources present in the CF lung, in order to multiply and produce virulence factors. Such virulence factors include exotoxin A, hemolysin, phospholipase C, pigments, proteases, exoenzyme S, and leukocidin [Nester *et. al.*, 2007; Lindsey *et. al.*, 2008; Silo-Suh *et. al.*, 2005]. *P. aeruginosa* also adapts to the hypoxic environment of the CF lung during chronic infection, and it multiplies at a much slower rate [Boucher, 2004].

Hypermutability is another strategy utilized by *P. aeruginosa* to persist within the CF lung [Oliver *et. al.*, 2000]. This strategy assures bacterial plurality and increases the opportunity for various derivatives to adapt and survive in the hostile CF lung environment. In addition, gene expression is altered in chronic isolates compared to acute isolates, which intuitively indicates that the pathogen utilizes alternative mechanisms for chronic persistence compared to acute infection [Silo-Suh *et. al.*, 2005; Son *et. al.*, 2007]. For example, *aceA*, which encodes for the enzyme isocitrate lyase, is permanently upregulated in the chronic CF isolate FRD1, which contributes to high levels of HCN production [Hagins *et. al.*, 2009].

The most notable virulence factor overproduced by chronic isolates of *P. aeruginosa* is alginate, which is believed to facilitate biofilm formation and persistence of the pathogen in the CF lung [Govan *et. al.,* 1996]. Evidence indicates that both alginate and the formation of biofilms confer protection on *P. aeruginosa* against antibiotics and neutrophils [Govan *et. al.,* 1996]. Another example of altered gene expression in chronic isolates of *P. aeruginosa* occurs with *zwf,* which encodes the enzyme glucose-6-phophate dehydrogenase (G6PDH). G6PDH is more active in FRD1 than PAO1, and a mutation in *zwf* leads to a dramatic decrease in alginate production in FRD1 [Silo-Suh *et. al.,* 2005]. Disruption of G6PDH may lead to decreased amounts of fructose-6-phosphate (F6P), which is the primary precursor for alginate biosynthesis [Silo-Suh *et. al.,* 2005]. The published study proposed that *P. aeruginosa* undergoes an adaptive change in the CF lung, which de-regulates *zwf* expression [Silo-Suh *et. al.,* 2005]. Relaxed control of *zwf* can be advantageous to *P. aeruginosa* survival in the CF lung for several reasons. Highly unregulated *zwf* leads to unregulated activity of G6PDH, which could provide the bacterial community with a metabolic advantage for survival in the CF lung [Silo-Suh *et. al.,* 2005]. Also, *zwf* seems to be required for resistance of *P. aeruginosa* to CF sputum [Silo-Suh *et. al.,* 2005]. Intuitively, evidence from this study and many others indicates that certain genes may be preferentially required for acute infection, while other genes are preferentially required for chronic persistence.

Chronic Virulence Factors Produced by FRD1

During the chronic persistence of *P. aeruginosa* in the CF lung, many virulence factors produced during acute infection are repressed, which suggests that these products are

dispensable during long-term infection by *P. aeruginosa* [Lindsey *et. al.*, 2008]. Most, but not all, virulence factors are classified as secondary metabolites, because they are not involved in the bacterium's energy metabolism nor the essential biosynthetic reactions required for survival [Salyers *et. al.*, 2002]. They are produced during the stationary phase of growth, and they are very costly in terms of energy requirements. During *P. aeruginosa*'s stage of acute infection in the CF lung, certain virulence factors are amply produced, such as pyoverdine. HCN is another key virulence factor secreted by *P. aeruginosa* that has been detected in CF sputum, and its presence has been associated with decreased pulmonary function [Ryall *et. al.*, 2008; Sanderson *et. al.*, 2008].

There are several other virulence factors upregulated during chronic persistence, such as pyocyanin and rhamnolipid. Pyocyanin is a blue-green pigmented phenazine antibiotic that generates oxygen radicals and damages the surrounding cells, proteins, and various other molecules [Cox, 1986]. Rhamnolipids are surface-active amphipathic molecules, and they comprise the main constituents of *P. aeruginosa* biosurfactant [Davey *et. al.*, 2003]. Rhamnolipids are believed to be responsible for maintaining biofilm channel structure and preventing biomass accumulation in the channels [Davey *et. al.*, 2003]. In contrast, other virulence factors are deregulated during chronic persistence, such as pili and flagella. These two virulence factors aid in initiating the initial acute infection by allowing the pathogen to bind to the respiratory epithelia, but they also aid in mediating chemotaxis and motility during the course of the infection [Saiman *et. al.*, 1990].

Another virulence factor that is highly upregulated during chronic persistence is the exopolysaccharide alginate. Once the bacterium is acclimated to the CF lung, *P. aeruginosa* acquires what is referred to as the mucoid phenotype. This phenotype is caused by a variety of mutations in the *P. aeruginosa* genome, most often by a defect in *mucA*, which encodes for an anti-sigma factor that is involved in regulation of AlgT, also known as AlgU [Malhotra *et. al.,* 2000]. AlgT is required for the activation of genes involved in alginate biosynthesis. The disruption in *mucA* causes the genes involved in alginate biosynthesis to be upregulated; therefore, copious amounts of alginate are produced [Malhotra *et. al.,* 2000]. Alginate aggregates around the bacteria and is a component of *P. aeruginosa* biofilms that pervades the CF lung, preventing antibiotics, antibodies, oxygen radicals, and macrophages from accessing the bacteria. As a result of becoming mucoid, *P. aeruginosa* also loses the genes responsible for flagellin synthesis, and it becomes non-motile [Tart *et. al.*, 2006]. Overall, the bacteria located within the biofilm are less metabolically active, which assists in the infection strategy of *P. aeruginosa* to avoid attack by the host immune system.

algT is emerging as a putative "switch" in *P. aeruginosa* physiology, by turning off genes involved in acute infection and turning on genes involved in chronic infection [Hagins *et. al.*, 2009]. Both alginate and HCN production are upregulated in chronic isolates of *P. aeruginosa* via AlgT [Wozniak *et. al.*, 1994; Sanderson *et. al.*, 2008]. AlgT induces AlgR expression, which in turn, induces the *hcnABC* gene cluster, effectively increasing production of HCN [Carterson *et. al.*, 2004]. AlgT also positively upregulates *dadA,* a gene involved in alanine catabolism [Wood *et. al.*, 2009]. However, the inactivation of

dadA leads to decreased levels of HCN, but no one has definitively shown the necessity of HCN for virulence in CF isolates of *P. aeruginosa in vivo* [Ryall *et. al.,* 2008]. Proposed functions for HCN in *P. aeruginosa* pathogenesis include nutrient liberation from host cells, microbial population control within the CF lung, and possibly the generation of energy [Williams *et. al.*, 2007]. Clearly, the roles of HCN and the *dad* operon in *P. aeruginosa* pathogenesis must be more definitively characterized.

Alanine Catabolism in *Pseudomonas aeruginosa* and Other Organisms

Alanine is a preferred substrate for *P. aeruginosa* within the CF lung, and high concentrations have been noted in this environment [Boulette *et. al.*, 2009]. Alanine is catabolized by the *dad* operon, which is comprised of three genes identified as *dadA*, PA5303, and *dadX* [Boulette *et. al.*, 2009]. PA5303 encodes a putative endoribonuclease-translation inhibitor, but the significance of this gene has not been determined, while *dadX* encodes an alanine racemase that converts L-alanine into D-alanine, but also functions in the racemization of D-alanine into L-alanine [Boulette *et. al.*, 2009]. *dadX* is not believed to be involved in D-alanine production for cell wall biosynthesis, because *P. aeruginosa* contains another alanine racemase encoded by *alr*. Evidence indicates that *alr* is not involved in alanine catabolism, but it is involved in cell wall biosynthesis [Wild *et. al.*, 1985].

dadA encodes a D-amino acid dehydrogenase and oxidatively deaminates D-alanine into pyruvate and ammonia [Boulette *et. al.*, 2009]. D-amino acid dehydrogenase is a well-studied enzyme from a variety of organisms. It contains flavin adenine dinucleotide

(FAD) and a non-heme iron in its active center [Lobocka *et. al.*, 1994]. It is also membrane-bound and directly linked to the respiratory chain. DadA has a highly hydrophobic FAD-binding domain at its N-terminal end, which is consistent with the fact that D-amino acid dehydrogenase is bound by strongly hydrophobic interactions with the inner cell membrane [Lobocka *et. al.*, 1994]. Previous studies identified *dadA* to be upregulated in chronic isolates of *P. aeruginosa* [Boulette *et. al.*, 2009; Son *et. al.*, 2007]. Experimental evidence shows that *dadA* is required for the overall optimal competitive fitness of acute isolates of *P. aeruginosa* in a rat lung model of infection, suggesting alanine catabolism is critical for *P. aeruginosa* during infection [Boulette *et. al.*, 2009].

dadA is required for optimal HCN production by CF isolates of *P. aeruginosa*, which may impact the bacterium's ability to persist during infection (Figure 1.2) [Hagins *et. al.*, 2009]. FRD1*dadA* mutants are reduced for HCN production, which can be restored by the addition of glyoxylate to the growth medium. However, the addition of glycine back to the growth medium does not restore HCN production to FRD1*dadA* [Hagins *et. al.*, 2009]. These results suggests that *dadA* somehow plays a role in the conversion of glyoxylate to glycine, and it is consistent with the elevated levels of glycine produced by FRD1, as compared to PAO1, under the specific growth conditions. Glycine is the preferred substrate for the production of HCN by *P. aeruginosa* (Figure 1.2). D-amino acid dehydrogenase reduces ubiquinone analogs in the presence of D-alanine, so oxidative deamination driven by this enzyme supplies the energy needed for active transport [Lobocka *et. al.*, 1994].

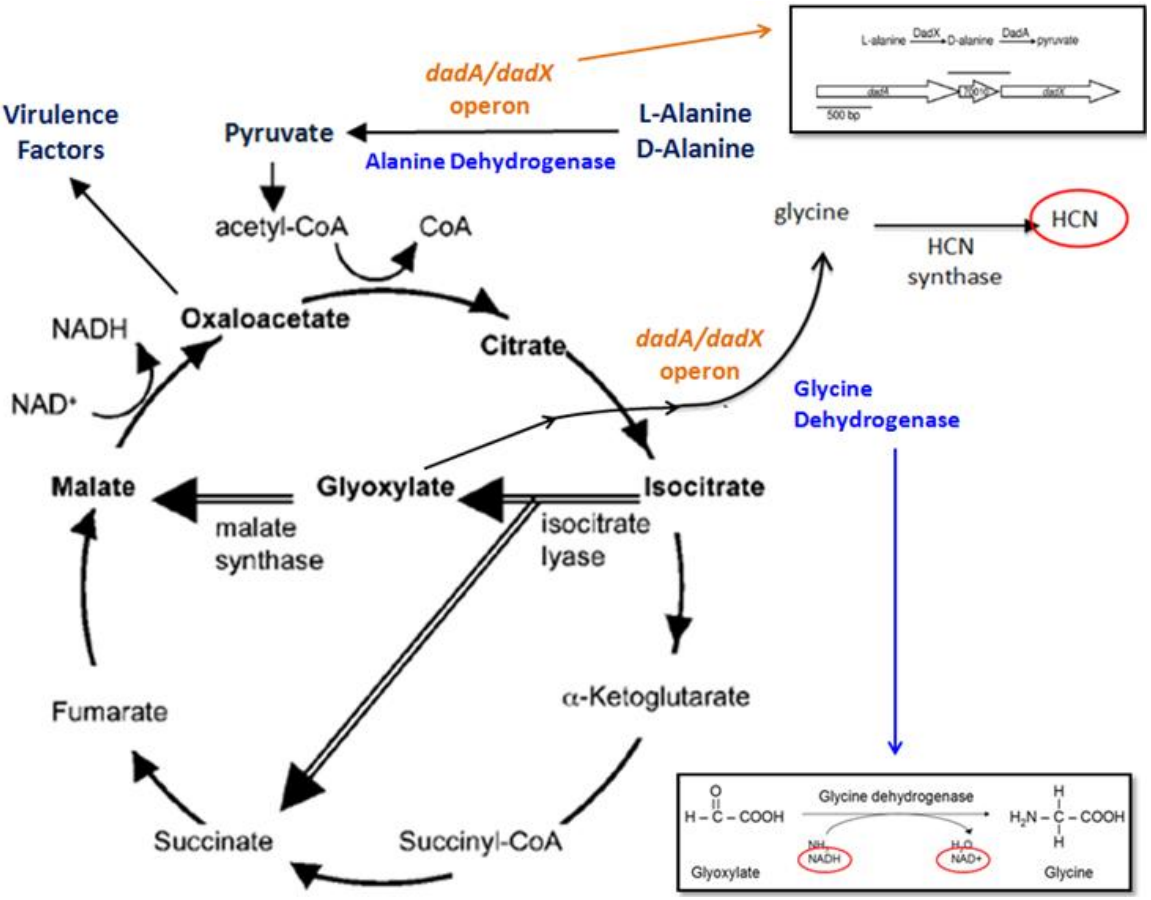

Figure 1.2. Theoretical mechanisms of action of the *dad* operon. Figure is adapted from Linsdey *et. al.* (2008) and Boulette *et. al.* (2009).

