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Novel Role of Pseudomonas Aeruginosa LptD Operon

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Recommended Citation

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

NOVEL ROLE OF *PSEUDOMONAS AERUGINOSA LPTD* OPERON

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Sundar Pandey

2018

To: Dean Michael R. Heithaus
      College of Arts, Sciences and Education

This dissertation, written by Sundar Pandey, and entitled Novel Role of *Pseudomonas aeruginosa lptD* Operon, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2018

ACKNOWLEDGMENTS

ABSTRACT OF THE DISSERTATION

NOVEL ROLE OF *PSEUDOMONAS AERUGINOSA LPTD* OPERON

by

Sundar Pandey

Florida International University, 2018

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Professor Kalai Mathee, Major Professor

Pseudomonas aeruginosa is an opportunistic pathogen that infects cystic fibrosis (CF) patients contributing to their high morbidity and mortality. *P. aeruginosa* undergoes a phenotypic conversion in the CF lung, from nonmucoid to mucoid, by constitutively producing a polysaccharide called alginate. These mucoid strains often revert to nonmucoid *in vitro* due to second-site suppressor mutations. We hypothesized that mapping these mutations would lead to the identification of novel genes involved in alginate production. In a previous study, a mucoid strain, PDO300 (PAO*mucA22*), was used to isolate suppressors of alginate phenotype (*sap*). One of the uncharacterized nonmucoid revertants, *sap27*, is the subject of this study. The mucoid phenotype in *sap27* was restored by pMO012217 from a minimal tiling path cosmid library. The cosmid pMO012217 harbors 18 *P. aeruginosa* open reading frames (ORF). The cosmid was mutagenized with a transposon to map the contributing gene. It was mapped to *lptD* (PA0595) encoding lipopolysaccharide transport protein. *E. coli* LptD transports lipopolysaccharide to the outer leaflet of the outer membrane. The Alg$^+$ phenotype was restored upon complementation with *P. aeruginosa lptD* alone, suggesting that *sap27* likely harbor a chromosomal mutation in *lptD*. Sequencing analysis of *sap27* showed the

presence of a mutation not in *lptD* but in *algO*, which encodes a periplasmic protease protein. This suggests LptD is able to bypass an *algO* mutation by positively regulating alginate production. The *lptD* is a part of a three-gene operon *lptD-surA-pdxA*. SurA is an essential protein for survival in starvation and a major chaperone protein for all outer membrane proteins and PdxA is a NAD-dependent dehydrogenase and is involved in the vitamin B_6 biosynthetic pathway. Pyridoxal 5'-phosphate (PLP) is the active form of vitamin B_6. *P. aeruginosa* grown in a media supplemented with PLP increased production of pyocyanin, a virulence factor. The PLP and aromatic amino acids are synthesized from a common precursor chorismic acid. We demonstrated an increase in pyocyanin production when the bacteria were cultured supplemented by the aromatic amino acids phenylalanine. We concluded that the *lptD* operon plays a role in the *P. aeruginosa* virulence by regulating alginate and pyocyanin production.

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LIST OF SYMBOLS

Symbol	Term/unit
α	Alpha
β	Beta
σ	Sigma
Δ	Delta (deletion)
°C	Degree Celsius
μg	Microgram
μL	Microliter
%	Percentage

LIST OF ABBREVIATIONS/ACRONYMS

Term	Abbreviation/Acronyms
Adenosine triphosphate	ATP
Alginate	Alg
Base pair	bp
Basic local alignment search tool	BLAST
bis-(3'-5')-cyclic-guanosine monophosphate	c-di-GMP
Community-acquired pneumonia	CAP
Cystic Fibrosis	CF
Cystic Fibrosis Transmembrane Conductance Regulator gene	*cftr*
Cystic Fibrosis Transmembrane Conductance Regulator protein	CFTR
Deoxyribonucleic acid	DNA
Ethylenediaminetetraacetic acid	EDTA
Extracytoplasmic function	ECF
Forced expiratory volume	FEV
Gravitational force	*g*
Hour	hr
Luria Bertani	LB
Lipopolysaccharide	LPS
Lipopolysaccharide transport protein D gene	*lptD*
Lipopolysaccharide transport protein D protein	LptD
Milliliter	mL
Mucoid	Muc

Sodium chloride	NaCl
Outer membrane	OM
Open reading frame	ORF
Pseudomonas isolation agar	PIA
Pyridoxal 5'-phosphate	PLP
Polypeptide transport-associated	POTRA
Polymerase chain reaction	PCR
Pseudomonas quinolone signaling	PQS
Quantitative PCR	qPCR
Quorum sensing	QS
Regulated intramembrane proteolysis	RIP
Regulator of sigma factor E	Rse
RNA polymerase sigma-E factor	RpoE
Transmembrane helix prediction hidden Markov model	TMHMM