Induction of the *dad* operon is dependent upon the presence of L- or D-alanine, but in *Escherichia coli*, it is also dependent on the transcriptional regulator Lrp, otherwise known as the leucine responsive regulatory protein [Boulette *et. al.*, 2009]. Lrp is involved in the regulation of enzymes involved in amino acid biosynthesis and metabolism. Specifically, Lrp is a global regulator of the feast/famine regulatory protein family. It can bind DNA specifically or nonspecifically, and alone or in the presence of other transcriptional regulators, such as the cyclic AMP receptor protein (Crp) [Zhi *et. al.*, 1999]. In *E. coli*, Lrp transcriptionally activates and represses *dadA* and *dadX*, respectively, by binding to the activation sites and masking the repressor sites when in

the presence of L- or D-alanine. Lrp is also required for induction of *gcv*, which is involved in the glycine cleavage system in *E. coli* [Ghrist *et. al.*, 1995].

In contrast to the situation in *E. coli*, Lrp is only a transcriptional activator in *P. aeruginosa* in the presence of L- or D-alanine [Boulette *et. al.*, 2009]. The *dad* operon is significantly repressed by insertional inactivation of *lrp*, but activity of the operon is fully restored by expression of *lrp* in *trans* [Boulette *et. al.*, 2009]. In addition, Lrp regulates the expression of chaperones, such as DnaK, GroEL, and GroES, as well as the suppressor protein DksA in *P. aeruginosa* [Ghrist *et. al.*, 1995].

Recent studies have identified an additional transcriptional regulator of the *dad* operon, characterized as DadR. Expression of the *dad* operon requires DadR [Boulette *et. al.*, 2009], which is known to be a transcriptional activator of the Lrp family [Brinkman *et. al.*, 2003; de los Rios *et. al.*, 2007]. Specifically, DadR is a transcriptional activator of the *dadA* promoter, and the affinity of its binding increases three-fold in the presence of L-alanine, but not D-alanine [He *et. al.*, 2011]. Mutants devoid of a functional DadR cannot catabolize L-alanine as a sole carbon source, as well [Chou *et. al.*, 2008]. Multiple DadR-DNA binding complexes are located in the *dadA* regulatory region, indicating that this transcriptional regulator plays a pivotal role in induction of the *dad* operon in the presence of L-alanine [He *et. al.*, 2011].

Alanine is predicted to be a very important carbon source for *P. aeruginosa* proliferation *in vivo*. Previous studies have shown that *P. aeruginosa* prefers L-alanine over many

other carbon sources present in the CF lung [Palmer *et. al.*, 2007]. In addition, other studies have shown that the mRNA levels of both *dadA* and *dadX* are highly elevated in a peritoneal rat lung infection model [Palmer *et. al.*, 2007] and during *in vitro* growth in CF sputum [Palmer *et. al.*, 2005]. Therefore, *in vitro* and *in vivo* competition assays have been performed between wild type CF isolates of *P. aeruginosa* and *dadA*$^{-}$ mutants. These assays indicate that *in vitro* and *in vivo*, the *dadA* mutants are reduced for competitive fitness when compared to the wild type strains [Boulette *et. al.*, 2009]. Given that *P. aeruginosa* is capable of catabolizing many different carbon sources in the lung environment, it is interesting that the loss of this single catabolic enzyme involved in alanine utilization decreases the overall competitive fitness of *P. aeruginosa*. Therefore, a better understanding of the role that *dadA* plays in the chronic persistence of *P. aeruginosa* infections is imperative for developing improved antimicrobials for victims of CF. To date, the role of *dadA* in the virulence of other microbial pathogens has not been elucidated.

Summary

P. aeruginosa infections remain the leading cause of morbidity and mortality of CF patients. Many other bacteria infect the lungs of CF patients alongside *P. aeruginosa*, such as *S. aureus* and *B. cepacia* [Mashburu *et. al.*, 2005]. However, biofilm formation and the secretion of virulence factors by *P. aeruginosa* exacerbate the CF disorder and contribute to an early demise in CF patients. In addition, CF-adapted isolates of *P. aeruginosa* utilize novel strategies to persist in the CF lung. A lack of effective antibiotics against *P. aeruginosa* is responsible for our inability to treat these devastating

infections. Therefore, alternative therapeutics must be developed to treat CF. A better understanding of the strategies utilized by *P. aeruginosa* to persist in the CF lung will enhance development of novel therapeutics for CF patients.

Interfering with bacterial metabolism *in vivo* can be a very useful strategy for future novel antibiotic therapies. Inactivation of specific carbon catabolic pathways in other bacterial pathogens has been shown to be efficacious in reducing the lethality of disease caused by those pathogens. For example, mutants of *Campylobacter jejuni*, a common poultry pathogen, that are unable to catabolize L-serine in the chicken gut are markedly reduced for colonization [Velayudhan *et. al.*, 2004]. In addition, *Legionella pneumophila* mutants that lack the ability to utilize threonine cannot replicate within alveolar macrophages of the lung [Sauer *et. al.*, 2005]. Therefore, central metabolism is not only critical for carbon and nitrogen source utilization, but also for the production of potential virulence factors present in many bacterial pathogens.

Virulence factors play a very important role in *P. aeruginosa* pathogenesis. Some virulence factors are required to initiate acute infections, while others function to maintain long-term chronic infections. D-amino acid dehydrogenase encoded by *dadA* appears to play important roles in both the acute and chronic stages of *P. aeruginosa* infection. Consequently, we propose to better characterize the role of the *dad* operon in *P. aeruginosa* physiology, in order to evaluate it as a potential therapeutic target.

References

Armougom, F., F. Bittar, N. Stremler, J. M. Rolain, C. Robert, J. C. Dubus, J. Sarles, D. Raoult & B. La Scola. 2009. Microbial diversity in the sputum of a cystic fibrosis patient studied with 16S rDNA pyrosequencing. *Eur J Clin Microbiol Infect Dis* **28:**1151-1154.

Barth, A. L., & T. L. Pitt. 1996. The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. *J Med Microbiol* **45:**110-119.

Boat, T. F., M. J. Welsh & A. L. Beaudet. 1989. *The Metabolic Basis of Inherited Disease*. New York, NY: McGraw Hill.

Boucher, R. C. 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. *Eur Respir J* **23:**146-158.

Boulette, M. L., P. J. Baynham, P. A. Jorth, I. Kukavica-Ibrulj, A. Longoria, K. Barrera, R. C. Levesque & M. Whiteley. 2009. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation *in vivo*. *J Bacteriol* **191:**6329-6334.

Brinkman, A. B., T. J. Ettema, W. M. de Vos & J. van der Oost. 2003. The Lrp family of transcriptional regulators. *Mol Microbiol* **48:**287-294.

Carterson, A. J., L. A. Morici, D. W. Jackson, A. Frisk, S. E. Lizewski, R. Jupiter, K. Simpson, D. A. Kunz, S. H. Davis, J. R. Schurr, D. J. Hassett, and M. J. Schurr. 2004. The transcriptional regulator AlgR controls cyanide production in *Pseudomonas aeruginosa*. *J Bacteriol* **186:**6837-6844.

Chou, H. T., D. J. Kwon, M. Hegazy & C. D. Lu. 2008. Transcriptome analysis of agmatine and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **190:**1966-1975.

Cohn, J. A. 2005. Reduced CFTR function and the pathobiology of idiopathic pancreatitis. *J Clin Gastroenterol* **39(4 Suppl 2):**S70-7.

Costerton, W., R. Veeh, M. Shirtliff, M. Pasmore, C. Post & G. Ehrlich. 2003. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* **112:**1466-1477.

Cox, C. D. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect Immun* **52:**263-270.

Davey, M. E., N. C. Calazza & G. A. O'Toole. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185:**1027-1036.

de los Rios, S. & J. J. Perona. 2007. Structure of the *Escherichia coli* leucine-responsive regulatory protein Lrp reveals a novel octameric assembly. *J Mol Biol* **366:**1589-1602.

Driscoll, J. A., S. L. Brody & M. A. Kollef. 2007. The epidemiology, pathogenesis, and treatment of *Pseudomonas aeruginosa* infections. *Drugs* **67:**351-368.

Ghrist, A. C. & G. V. Stauffer. 1995. The *Escherichia coli* glycine transport system and its role in the regulation of the glycine cleavage enzyme system. *Microbiol* **141:**133-140.

Govan, J. R. & V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60:**539-574.

Hagins, J. M., R. Locy & L. Silo-Suh. 2009. Isocitrate lyase supplies precursors for hydrogen cyanide production in a Cystic Fibrosis isolate of *Pseudomonas aeruginosa*. *J Bacteriol* **191:**6335-6339.

Hagins, J. M., J. Scoffield, S. J. Suh & L. Silo-Suh. 2011. Malate synthase expression is deregulated in the *Pseudomonas aeruginosa* cystic fibrosis isolate FRD1. *Can J Microbiol* **57:**189-195.

He, W., C. Li & C. D. Lu. 2011. Regulation and characterization of the *dadRAX* locus for D-amino acid catabolism in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **193:**2107-2115.

Hong, P. C., R. M. Tsolis & T. A. Ficht. 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. *Infect Immun* **68:**4102-4107.

Lindsey, T. L., J. M. Hagins, P. A. Sokol & L. A. Silo-Suh. 2008. Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiol* **154:**1616-1627.

Lobocka, M., J. Hennig, J. Wild & T. Klopotowski. 1994. Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. *J Bacteriol* **176:**1500-1510.

Malhotra, S., L. A. Silo-Suh, K. Mathee & D. E. Ohman. 2000. Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, DsbA. *J Bacteriol* **182:**6999-7006.

Mashburu, L. M., A. M. Jett, D. R. Akins & M. Whiteley. 2005. *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. *J Bacteriol* **187:**554-566.

Meyer, K. C., A. Sharma, R. Brown, M. Weatherly, F. R. Moya, J. Lewandoski & J. J. Zimmerman. 2000. Function and composition of pulmonary surfactant and surfactant-derived fatty acid profiles are altered in young adults with cystic fibrosis, *Chest* **118:**164-174.

Nester, E. W., D. G. Anderson, C. E. Roberts Jr. & M. T. Nester. 2007. *Microbiology*. New York, NY: McGraw-Hill.

Oliver, A., R. Canton, P. Campo, F. Baquero & J. Blazquez. 2000. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288:**1251-1253.

Palmer, K. L., L. M. Mashburu, P. K. Singh & M. Whiteley. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187:**5267-5277.

Palmer, K. L., L. M. Aye & M. Whiteley. 2007. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* **189:**8079-8087.

Poole, K. & R. Skirumar. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms, and clinical significance. *Curr Top Med Chem* **1:**59-71.

Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins & L. Tsui. 1989. Identification of the Cystic Fibrosis gene: cloning and characterization of complementary DNA. *Science* **245:**1066-1073.

Rogers, G. B., C. A. Hart, J. R. Mason, M. Hughes, M. J. Walshaw & K. D. Bruce. 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S rDNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* **41:**3548-3558.

Ryall, B., J. C. Davies, R. Wilson, A. Shoemark, & H. D. Williams. 2008. *Pseudomonas aeruginosa*, cyanide accumulation and lung function in CF and non-CF bronchiectasis patients. *Eur Resp J* **32:**740-747.

Saiman, L., K. Ishimoto, S. Lory & A. Prince. 1990. The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. *J Infect Dis* **161:**541-548.

Salyers, A. A. & D. D. Whitt. 2002. *Bacterial Pathogenesis*. Washington, D.C.: ASM Press.

Sanderson, K., L. Wescombe, S. M. Kirov, A. Champion & D. W. Reid. 2008. Bacterial cyanogenesis occurs in the cystic fibrosis lung. *Eur Respir J* **32:**329-333.

Sauer, J. D., M. A. Bachman & M. S. Swanson. 2005. The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *PNAS* **102:**9924–9929.

Silo-Suh, L., S. Suh, P. V. Phibbs & D. E. Ohman. 2005. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment can include deregulation of *zwf*, encoding glucose-6-phosphate dehydrogenase. *J Bacteriol* **187:**7561-7568.

Son, M. S., W. J. Matthews, Jr., Y. Kang, D. T. Nguyen & T. T. Hoang. 2007. *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of Cystic Fibrosis patients. *Infect Immun* **75:**5313-5324.

Tart, A. H., M. J. Blanks & D. J. Wozniak. 2006. The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J Bacteriol* **188:**6483-6489.

Velayudhan, J., M. A. Jones, P. A. Barrow & D. J. Kelly. 2004. L-serine catabolism via an oxygen-labile L-serine dehydrogenase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect Immun* **72:**260-268.

Wieland, C. W., B Siegmund, G. Senaldi, M. L. Vasil, C. A. Dinarello & G. Fantuzzi. 2002. Pulmonary inflammation induced by *Pseudomonas aeruginosa* lipopolysaccharide, phospholipase C, and exotoxin A: role of interferon regulatory factor 1. *Infect Immun* **70:**1352-1358.

Wild, J., J. Hennig, M. Lobocka, W. Walczak & T. Klopotowski. 1985. Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli* K12. *Mol Gen Genet* **198:**315-322.

Williams, H. D., J. E. Zlosnik, &B. Ryall. 2007. Oxygen, cyanide and energy generation in the cystic fibrosis pathogen *Pseudomonas aeruginosa*. *Adv Micro Physiol* **52:**1-71.

Wood, L. F. & D. E. Ohman. 2009. Use of cell wall stress to characterize sigma 22 (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. *Mol Microbiol* **72:**183-201.

Wozniak, D. J. & D. E. Ohman. 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR, algB,* and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *J Bacteriol* **176:**6007-6014.

Young, D., T. Hussell & G. Dougan. 2002. Chronic bacterial infections: living with unwanted guests. *Nat Immunol* **3:**1026-1032.

Zhi, J., E. Mathew & M. Freundlich. 1999. Lrp binds to two regions in the *dadAX* promoter region of *Escherichia coli* to repress and activate transcription directly. *Mol Microbiol* **32:**29-40.

Chapter 2

Initial Characterization of *dadA* in *Pseudomonas aeruginosa* Virulence Factor Production

Introduction

CF is an autosomal recessive genetic disorder that afflicts millions of people worldwide [Boat *et. al.*, 1989]. According to a survey completed in 2010 by the U.S. Department of Energy, Biological, and Environmental Research, one in 2,500 children in the United States is born with CF every year, and one in 20 people are asymptomatic carriers. CF is caused by a mutation in the gene that encodes for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). Defects in CFTR allow opportunistic pathogens the ability to invade, colonize, and proliferate within host respiratory systems [Riordan *et. al.*, 1989]. However, it is chronic *Pseudomonas aeruginosa* infections which remain the leading cause of lung dysfunction and mortality in CF patients. This opportunistic pathogen maintains the ability to metabolize a wide array of carbon and nitrogen sources, and its minimal nutrition requirements allow this pathogen to persist within very unfavorable environmental niches, such as the CF lung [Nester *et. al.*, 2007]. Upon initial invasion of the CF lung, *P. aeruginosa* causes an acute infection of the respiratory tract, which transitions into a chronic infection. During the process, *P. aeruginosa* undergoes several morphological changes, including acquisition of the mucoid

phenotype, loss of motility, increased biofilm capabilities, and reduced production of various virulence factors [Driscoll *et. al.*, 2007].

P. aeruginosa, like other microbial pathogens, utilizes different strategies to cause acute infections than to maintain chronic infections within hosts [Costerton *et. al.*, 2003; Hong *et. al.*, 2000; Young *et. al.*, 2002]. Acute and chronic isolates of *P. aeruginosa* display a differential preference for carbon sources, suggesting that the bacterium alters its metabolic pathways during chronic infection of the CF lung. For example, transcriptome studies indicate that a CF isolate of *P. aeruginosa* primarily utilizes amino acids and lipids as carbon sources when grown in CF sputum, while an acute wound isolate primarily uses amino acids [Palmer *et. al.*, 2005; Son *et. al.*, 2007]. In addition, regulatory control of several central metabolic enzymes has been shown to be altered in the CF isolate, FRD1, compared to the acute isolate, PAO1 [Lindsey *et. al.*, 2008; Hagins *et. al.*, 2009]. Not surprisingly, therapeutic treatments that are successful against acute infections are often ineffective at treating chronic infections. Several drug-based approaches are being investigated, but current antimicrobial therapies are ineffective at treating the chronic infections maintained by *P. aeruginosa* within the CF lung. Therefore, a better understanding of the chronic virulence mechanisms utilized by *P. aeruginosa* is critical for the development of improved therapies in treating CF.

Previous studies identified *dadA*, a gene encoding a D-amino acid dehydrogenase, to be upregulated in chronic isolates of *P. aeruginosa* [Boulette *et. al.*, 2009]. In addition, *dadA* is required for optimal hydrogen cyanide (HCN) production by *P. aeruginosa*, and

HCN concentrations in the CF lung correlate with pulmonary damage [Hagins *et. al.*, 2009; Ryall *et. al.*, 2008; Sanderson *et. al.*, 2008]. Taken together, the results suggest a role for DadA in persistence of *P. aeruginosa* during chronic infection of CF patients. However, *dadA* is also required for the overall optimum competitive fitness of *P. aeruginosa* in a rat lung model of infection, suggesting it is also required for acute infection [Boulette *et. al.*, 2009]. Disruption of the *dad* operon with an insertion in *dadA* in FRD1 produces a pleiotropic phenotype, suggesting the loss of additional virulence factors in the bacterium. Morphologically, this mutant appears to be defective for the production of alginate, pyocyanin, and pyoverdine upon visualization of bacterial growth on agar plates (Figure 2.1). However, the mechanism underlying these phenotypes is not immediately obvious. *dadA* is located with *dadX* in an operon, and both are required by *P. aeruginosa* to catabolize alanine as a carbon source [Boulette *et. al.*, 2009]. *dadX* encodes an alanine racemase that interconverts L-alanine into D-alanine and vice versa, while the D-amino acid dehydrogenase oxidatively deaminates D-alanine into pyruvate and ammonia [Boulette *et. al.*, 2009]. In order to determine whether disruption of this pathway is of therapeutic value, it is important to gain a better understanding of the role of this operon in *P. aeruginosa* virulence and physiology.

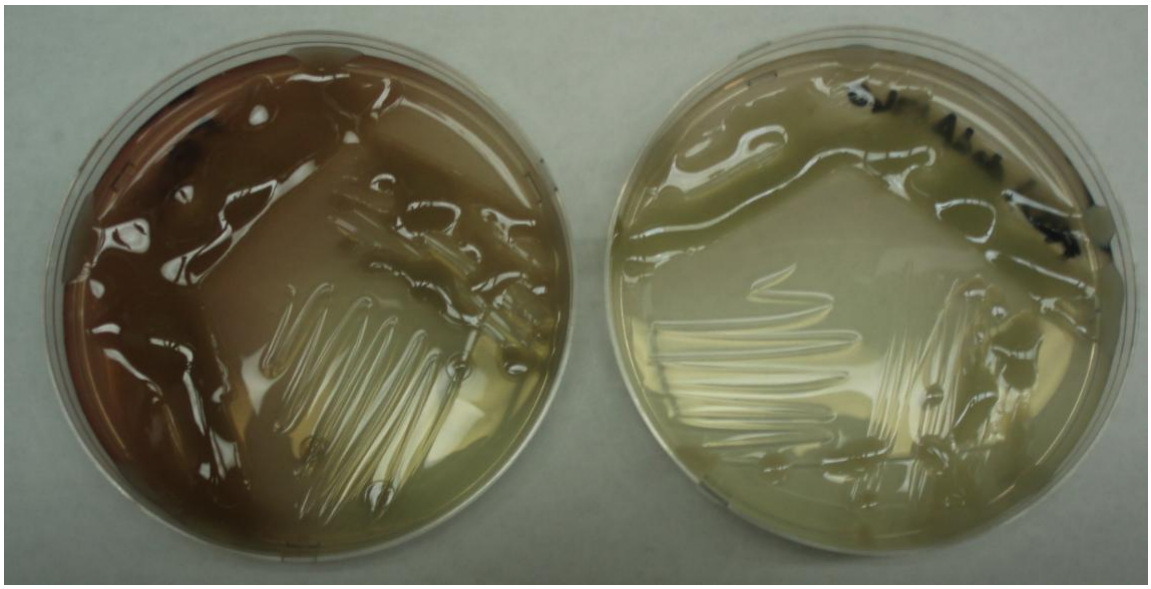

Figure 2.1. Phenotypic comparison of FRD1 and FRD1*ΔdadA*. A three day-old culture of FRD1 is on the left, and a three day-old culture of FRD1*ΔdadA* is on the right. Photograph was taken by Kathryn Oliver.

In order to better understand the increased significance of *dadA* in *P. aeruginosa*, we characterized the contribution of the *dad* operon to virulence factor production by FRD1 (chronic CF isolate) and PAO1 (acute wound isolate). In this study, we demonstrate that *dadA* is required for optimal production of the virulence factors pyocyanin, pyoverdine, rhamnolipid, and alginate by FRD1, as well as optimal biofilm formation. Using an alfalfa seedling model of infection, we also illustrate that *dadA* is required for the overall optimal virulence of FRD1 *in vivo*. In contrast, *dadA* is required only for optimal rhamnolipid production by PAO1. Intracellular levels of L- and D-alanine were quantitated in an attempt to explain the *dadA*$^{-}$ phenotype, but the data was inconclusive. Taken together, the results indicate that *dadA* plays a pleiotropic role in the production of important virulence factors by CF isolates 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 noted, bacteria were cultured in Luria Bertani (LB) broth at 37°C. All amino acids added to growth media were used at 20mM. *P. aeruginosa* was cultured in 1% (w/v) peptone broth supplemented with 1% (w/v) NaCl and 1% (w/v) glycerol for the pyocyanin assay and in King's B medium for the pyoverdine assay as previously described [Essar *et. al.,* 1990]. A 1:1 mixture of LB-agar and Pseudomonas Isolation Agar (PIA) was used to select for *P. aeruginosa* transconjugants and for counter-selection of *E. coli* after tri-parental mating. Solidified media contained 1.5% (w/v) Bacto Agar (Difco; Becton Dickinson). Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: 100 µg/mL ampicillin, 20 µg/mL gentamicin, and 50 µg/mL kanamycin for *E. coli*; 180 µg/mL gentamicin, 180 µg/mL carbenicillin, 100 µg/mL tetracycline, 800 µg/mL kanamycin for *P. aeruginosa*.

To monitor growth over a 24-hour period, bacterial cultures were grown in 24-well microtiter plates, and the OD_{600} was obtained every 15 minutes using a BioTek Synergy HT plate reader (BioTek, Winooski, VT). A Shimadzu UV-1601 Spectrophotometer using 1 cm path length cells was used to record UV-Vis absorption spectra.

DNA manipulations, transformations, and conjugations. The host strain used routinely for cloning was *E. coli* strain DH10B. Electroporation was used to introduce DNA into *E. coli*, and tri-parental conjugation was used to introduce DNA into *P.*

aeruginosa as previously described [Suh *et. al.*, 1999]. For PCR amplification of DNA, either *Taq* (New England Biolabs, Beverly, MA) or *Pfu* (Stratagene, La Jolla, CA) were used. Oligonucleotides were purchased from Integrated DNA technologies (Coralville, IA). Restriction enzymes were also purchased from New England Biolabs. DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen, Valencia, CA). Plasmids were purified using the QIAprep Spin Miniprep columns (Qiagen).

Construction of *P. aeruginosa ΔdadA*, *dadX*, and double (*ΔdadAdadX*) mutants. In-frame deletion constructs of *dadA* were generated, using PCR-driven spliced overlap extension, and placed on a suicide plasmid. Tri-parental mating was used to introduce the plasmid carrying the altered *dadA* gene into FRD1 and PAO1. Homologous recombination with a double crossover between the plasmid and chromosomal DNA resulted in a replacement of the wild type *dadA* gene with the deleted allele, yielding FRD1*ΔdadA* and PAO1*ΔdadA* mutants, respectively.

To generate *dadX* mutants of *P. aeruginosa*, the suicide plasmid pLS1934 was constructed: a DNA sequence containing approximately 500 bp upstream and downstream of the *dadX* coding sequence was PCR amplified from FRD1 with *Pfu* and cloned into the *Sma*I site of pBluescript K(+). The resulting plasmid was digested with *Sph*I, and the internal *dadX* fragment was removed and replaced with the *aacC1* gene encoding gentamicin resistance as an *Sph*I fragment [Schweizer, 1993]. An origin of transfer (m*oriT*) from pUC19 on a *Hind*III fragment was then introduced into the plasmid

[Suh *et. al.*, 2004]. pLS1934 was transferred into FRD1 and PAO1 by tri-parental mating. Gentamicin-resistant and carbenicillin-sensitive clones were isolated, indicating a double crossover via homologous recombination. Replacement of the wild-type *dadX* gene with the *dadX101::aacC1* allele was verified by PCR. Mutants were designated FRD1*dadX* and PAO1*dadX*, respectively.

To generate the *ΔdadAdadX* double mutants of *P. aeruginosa*, pLS1934 was moved into the already-constructed FRD1*ΔdadA* and PAO1*ΔdadA* mutants via tri-parental conjugation. This allowed the altered *dadX* gene to recombine into the FRD1*ΔdadA* and PAO1*ΔdadA* chromosomes, effectively inactivating the *dadX* gene in conjunction with the already-inactivated *dadA* gene. Transconjugants were isolated on PIA containing gentamicin, verified via PCR analysis, and designated FRD1*ΔdadAdadX* and PAO1*ΔdadAdadX*, respectively.