CHAPTER 1

Posttranslational Regulation of Antisigma Factors of RpoE: A Comparison Between the

Escherichia coli and *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa, a rod-shaped Gram-negative bacterium, is an opportunistic human pathogen associated with skin and soft tissue infections, sepsis, and nosocomial infection. The bacterium can establish chronic infection and causes demise of people with cystic fibrosis. One of the virulence factors of the *P. aeruginosa* is an exopolysaccharide alginate. Alginate is synthesized in response to environmental conditions. The biosynthesis of alginate in *P. aeruginosa* is a highly regulated process where an extracytoplasmic sigma factor AlgT/U plays a key role. The increased alginate producing phenotype of *P. aeruginosa* is known as mucoid phenotype. The mechanism of mucoid phenotype in *P. aeruginosa* is extensively studied where non-mucoid to mucoid transition is the result of the mutation of inner membrane protein MucA. The degradation of MucA is a sequential process which is known as regulated intramembrane proteolysis (RIP). The RIP mechanism is well conserved in Gram-negative bacteria. This chapter describes the RIP cascade in *P. aeruginosa* and compares it with *Escherichia coli*.

Pseudomonas aeruginosa

In 1882, Carle Gessard isolated an organism that causes a blue-green coloration of wound dressings and named the microbe as *Bacillus pyocyaneus*, which is now known as *P. aeruginosa* (Gessard, 1882; Webster and Bernheim, 1936). The *P. aeruginosa* is a Gram-negative, rod-shaped (Figure 1.1) measuring 0.5-0.8 µm by 1.5-3.0 µm, typically with monotrichous flagellum and multiple pili for locomotion.

P. aeruginosa is a biochemically versatile microorganism that can adapt to different ecological niches ranging from soil, water, plants and humans (Holloway, 1955; Rahme *et al.*, 1995). In hospitals, the bacterium can colonize sinks, humidifiers, respirators and sometimes it can be found on the hands of medical personnel (Aiello *et al.*, 2003; Hota *et al.*, 2009; Jadhav *et al.*, 2013). *P. aeruginosa* is an aerobic gammaproteobacterium but it can grow in oxygen-limited conditions by using nitrate or arginine as the terminal electron
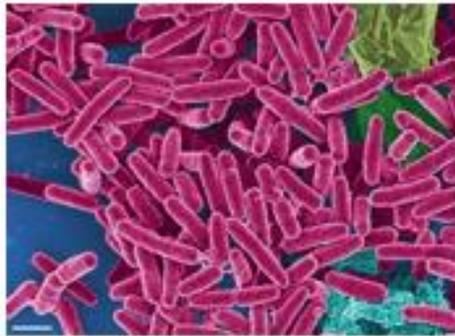
Figure 1.1. An electron micrograph of *P. aeruginosa.* (Courtesy: http://www.waterscans.rs/)

acceptor (van Hartingsveldt and Stouthamer, 1973). *P. aeruginosa* does not usually establish infection in normal humans but the bacterium can become an opportunistic pathogen and establish disease condition in patients with impaired host defense. It can cause both acute and chronic infection.

Acute infection by *P. aeruginosa*

i. **Acute pneumonia in hospitalized and in particularly mechanically ventilated patients**

    P. aeruginosa along with *Staphylococcus aureus* are the most common bacteria that cause ventilator-associated pneumonia with a high mortality rate of 13.5%

(Rello *et al.*, 1996). *P. aeruginosa* can contribute to community-acquired, hospital-acquired, and healthcare-associated pneumonia (Fujii *et al.*, 2014). *P. aeruginosa* is associated with 92% of community-acquired pneumonia (CAP) with a mortality rate of 33% (Hatchette *et al.*, 2000). In the case of CAP, younger patients recover better than older patients. In fatal cases, patients die because of septic shock due to organ failure (Huhulescu *et al.*, 2011; Maharaj *et al.*, 2017). In cases of recovery, the complications could still arise resulting in parenchymal scarring and recrudescence, which require repeated antibiotic treatment (Maharaj *et al.*, 2017).

ii.   **Sepsis in patients with extensive burns**

Fever diarrhea, pneumonia, skin lesion, and shock are the most common symptom of sepsis caused by *P. aeruginosa* (Grisaru-Soen *et al.*, 2000; Huang *et al.*, 2002). The mortality rate of *P. aeruginosa* associated sepsis in children ranges from 20% - 50% (Huang *et al.*, 2002).

iii.   **Corneal infection in individuals wearing contact lenses**

Gram-negative bacteria and fungi are the dominant pathogens of corneal infection in tropical or sub-tropical climates (Lichtinger *et al.*, 2012; Stapleton *et al.*, 2012). *P. aeruginosa* is the most common organism in contact lens-related disease (Stapleton *et al.*, 2012). It has been proposed that the lens, storage case, and ocular environment provide a favorable niche for the survival of *P. aeruginosa* (Stapleton *et al.*, 2012). It has been shown that *exoU* expressing phenotype is highly selected in contact-lens related corneal keratitis compared to non-contact lens-related keratitis (Choy *et al.*, 2008).