Complementation of the *dadA* and *dadX* mutants. To complement the *dadA* mutant, the *dadA* gene was PCR-amplified from FRD1 using *Pfu*. The PCR fragment was cloned into pLS1793. The m*oriT* was inserted at the *Hind*III site on the plasmid, after which the plasmid was transformed into *E. coli*. pLS1793 was then transferred to FRD1*ΔdadA* and PAO1*ΔdadA* in *trans* via tri-parental conjugation. The transconjugants were selected on PIA containing kanamycin, and the resulting colonies were verified using PCR. The complemented FRD1*ΔdadA* and PAO1*ΔdadA* mutants were designated FRD1*ΔdadAC* (KO46) and PAO1*ΔdadAC* (KO48), respectively.

To complement the *dadX* mutant, the *dadX* gene was PCR-amplified from FRD1 using *Pfu*. The PCR fragment was cloned into pBluescript K(+) at a *Sma*I site, resulting in the plasmid pKO13. The m*oriT* was inserted at the *Hind*III site on the plasmid, resulting in pKO57, after which pKO57 was transformed into *E. coli*. pSS124, containing an *oriV* on mSF that was more compatible with *P. aeruginosa*, was cloned into pKO57 at an *Xba*I site, resulting in the plasmid pKO60. pKO60 was then transferred to FRD1*dadX* and PAO1*dadX* in *cis* via tri-parental conjugation. The transconjugants were selected on PIA containing carbenicillin, and the resulting colonies were verified using PCR. The complemented FRD1*dadX* and PAO1*dadX* mutants were designated FRD1*dadXC* (KO50) and PAO1*dadXC* (KO49), respectively.

Construction of *dadA* transcriptional fusions. The *dadA::lacZ* transcriptional fusion was constructed using the *dadA* gene fragment obtained from FRD1 using PCR-amplification with *Pfu*. This PCR fragment was then cloned into pSS223 [Suh *et. al.,* 2004]. The resulting plasmid (pLS1951) was then conjugated into FRD1 and PAO1. Carbenicillin resistant colonies were selected and verified for the presence of the *dadA::lacZ* fusion by PCR analysis.

Strain or Plasmid	Genotype, Relevant Characteristics	Source
Strains		
FRD1	CF isolate, mucoid	Ohman *et. al.* (1981)
PAO1	Wound isolate, nonmucoid	Holloway *et. al.* (1979)
FRD1Δ*dadA* (LS1938)	FRD1 with in-frame deletion of *dadA*	Dr. L. Silo-Suh
PAO1Δ*dadA* (LS1940)	PAO1 with in-frame deletion of *dadA*	Dr. L. Silo-Suh
FRD1*dadX* (LS1943)	FRD1*dadA101::aacC1*	Dr. L. Silo-Suh
PAO1*dadX* (LS1941)	PAO1*dadA101::aacC1*	Dr. L. Silo-Suh
FRD1Δ*dadAdadX* (KO39)	FRD1Δ*dadA* + *dadA101::aacC1*	This study
PAO1Δ*dadAdadX* (KO42)	PAO1Δ*dadA* + *dadA101::aacC1*	This study
FRD1Δ*dadAC* (KO46)	FRD1Δ*dadA* complemented in *trans* for *dadA*	This study
PAO1Δ*dadAC* (KO48)	PAO1Δ*dadA* complemented in *trans* for *dadA*	This study
FRD1*dadXC* (KO50)	FRD1*dadX* complemented in *cis* for *dadX*	This study
PAO1*dadXC* (KO49)	PAO1*dadX* complemented in *cis* for *dadX*	This study
Plasmids		
pLS214	pUC19 with m*oriT* at *Hind*III	Suh *et. al.* (2004)
pLS93	pUCGm	Schweizer (1993)
pKO13	*dadX101* in pBluescript K(+) at *Sma*I	This study
pKO57	m*oriT* in pKO13 at *Hind*III	This study
pSS124	*oriV* on mSF	Suh *et. al.* (2004)
pKO60	*oriV* in pKO57 at *Xba*I	This study
pLS1934	*dadX101::aacC1* in pKO13	Dr. L. Silo-Suh
pLS1793	*dadA* complementing plasmid with FRD1 *dadA*	Dr. L. Silo-Suh

pLS1952	*dadX* complementing plasmid with FRD1 *dadX*	Dr. L. Silo-Suh
pSS223	*lacZ* transcriptional fusion plasmid	Suh *et. al.* (2004)
pLS1951	*dadA::lacZ* transcriptional fusion in pSS223	Dr. L. Silo-Suh

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

Biochemical assays. Stationary phase cultures of *P. aeruginosa* were used for all biochemical assays unless otherwise indicated. Pyocyanin was purified and measured from 21-hour cultures as previously described [Essar *et. al.*, 1990]. Pyoverdine levels were measured as previously described by Suh *et. al.* (1999). Rhamnolipid was purified and measured as previously described [Du Plessis, 2005]. Alginate was isolated from *P. aeruginosa* culture supernatants dialysed against distilled water as previously described [Suh *et. al.*, 1999], and alginate levels were quantified using the carbazole method [Knutson *et. al.*, 1968] with *Macrocystis pyrifera* alginate (Sigma-Aldrich) used as the standard. The β-galactosidase assay was also performed as previously described [Miller, 1972].

Biofilm growth. Measurement of static biofilm activity was performed as previously described [Lindsey *et. al.*, 2008]. *P. aeruginosa* was grown overnight in LB broth, diluted, and adjusted to an approximate OD_{600} of 0.5. From this culture, 5 µL was inoculated into 125 µL of fresh LB broth in a 96-well microtiter plate. The plate was incubated for 15 hours at 37°C, after which crystal violet was used to stain the cells for optical density readings.

Alfalfa seedling infection assay. Alfalfa seeds of variety 57Q77, a wild-type strain not bred for pest resistance, were kindly provided by Pioneer Hi-Bred International. The alfalfa assay was performed as previously described [Silo-Suh *et. al.*, 2002]. FRD1, PAO1, and their derivatives were inoculated onto wounded alfalfa seedlings using approximately 10^4 c.f.u. per seedling. Water agar plates were sealed with Parafilm and

incubated at 30°C for 5 days without light. The plates were allowed to incubate an additional 24 hours at room temperature with light, after which disease symptoms were scored by visual inspection. All seedlings with visible maceration symptoms were scored positive for infection. All bacterial strains were inoculated on 40 seedlings for each of three experiments.

Amino acid assay. *P. aeruginosa* cells were harvested from stationary phase cultures, resuspended in TE buffer (pH 7.0), and sonicated to break open the cells. The suspension was centrifuged to remove membrane fractions, after which the cell-free extracts were collected from the supernatant. The cell-free extracts were then derivatized with L-FDAA (*N*-(2,4-dinitro-5-fluoro-phenyl)-L-alanineamide) as previously described [Kolodkin-Gal *et. al.*, 2010]. These derivatives were analyzed by Ultra Performance Light Chromatography / Mass Spectrophotometry (UPLC/MS) with electrospray ionization (ESI) in the positive ion mode using a gradient solvent system from 30% to 100% acetonitrile with 0.1% formic acid over 15 minutes (Waters ACQUITY UPLC/ Q-TOF Premier quadrupole time-of-flight MS, ACQUITY UPLC BEH reverse-phase C18 column, 1.0 mm × 50 mm, 1.7μM) (Waters Corporation, Milford, MA). The retention times of L-FDAA-L-alanine and L-FDAA-D-alanine were compared with L-FDAA-authentic standards of L- and D-alanine. Total protein content in the cell-free extracts was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA), and the samples were normalized using the L- or D-alanine concentration (mM) per unit of protein (μg).

Results

dadA and *dadX* are in an operon together, and *dadA* is located upstream of *dadX* (Figure 2.2.) In order to ensure that our data did not result from downstream polar effects, a clean deletion in *dadA* was made, designated *ΔdadA*, which was used to access the role of *dadA* in both FRD1 and PAO1. However, an insertion in *dadX* with the use of an antibiotic cassette was generated to access the role of this gene in both FRD1 and PAO1.

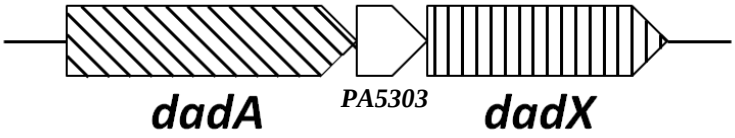

Figure 2.2. Schematic of the *dad* operon. This figure was created by Kathryn Oliver.

FRD1*ΔdadA* and PAO1*ΔdadA* are not significantly defective for growth.

Disruption of *dadA*, but not *dadX*, has a slight effect on the growth of both FRD1 (Figure 2.3) and PAO1 (Figure 2.4). The PAO1*ΔdadA* growth defect manifests primarily in stationary phase, while FRD1*ΔdadA* is affected throughout the growth cycle. The observed growth defect caused by loss of *dadA* is likely due to the buildup of the toxic intermediate D-alanine [Fox *et. al.*, 1944]. This is supported by the observation that disruption of *dadX*, which interconverts L-alanine to D-alanine, does not greatly affect growth of either isolate. More importantly, the growth defect observed for the *dadA* mutants should not severely affect virulence factor production by either isolate, and it should affect the isolates equally by using stationary phase cultures for analysis. In addition, the FRD1 double mutant is not deficient for growth (Figure 2.3). However, the defect in growth that is seen in the PAO1 double mutant presents itself primarily during

stationary phase, which is similar to what is observed in the PAO1*ΔdadA* mutant (Figure 2.4).

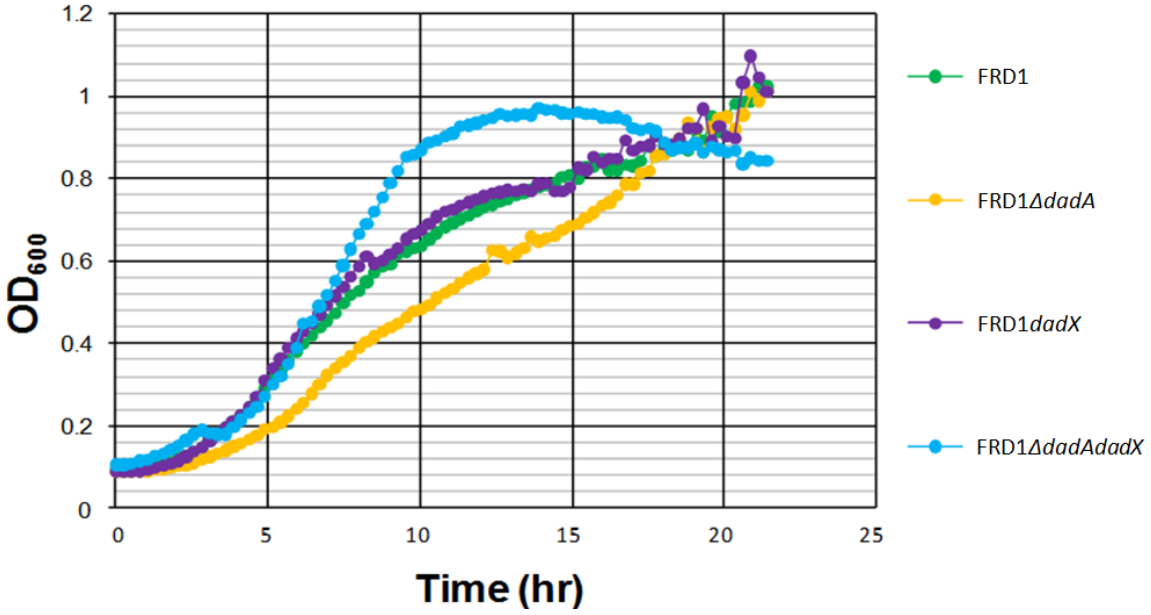

Figure 2.3. Effect of *dadA* and *dadX* disruption on FRD1 grown in LB broth. OD_{600} readings were taken every 15 minutes for a 24-hour period.

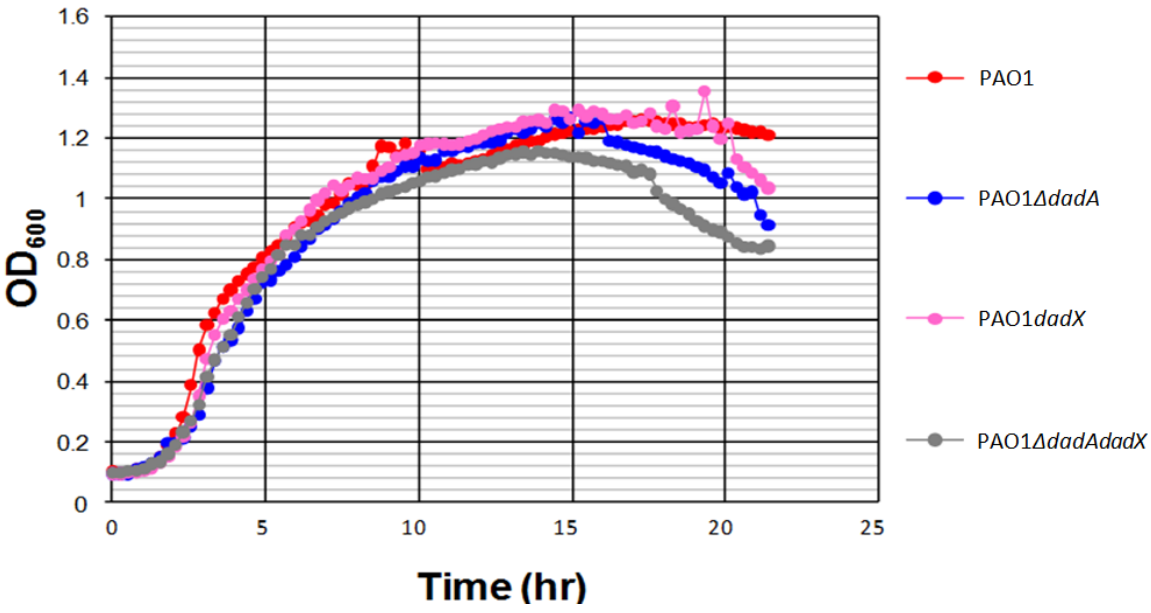

Figure 2.4. Effect of *dadA* and *dadX* disruption on PAO1 grown in LB broth. OD_{600} readings were taken every 15 minutes for a 24-hour period.

DadA is required for optimal production of pyocyanin, pyoverdine, rhamnolipid, and alginate.

Disruption of *dadA* in FRD1 causes a 40% decrease in pyocyanin production (Figure 2.5), a four-fold decrease in pyoverdine production (Figure 2.6), a two-fold decrease in rhamnolipid production (Figure 2.8), and a 40% decrease in alginate production (Figure 2.10). In contrast, loss of *dadA* by PAO1 causes only a slight 2.3-fold decrease in pyoverdine production (Figure 2.7), a 5.4-fold decrease in rhamnolipid production (Figure 2.9), and has no effect on pyocyanin production (Figure 2.5). Taken together, mutations in *dadA* affect virulence factor production by the chronic isolate more severely than the acute isolate of *P. aeruginosa*.

dadX does not seem to play a role in the production of the given virulence factors by FRD1, but it does seem to play a role in rhamnolipid production by PAO1 (Figure 2.9). Finally**,** the double mutants resemble the FRD1*ΔdadA* mutant for reduction of pyoverdine and rhamnolipid production, suggesting a mechanism other than the buildup of D-alanine is responsible for lack of virulence factor production by these *P. aeruginosa* derivatives.

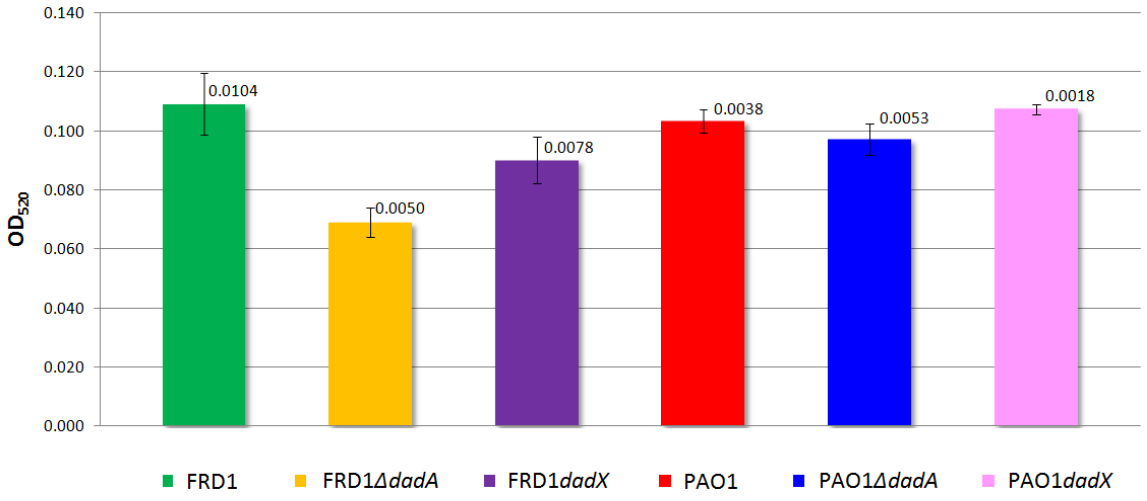

Figure 2.5. *dadA* is required for optimal pyocyanin production in FRD1, but not PAO1. Values represent the average of three experiments with standard errors given for each isolate.

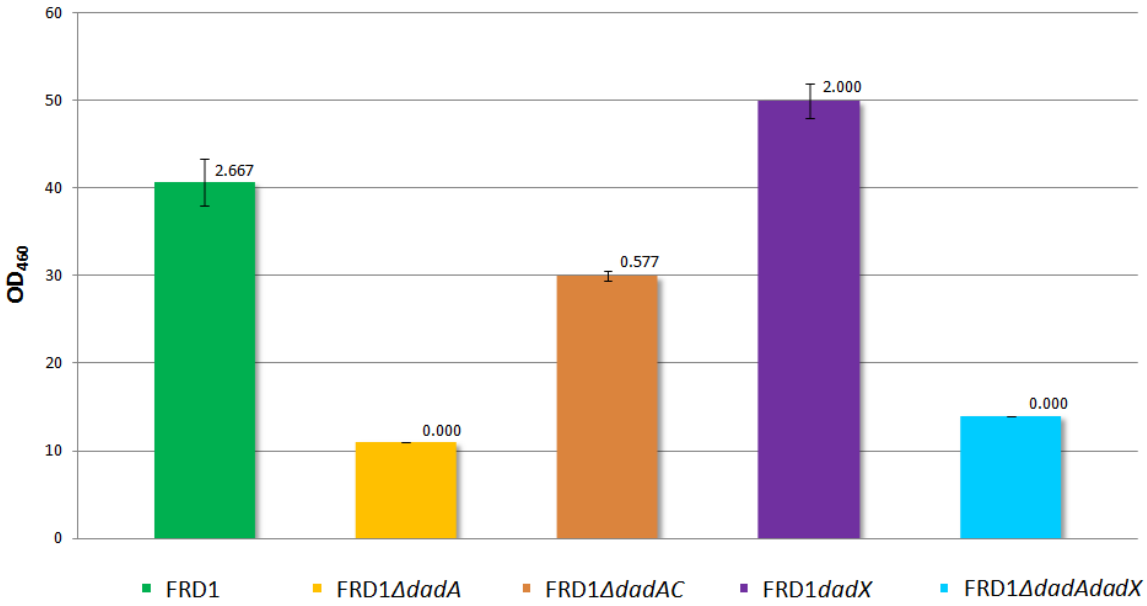

Figure 2.6. *dadA*, but not *dadX*, is required for optimal production of pyoverdine in FRD1. Values represent the average of three experiments with standard errors given for each isolate.

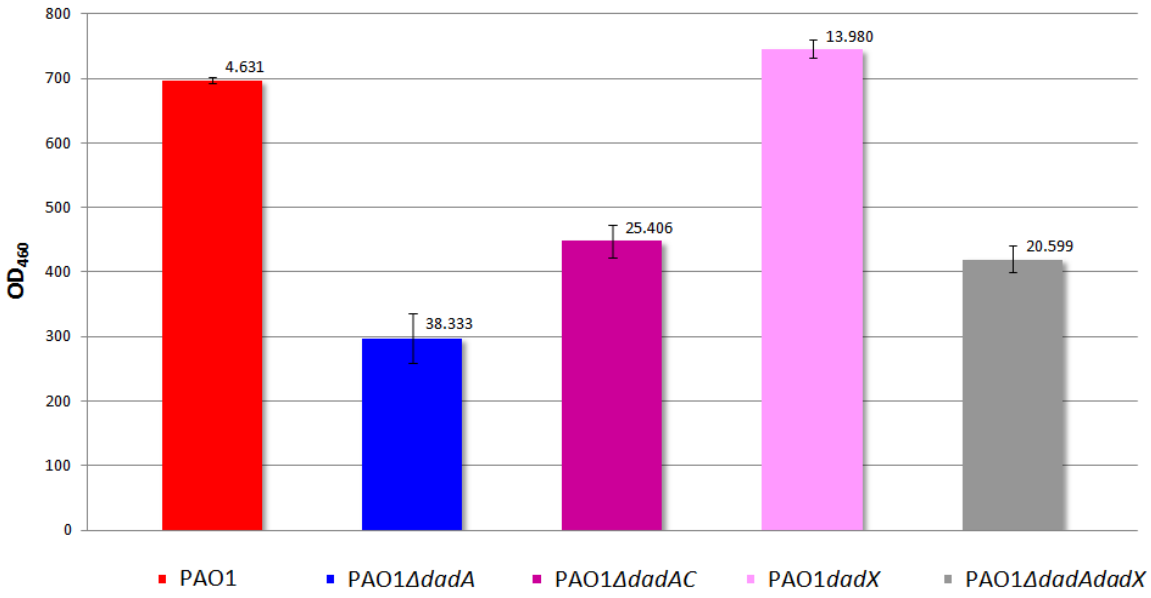

Figure 2.7. *dadA*, but not *dadX*, is required for optimal production of pyoverdine in PAO1. Values represent the average of three experiments with standard errors given for each isolate.

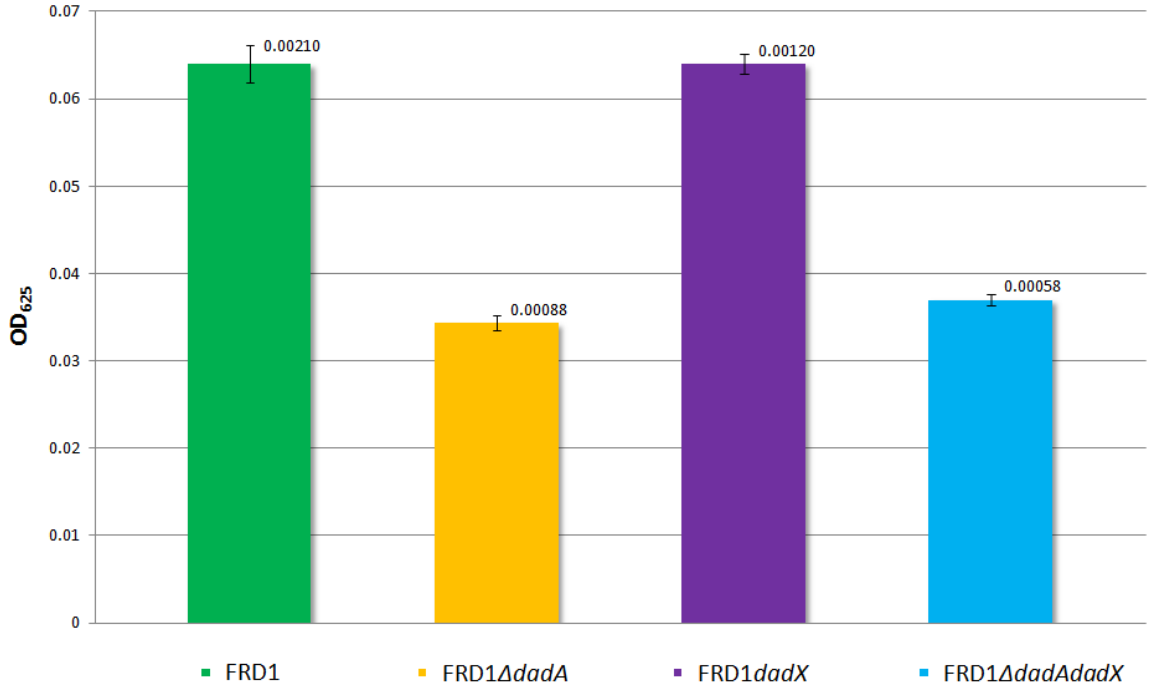

Figure 2.8. Rhamnolipid production by FRD1 and its derivatives. Values represent the average of three experiments with standard errors given for each isolate.

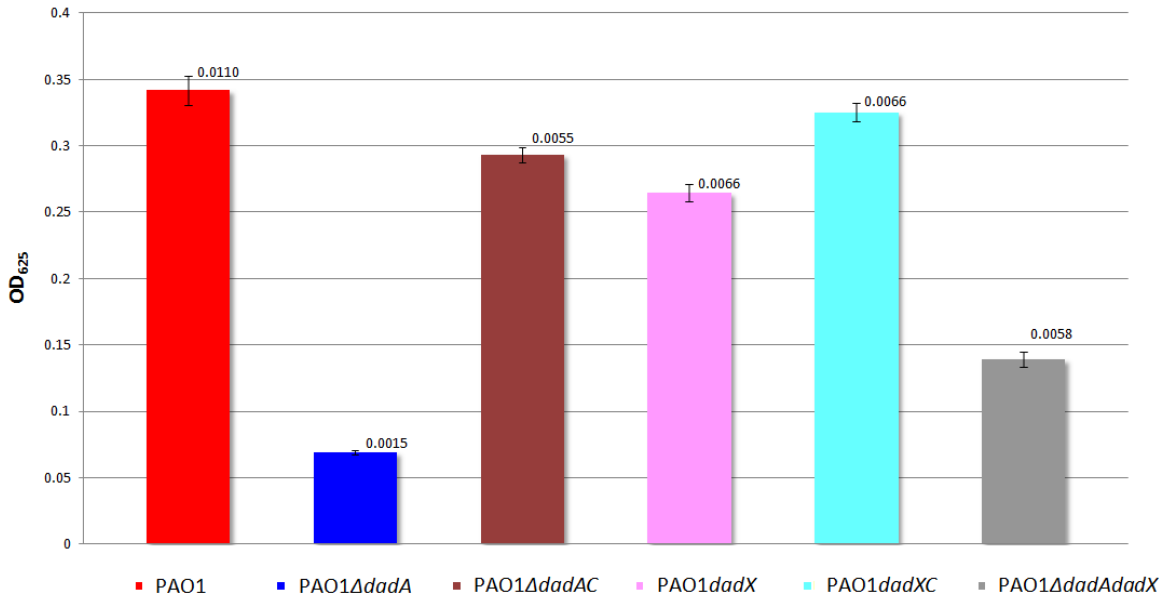

Figure 2.9. Rhamnolipid production by PAO1 and its derivatives. Values represent the average of three experiments with standard errors given for each isolate.

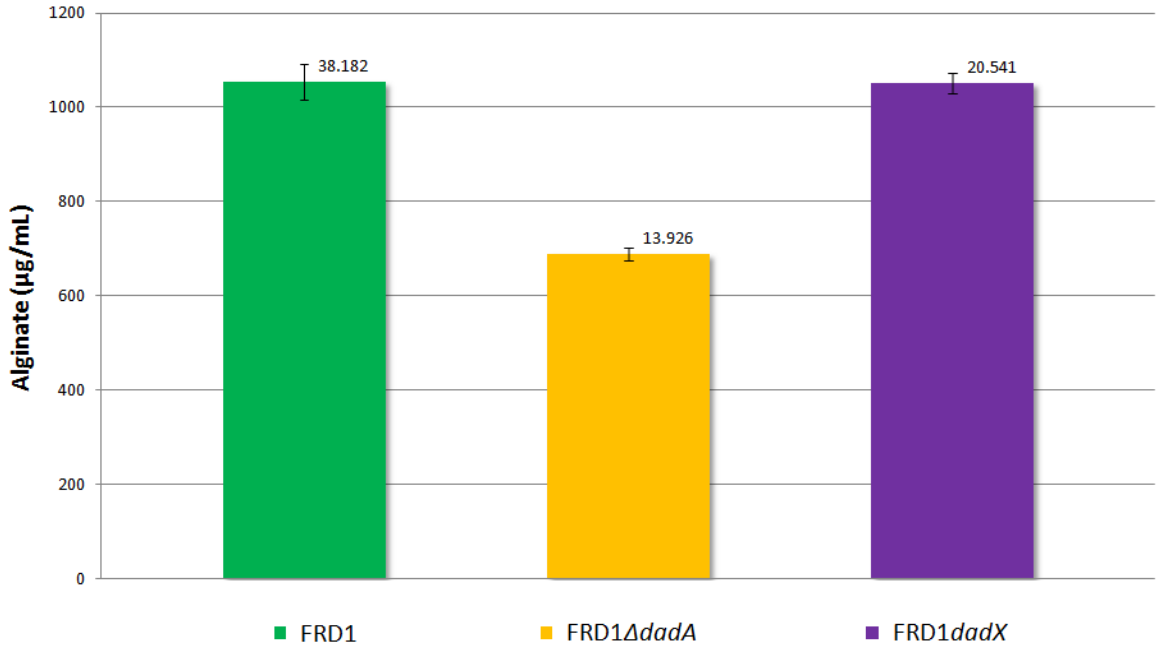

Figure 2.10. *dadA* is required for optimal alginate production by FRD1. Values represent the average of three experiments with standard errors given for each isolate.

DadA is required for optimal biofilm formation.

Disruption of *dadA* in FRD1 causes a three-fold decrease in biofilm activity, which is not restored to wild-type levels by the addition of L-alanine, D-alanine, or glycine to the medium (Figure 2.11). DadA does not appear to be required for biofilm formation by PAO1 (Figure 2.12), and neither is DadX by either FRD1 or PAO1 (Figures 2.11-2.12). It was shown recently that high levels of D-amino acids can lead to biofilm disassembly in *P. aeruginosa* [Kolodkin-Gal *et. al.*, 2010]. This is consistent with our hypothesis that the *ΔdadA* phenotype causes a buildup of intracellular D-alanine, and provides a possible explanation for reduced biofilm formation by FRD1*ΔdadA*, compared to FRD1.

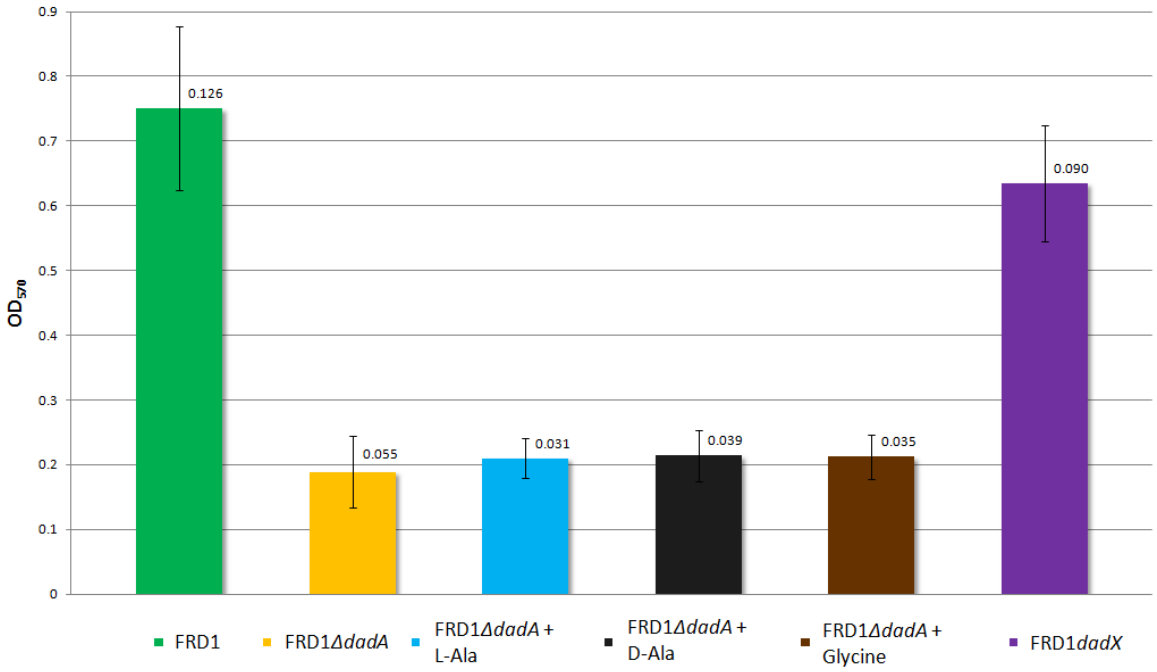

Figure 2.11. Optimal biofilm formation by FRD1 requires *dadA*. Values represent the average of two experiments with standard errors given for each isolate.

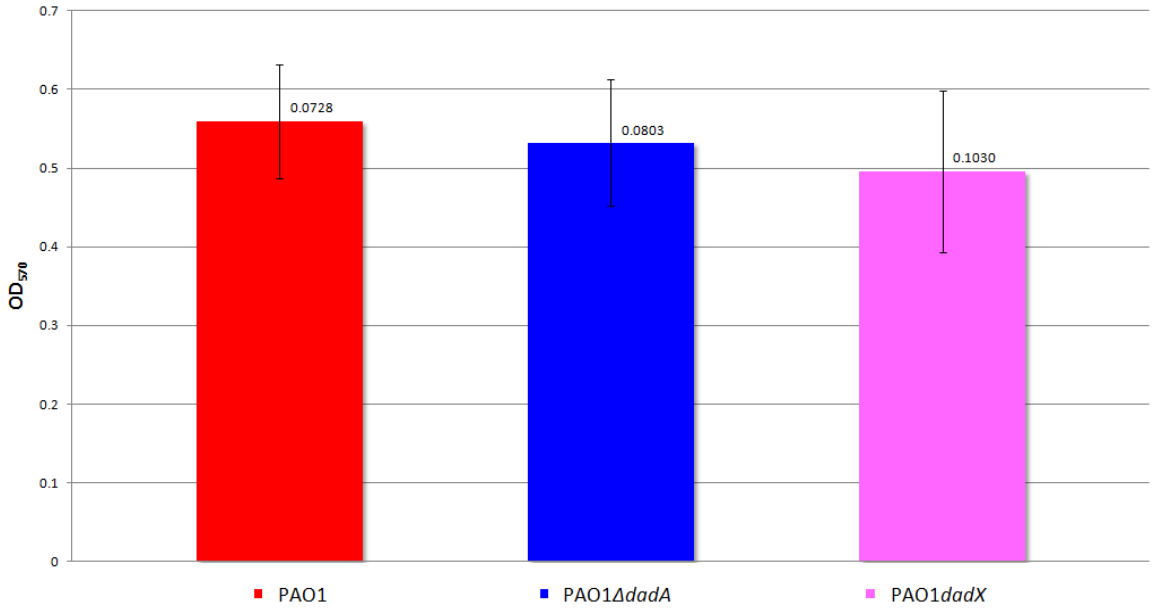

Figure 2.12. *dadA* is not required for optimal biofilm formation by PAO1. Values represent the average of two experiments with standard errors given for each isolate.

dadA is upregulated in FRD1 compared to PAO1.

Transcription of *dadA* rises during log phase in both FRD1 and PAO1, and it decreases during stationary phase (Figure 2.13). However, initial expression of *dadA::lacZ* is higher in FRD1 compared to PAO1, and the decrease during stationary phase occurs earlier, leading to a steeper decline in late stationary phase. Interestingly, this is the time period at which virulence factor production was measured for this study. If reduced virulence factor production is caused by high D-alanine concentrations, then we would have expected to observe higher expression of *dadA* in FRD1, compared to PAO1, during stationary phase. The potential discrepancy may be explained by increased stability of DadA activity in FRD1 compared to PAO1, and would suggest a lack of correlation of *dadA* expression with protein activity. To verify these results and others, it is critical to determine relative intracellular levels of D-alanine in FRD1 and PAO1.

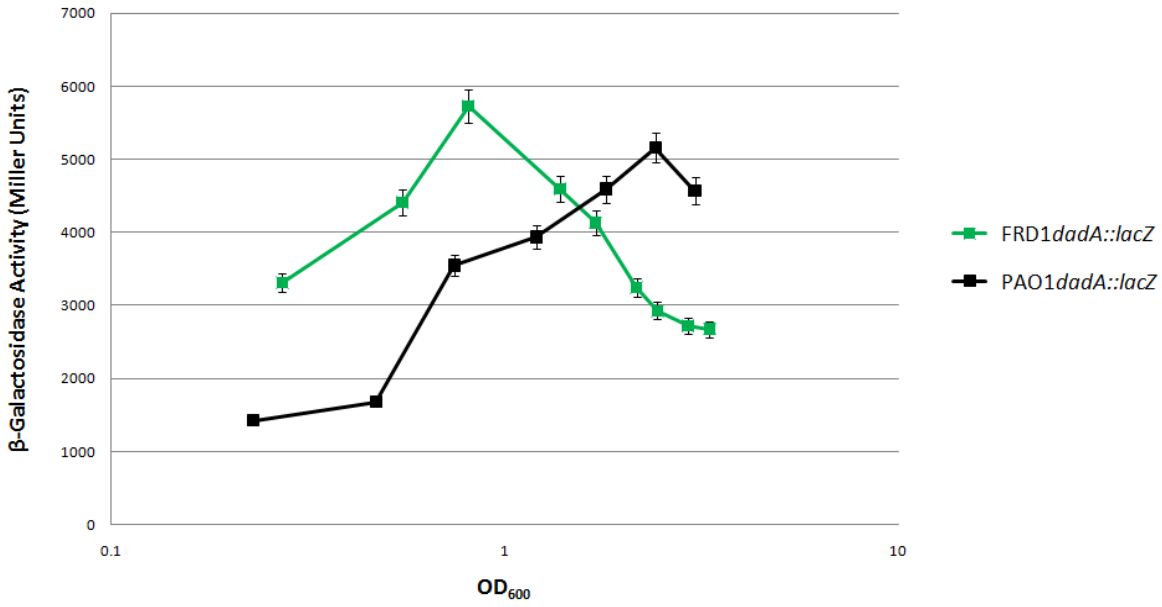

Figure 2.13. Expression of *dadA::lacZ* by FRD1 and PAO1. Values represent the average of three experiments with a standard error of 5%.

DadA is required for optimal virulence of FRD1 in an alfalfa model of infection. Disruption of *dadA* in FRD1 leads to a 43% decrease in virulence on alfalfa, whereas disruption of *dadX* has no effect (Figure 2.14). Interestingly, neither disruption of *dadA* nor *dadX* has any effect on the virulence of PAO1. Therefore, it is likely that reduced virulence of FRD1*ΔdadA* compared to the parental isolate may be a consequence of reduced virulence factor production. These findings are consistent with the hypothesis that chronic and acute isolates of *P. aeruginosa* utilize different strategies to cause infections.

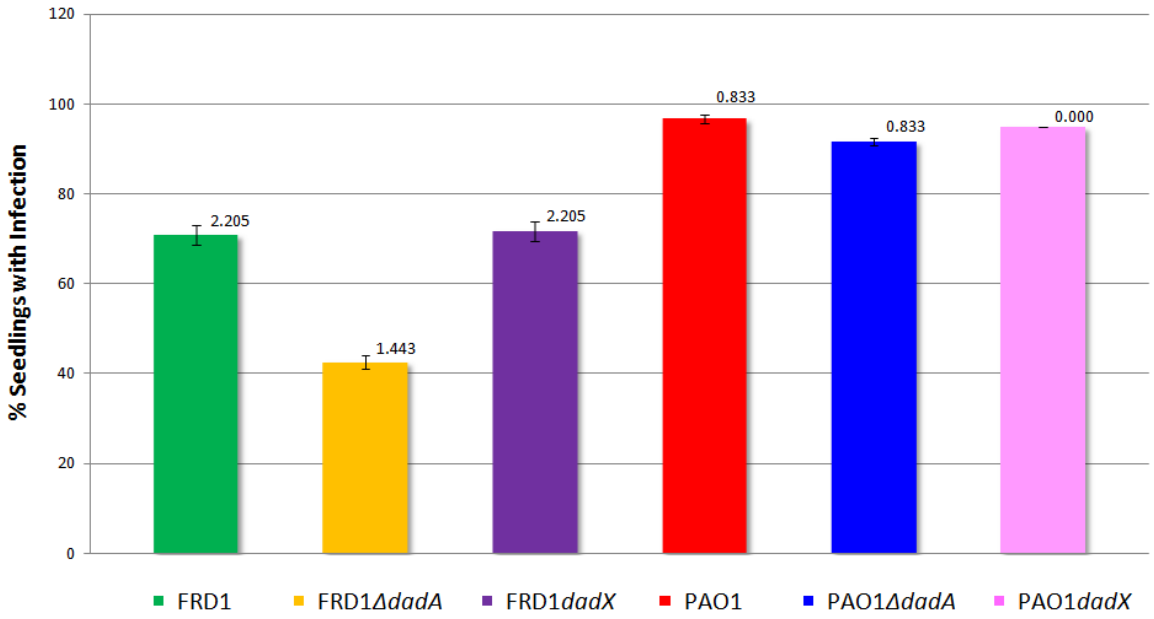

Figure 2.14. Rate of infection of alfalfa seedlings by FRD1, PAO1, and their derivatives. Values represent the average of three experiments with standard errors given for each isolate.

Quantitation of Alanine

Using Mass Spectrophotometry (MS), intracellular levels of alanine were quantitated in the parental and mutant derivatives of *P. aeruginosa* (data not shown). We observed no significant difference in alanine levels between FRD1 and FRD1*ΔdadA*, nor between PAO1 and its derivatives. However, we are unsure of the reliability of the MS used; therefore, we do not believe this data to be conclusive.

Ultra Performance Light Chromatography / Mass Spectrophotometry (UPLC/MS) was performed, with the use of a gradient solvent system, to differentiate between L- and D-alanine in the same samples, similar to the technique used by Kolodkin-Gal *et. al.* (2010). We hypothesize that D-alanine levels should be higher in the *ΔdadA* mutants compared to their parental strains, due to the cell's inability to convert D-alanine into pyruvate.

Similarly, L-alanine levels in the *dadX* mutants should be higher when compared to their parents, due to the cell's inability to convert L-alanine into D-alanine. However, the results gathered from this experiment were inconclusive, due to the fact that L- and D-alanine levels appeared to be greatly scattered between repeated experiments, and these levels did not correlate with the previous data gathered on total alanine concentrations (data not shown).

Discussion

In order to develop alternative antimicrobial treatments to combat chronic infection in CF patients, it is essential to better understand the persistence and adaptive mechanisms utilized by *P. aeruginosa*. In this study, the role of the *dad* operon in virulence factor production by *P. aeruginosa* was investigated. We focused on this operon, because it is upregulated in *P. aeruginosa* growing in the CF lung and is required for optimal HCN production by some CF adapted isolates. In addition, the *dad* operon is required for catabolism of alanine, which appears to be a major carbon source for *P. aeruginosa* within the CF lung.

This study illustrates that disruption of *dadA* in a CF adapted isolate of *P. aeruginosa* decreases production of several virulence factors, including pyocyanin, pyoverdine, rhamnolipid, alginate, and biofilm formation. Consistent with this observation, FRD1*Δdad*A mutants are reduced for virulence in an alfalfa model system of infection compared to the wild type. Conversely, loss of *dadA* has a minimal effect on virulence factor production by an acute isolate of *P. aeruginosa* and does not affect virulence of

this isolate in the plant model system of infection. Taken together, *dadA* appears to be critical for CF *P. aeruginosa* virulence, but not for acute *P. aeruginosa* virulence.

Our lab previously showed that disruption of *dadA* reduces HCN production in FRD1 [Hagins *et. al.*, 2009]. This result suggests that the D-amino acid dehydrogenase encoded by *dadA* is responsible for the conversion of glyoxylate to glycine, which can then be converted to HCN by HCN synthase. HCN has been detected in CF sputum, and it is associated with decreased pulmonary function [Ryall *et. al.*, 2008]. FRD1 produces increased levels of HCN compared to PAO1, due to increased transcription of the *hcn* gene cluster and increased glyoxylate concentrations [Carterson *et. al.*, 2004; Hagins *et. al.*, 2009]. Therefore, *dadA* plays key roles in the central metabolism and the production of essential virulence factors in CF isolates of *P. aeruginosa*.

The most likely explanation for the pleiotropic effect a disruption of *dadA* has on *P. aeruginosa* physiology is the toxic buildup of D-alanine caused by the alanine racemase encoded by *dadX*. Previous studies have shown that high intracellular levels of D-amino acids can be toxic to bacterial cells [Kolodkin-Gal *et. al.*, 2010]. However, even in the double FRD1*ΔdadAdadX* mutant, we observe reduced rhamnolipid and pyoverdine production, suggesting an alternative mechanism is responsible for these phenotypes.

An alternative hypothesis that attempts to explain the role of *dadA* in the overall physiology of *P. aeruginosa* is that *dadA* is responsible for redox balancing. During the conversion of glyoxylate to glycine via the D-amino acid dehydrogenase encoded by

dadA, NAD+ is reduced to NADH. Maintenance of intracellular levels of NAD+ and NADH are extremely important to the cell. If the balance between the two is not properly maintained, then the cell does not have enough reducing power to carry out its central metabolic pathways or biosynthetic reactions. FRD1*ΔdadA* could be utilizing an enzyme, such as glycine dehydrogenase, to maintain redox balance. On a broader scale, glycine dehydrogenase may be a common determinant for chronic persistence by bacterial pathogens. Genes encoding for this enzyme are upregulated in both *Mycobacterium* species and *Brucella abortus* during persistent infections [Hong *et. al.,* 2000; Lim *et. al.,* 1999; Muttucumaru *et. al.,* 2004]. However, the ultimate significance of glycine dehydrogenase during chronic persistence has not been determined.

The *dad* operon is regulated by intracellular L- and D-alanine levels via the effect it exerts on the transcriptional regulator leucine-responsive regulatory protein (Lrp). Lrp is a global regulator of the feast/famine regulatory protein family, and it has been shown to be a transcriptional regulator of the *dad* operon in the presence of L- or D-alanine in *P. aeruginosa* [Boulette *et. al.,* 2009]. In *E. coli,* Lrp either represses or induces transcription of the *dad* operon [Zhi *et. al.,* 1999]. If this type of regulation is present in *P. aeruginosa,* it would provide a plausible explanation for reduced expression of *dadA* during mid-log phase in FRD1. This would be contingent upon a buildup of D-alanine within the cell, which would activate Lrp to repress the *dad* operon. Similarly, high levels of alanine could induce Lrp to transcriptionally activate the *dad* operon, and high levels of alanine are known to be present in the CF lung environment.

However, recent evidence shows that the *dad* operon, specifically the *dadA* promoter, is transcriptionally activated by another protein, designated as DadR [He *et. al.*, 2011]. DadR is a transcriptional activator of the Lrp family of proteins [Brinkman *et. al.*, 2003; de los Rios *et. al.*, 2007], and it is induced by several different L-amino acids, the strongest inducer being L-alanine [He *et. al.*, 2011]. From this study, it is hypothesized that L-alanine serves as a major reservoir for carbon and nitrogen storage in *P. aeruginosa*, due to high levels of L-alanine that are generated by pyruvate-dependent transaminases during metabolism. This hypothesis is supported by the observation that the *dadA* promoter is constitutively induced in a *dadX* mutant, where intracellular levels of L-alanine are expected to accumulate without conversion into D-alanine, thereby inducing DadR to activate the *dadA* promoter [He *et. al.*, 2011].

Overall, an intact DadA has been shown to be beneficial for *P. aeruginosa* for virulence in the alfalfa model presented in this study, competitive fitness in a peritoneal rat lung model of infection [Boulette *et. al.*, 2009], and biofilm formation [Kolodkin-Gal *et. al.*, 2010]. This protein serves as a critical component of alanine catabolism in both chronic and acute isolates of *P. aeruginosa*, but it is upregulated in chronic isolates compared to the acute isolates. This information supports other evidence indicating that chronic isolates of *P. aeruginosa* utilize different metabolic strategies for persistence compared to the acute isolates, which renders these metabolic pathways attractive targets for therapeutic treatments. The significance of alanine catabolism in chronic *P. aeruginosa* infections is still poorly understood, but it does seem to be an integral part of *P. aeruginosa* virulence factor production. Specifically, our study suggests that *dadA* plays

a greater role in the production of several virulence factors by a chronic isolate of *P. aeruginosa*, compared to an acute isolate, and disruption of *dadA* leads to the buildup of toxic levels of D-alanine. It remains to be elucidated whether D-alanine levels, specifically, correlate with virulence factor production.

Interfering with bacterial metabolism *in vivo* can be a very useful strategy for future novel antibiotic therapies. Inactivation of specific carbon catabolic pathways in other bacterial pathogens has been shown to be efficacious in reducing the lethality of disease caused by those pathogens. For example, mutants of *Campylobacter jejuni*, a common poultry pathogen, that are unable to catabolize L-serine in the chicken gut are markedly reduced for colonization [Velayudhan *et. al.*, 2004]. In addition, mutants of the pulmonary pathogen *Legionella pneumophila* that lose their ability to utilize threonine cannot replicate within the alveolar macrophages of the lung [Sauer *et. al.*, 2005]. Therefore, central metabolism is not only critical for carbon and nitrogen source utilization, but also for the production of potential virulence factors present in many bacterial pathogens.

References

Boat, T. F., M. J. Welsh & A. L. Beaudet. 1989. *The Metabolic Basis of Inherited Disease*. New York, NY: McGraw Hill.

Boulette, M. L., P. J. Baynham, P. A. Jorth, I. Kukavica-Ibrulj, A. Longoria, K. Barrera, R. C. Levesque & M. Whiteley. 2009. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation *in vivo*. *J Bacteriol* **191:**6329-6334.

Brinkman, A. B., T. J. Ettema, W. M. de Vos & J. van der Oost. 2003. The Lrp family of transcriptional regulators. *Mol Microbiol* **48:**287-294.

Carterson, A. J., L. A. Morici, D. W. Jackson, A. Frisk, S. E. Lizewski, R. Jupiter, K. Simpson, D. A. Kunz, S. H. Davis, J. R. Schurr, D. J. Hassett, and M. J. Schurr. 2004. The transcriptional regulator AlgR controls cyanide production in *Pseudomonas aeruginosa*. *J Bacteriol* **186:**6837-6844.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. & Ehrlich, G. 2003. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* **112:**1466-1477.

de los Rios, S. & J. J. Perona. 2007. Structure of the *Escherichia coli* leucine-responsive regulatory protein Lrp reveals a novel octameric assembly. *J Mol Biol* **366:**1589-1602.

Driscoll, J. A., S. L. Brody & M. A. Kollef. 2007. The epidemiology, pathogenesis, and treatment of *Pseudomonas aeruginosa* infections. *Drugs* **67:**351-368.

Du Plessis, D. J. F. 2005. *Regulation of rhamnolipid biosynthesis in the Pseudomonas aeruginosa PAOI biofilm population*, MSc dissertation, University of Pretoria, Pretoria, viewed 18 April 2011 < http://upetd.up.ac.za/thesis/available/etd-08182008-085625/ >.

Essar, D. W., L. Eberly, A. Hadero & I. P. Crawford. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* **172:**884-900.

Fox, S. W., M. Fling, & C. N. Bollenback. 1944. Inhibition of bacterial growth by D-leucine. *J Biol Chem* **155:**465-468.

Hagins, J. M., R. Locy & L. Silo-Suh. 2009. Isocitrate lyase supplies precursors for hydrogen cyanide production in a Cystic Fibrosis isolate of *Pseudomonas aeruginosa*. *J Bacteriol* **191:**6335-6339.

He, W., C. Li & C. D. Lu. 2011. Regulation and characterization of the *dadRAX* locus for D-amino acid catabolism in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **193:**2107-2115.

Holloway, B. W., V. Krishnapillai & A. F. Morgan. 1979. Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* **43:**73-102.

Hong, P. C., Tsolis, R. M. & Ficht, T. A. 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. *Infect Immun* **68:**4102-4107.

Knutson, C. A. & A. Jeanes. 1968. A new modification of the carbazole analysis: application to heteropolysaccharides. *Anal Biochem* **24:**470-481.

Kolodkin-Gal, I., D. Romero, J. Clardy, R. Kolter, & R. Losick. 2010. D-amino acids trigger biofilm disassembly. *Science* **328:**627-629.

Lim, A., M. Eleuterio, B. Hutter, B. Murugasu-Oei & T. Dict. 1999. Oxygen depletion-induced dormancy in *Mycobacterium bovis* BCG. *J Bacteriol* **181:**2252-2256.

Lindsey, T. L., J. M. Hagins, P. A. Sokol & L. A. Silo-Suh. 2008. Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiol* **154:**1616-1627.

Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Muttucumaru, D. G., G. Roberts, J. Hinds, R. A. Stabler & T. Parish. 2004. Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis (Edinb)* **84:**239-246.

Nester, E. W., D. G. Anderson, C. E. Roberts Jr. & M. T. Nester. 2007. *Microbiology*. New York, NY: McGraw-Hill.

Ohman, D. E. & A. M. Chakrabarty. 1981. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect Immun* **33:**142-148.

Palmer, K. L., L. M. Mashburu, P. K. Singh & M. Whiteley. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187:**5267-5277.

Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins & L. Tsui. 1989. Identification of the Cystic Fibrosis gene: cloning and characterization of complementary DNA. *Science* **245:**1066-1073.

Ryall, B., J. C. Davies, R. Wilson, A. Shoemark, & H. D. Williams. 2008. *Pseudomonas aeruginosa*, cyanide accumulation and lung function in CF and non-CF bronchiectasis patients. *Eur Resp J* **32:**740-747.

Sanderson, K., L. Wescombe, S. M. Kirov, A. Champion & D. W. Reid. 2008. Bacterial cyanogenesis occurs in the cystic fibrosis lung. *Eur Respir J* **32:**329-333.

Sauer, J. D., M. A. Bachman & M. S. Swanson. 2005. The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *PNAS* **102:**9924–9929.

Schweizer, H. D. 1993. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. *Biotechniques* **15:**831-833.

Silo-Suh, L., S. J. Suh, P. A. Sokol & D. E. Ohman. 2002. A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with Cystic Fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis. *PNAS* **99:**15699-15704.

Son, M. S., W. J. Matthews, Jr., Y. Kang, D. T. Nguyen, & T. T. Hoang. 2007. *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of Cystic Fibrosis patients. *Infection and Immunity* **75:**5313-5324.

Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. West & D. E. Ohman. 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181:**3890-3897.

Suh, S. J., L. Silo-Suh & D. E. Ohman. 2004. Development of tools for the genetic manipulation of *Pseudomonas aeruginosa*. *J Micriobiol Methods* **58:**203-212.

Velayudhan, J., M. A. Jones, P. A. Barrow & D. J. Kelly. 2004. L-serine catabolism via an oxygen-labile L-serine dehydrogenase is essential for colonization of the avian gut by *Campylobacter jejuni*. *Infect Immun***72:**260-268.

Young, D., Hussell, T. & Dougan, G. 2002. Chronic bacterial infections: living with unwanted guests. *Nat Immunol* **3:**1026-1032.

Zhi, J., E. Mathew & M. Freundlich. 1999. Lrp binds to two regions in the *dadAX* promoter region of *Escherichia coli* to repress and activate transcription directly. *Mol Microbiol* **32:**29-40.

Chapter 3

Conclusions and Future Directions

Conclusions

Understanding the mechanisms utilized by microbial pathogens for maintaining chronic infections over time is essential for the development of improved antimicrobial therapies. Chronic pathogens, such as *Pseudomonas aeruginosa*, utilize different survival strategies for persistence compared to survival strategies utilized to initiate acute infections. *P. aeruginosa* undergoes several adaptations within the cystic fibrosis (CF) lung during chronic infection, including decreased production of virulence factors necessary for the initiation of an acute infection [Smith *et. al.*, 2006]. Chronic isolates of *P. aeruginosa* also cannot cause infection in typical animal models used to study acute infections, so an alternative model system of infection based on alfalfa seedlings was developed to identify chronic virulence factors utilized by CF *P. aeruginosa* isolates [Silo-Suh *et. al.*, 2002]. Using this model system, I determined that *dadA* is a virulence factor for the CF isolate, FRD1, but not the acute isolate, PAO1. I initiated the characterization of the role of *dadA* in *P. aeruginosa* virulence, and the results suggest that *dadA* plays a greater role in virulence factor production by FRD1, compared to PAO1. It remains to be proven that disruption of *dadA* leads to the buildup of high levels of D-alanine, which may be toxic to the bacterium.

Prior to my analysis, the role of *dadA* in *P. aeruginosa* virulence factor production had not been characterized. I first determined that *dadA* was required for optimal production of the virulence factors pyocyanin, pyoverdine, rhamnolipid, alginate, and biofilm formation by FRD1, whereas *dadA* was only required for optimal rhamnolipid production by PAO1. In addition, expression of *dadA* was upregulated during logarithmic growth in FRD1 compared to PAO1, and disruption of *dadA* caused a two-fold decrease in virulence in an alfalfa seedling model of infection by FRD1, but not PAO1. These results suggest that *dadA* plays a much larger role in the physiology of chronic isolates of *P. aeruginosa* compared to the acute isolates. There are two possible benefits for upregulation of *dadA* in CF isolates of *P. aeruginosa*, including catabolism of alanine present in the CF lung and optimal production of many different virulence factors for protection and a competitive advantage over other microorganisms.

Amino acids are found in copious amounts in CF sputum, and many studies indicate that they are utilized as a carbon source by *P. aeruginosa* [Meyer *et. al.*, 2000; Palmer *et. al.*, 2005; Son *et. al.*, 2007]. Elevated levels of alanine have been documented in the CF lung, so it is intuitive that a selective advantage would be conferred on those chronic isolates of *P. aeruginosa* that can catabolize alanine efficiently over an extended length of time. My study is not the first to suggest that central metabolic pathways impact the virulence of *P. aeruginosa* and other pathogens. Lindsey *et. al.* (2008) showed that the glyoxylate pathway was required for optimal production of alginate and hydrogen cyanide (HCN) by a chronic CF isolate of *P. aeruginosa*. In addition, mutants of the pulmonary pathogen *Legionella pneumophila* that lose their ability to catabolize

threonine cannot replicate within the alveolar macrophages of the lung [Sauer *et. al.*, 2005]. However, this study does support the novel idea that central metabolic pathways are altered in *P. aeruginosa* during chronic infection, and that these alterations affect virulence factor production. Therefore, this study has much broader implications for understanding how chronic infections are established and maintained by chronic infecting bacterial pathogens.

Future Directions

The goal of my study was to initiate the characterization of the role of *dadA* in the virulence and physiology of a chronic CF isolate of *P. aeruginosa*. I determined that disruption of *dadA* has a pleiotropic effect on virulence factor production by the CF isolate of *P. aeruginosa*, and that this effect may be caused by a buildup of toxic D-alanine concentrations. However, reduced virulence factor production by the double FRD1*ΔdadAdadX* mutants suggests another mechanism plays a role in reduced virulence factor production by these derivatives. Therefore, the role of *dadA* in redox balancing should be investigated as a possible mechanism that impacts virulence factor production in FRD1.

In addition, regulation of the *dad* operon in FRD1 should be investigated. My study showed that regulation of the *dadA* operon is slightly altered in FRD1 compared to PAO1. DadR is a transcriptional activator of the *dadA* promoter, and it is most highly induced by intracellular levels of L-alanine in PAO1 [He *et. al.*, 2011]. Similarly, the leucine-responsive regulatory protein (Lrp) is a major regulator of the *dad* operon in

PAO1 [Boulette *et. al.*, 2009]. However, the role of DadR and Lrp in FRD1 is yet to be determined. Also, the exact mechanism by which Lrp activates or represses the *dad* operon should be identified, including if intracellular levels of D-alanine play any role in that regulatory mechanism. Additional studies to identify other regulators of *dadA* in *P. aeruginosa* are also needed. For this proposed study, PAO1 carrying the *dadA::lacZ* fusion can be subjected to transposon mutagenesis, after which mutants with altered β-galactosidase activity can be identified and characterized. If DadR or Lrp is not responsible for altered *dadA* in FRD1, then another regulator is likely altered in FRD1, which can be identified from this study.

Alternatively, FRD1 may have acquired an additional plasmid or pathogenicity island while in the CF lung, and this extra DNA may encode for a transcriptional activator or repressor of the *dad* operon. To date, three *P. aeruginosa* pathogenicity islands have been identified, including *P. aeruginosa* genomic island 1 (PAGI-1), PAGI-2, and PAGI-3 [Finnan *et. al.*, 2004]. In a study performed by Liang *et. al.* (2001), 85% of the clinical isolates tested contained PAGI-1. Such a find may explain the difference observed in the role of *dadA* in virulence factor production by FRD1, compared to PAO1.

A major goal of this study was to identify novel targets in chronic isolates of *P. aeruginosa* for improved antimicrobial therapies for CF patients. This study demonstrates that *dadA* is a critical component of virulence factor production by some *P. aeruginosa* isolates adapted to the CF lung. Additional studies of the metabolic pathways utilized by *P. aeruginosa* in the CF lung are essential to enhance our understanding of the

physiology and virulence of this opportunistic pathogen during its chronic persistence, and to possibly identify novel antimicrobial targets for victims of CF.

References

Boulette, M. L., P. J. Baynham, P. A. Jorth, I. Kukavica-Ibrulj, A. Longoria, K. Barrera, R. C. Levesque & M. Whiteley. 2009. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation *in vivo*. *J Bacteriol* **191:**6329-6334.

Finnan, S., J. P. Morrissey, F. O'Gara & E. F. Boyd. 2004. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. *J Clin Microbiol* **42:**5783-5792.

He, W., C. Li & C. D. Lu. 2011. Regulation and characterization of the *dadRAX* locus for D-amino acid catabolism in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **193:**2107-2115.

Liang, X., X. Q. Pham, M. V. Olson & S. Lory. 2001. Identification of a genomic island present in the majority of pathogenic isolates of *Pseudomonas aeruginosa. J Bacteriol* **183:**843-853.

Lindsey, T. L., J. M. Hagins, P. A. Sokol & L. A. Silo-Suh. 2008. Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiol* **154:**1616-1627.

Meyer, K. C., A. Sharma, R. Brown, M. Weatherly, F. R. Moya, J. Lewandoski & J. J. Zimmerman. 2000. Function and composition of pulmonary surfactant and surfactant-derived fatty acid profiles are altered in young adults with cystic fibrosis, *Chest* **118:**164-174.

Palmer, K. L., L. M. Mashburu, P. K. Singh & M. Whiteley. 2005. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187:**5267-5277.

Sauer, J. D., M. A. Bachman & M. S. Swanson. 2005. The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *PNAS* **102:**9924–9929.

Silo-Suh, L., S. J. Suh, P. A. Sokol & D. E. Ohman. 2002. A simple alfalfa seedling infection model for *Pseudomonas aeruginosa* strains associated with Cystic Fibrosis shows AlgT (sigma-22) and RhlR contribute to pathogenesis. *PNAS* **99:**15699-15704.

Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman, D. A. D'Argenlo, S. I. Miller, B. W. Ramsey, D. P. Speert, S. M. Moskowitz, J. L. Burns, R. Kaul & M. V. Olson. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *PNAS* **103:**8487-8492.

Son, M. S., W. J. Matthews, Jr., Y. Kang, D. T. Nguyen & T. T. Hoang. 2007. *In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of Cystic Fibrosis patients. *Infect Immun* **75:**5313-5324.