iv.   Urinary tract infection in patients with indwelling foley catheters

P. aeruginosa causes biofilm-mediated infections that includes catheter-associated urinary tract infections, ventilator-associated pneumonia, infections related to mechanical heart valves, stents, grafts, and sutures (Bharathi *et al.*, 2014; Bouchart *et al.*, 1997; Chan *et al.*, 2018; Dapas *et al.*, 2016; Edmiston *et al.*, 2013; Olejnickova *et al.*, 2014). *P. aeruginosa* is responsible for 12% of all nosocomial urinary tract infections (Cole *et al.*, 2014).

v.   Bacteremia and sepsis in immunocompromised patients, particularly neutropenic patients receiving cytotoxic therapies

A positive correlation has been shown between *P. aeruginosa* and neutropenic patients (Lee *et al.*, 2011; Lucas *et al.*, 2017; Righi *et al.*, 2017).

vi.   Post-surgical wound infection

Post-surgical wound infection occurs after surgical operation, may originate during the operation or may occur after the operation from sources in the ward or as a result of some complications (Mousa, 1997). The increase rate of *P. aeruginosa* infection has been associated in post-operative wound infections (McNeil *et al.*, 2001; Ranjan *et al.*, 2010)

vii.   Burn wound infection

P. aeruginosa is the most commonly isolated from burn wounds where the organism can cause sepsis (Glik *et al.*, 2017; Hsueh *et al.*, 1998).

Chronic infection by *P. aeruginosa*

Chronic infection is defined as an infection that persists in spite of therapy, and the host's immune and inflammatory response (Pressler *et al.*, 2011b). The common chronic lung infections caused by *P. aeruginosa* are in the lung of patients with chronic-bronchiectasis, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) (Finch *et al.*, 2015; Hilliam *et al.*, 2017; Lieberman and Lieberman, 2003; McAvoy *et al.*, 1989). CF and COPD have different causal origins, but they share the same relevant symptoms and bacterial colonization. The difference between CF and COPD patients is that the *P. aeruginosa* infections occur earlier in the case of the CF (Martinez-Solano *et al.*, 2008).

According to Leed's criteria, chronic *P. aeruginosa* infection in CF patients is defined when >50 % of sputum cultures are positive for *P. aeruginosa* in the preceding 12 months of routine checkups (Lee *et al.*, 2003). But the Leed's criteria are difficult to implement especially in young children because of the requirement of sputum samples and follow-up time. To solve the issue of sputum culture of *P. aeruginosa* it was hypothesized that *P. aeruginosa* chronic infection is associated with the dominance of the bacterium in the lungs while intermittently infected patients only showed low abundance of the pathogen. The qPCR technique can be used as good diagnostic tool to discriminate between intermittent and chronic infection (Boutin *et al.*, 2018). It has been accepted that $10^{3.4}$ colony-forming units (CFU) of *P. aeruginosa* as a chronic infection in the lung of CF patients and qPCR value for the bacterium is adjusted according to the CFU (Boutin *et al.*, 2018).

Cystic Fibrosis

Cystic Fibrosis (CF) is a debilitating autosomal recessive genetic disease that affects multiple organs resulting in exocrine pancreatic insufficiency in diabetes, malnutrition and impaired growth associated with gastrointestinal malabsorption, male infertility from congenital malformation of the vas deferens, and sinusitis (Coste *et al.*, 1995; Dray *et al.*, 2005; Yu *et al.*, 2012). CF is distributed worldwide (Farrell, 2008b; Prasad *et al.*, 2010; Silva Filho *et al.*, 2016; Stewart and Pepper, 2016). On the basis of live births, the highest incidence of the CF disease is found in Brazil especially in Euro Brazilians (1/1600) and the lowest incidence is found in the Japanese population (1/100,000-350,000) (Faucz *et al.*, 2010; Yamashiro *et al.*, 1997). The prevalence of the CF in the United States is one in 3500 and in the European Union is one in 2500 (Farrell, 2008a). Currently, 70,000 CF patients have been registered worldwide and 30,000 in the United States (2016).

Sweat glands microdissected from the skin of CF patients showed a low permeability of chloride ions leading to poor reabsorption of NaCl resulting in high NaCl concentrations in the sweat (Quinton, 1983). The inability of the sweat duct to re-absorb NaCl led to the identification of a defective locus on chromosome seven (Knowlton *et al.*, 1985; Tsui *et al.*, 1985; Wainwright *et al.*, 1985). Using the chromosome walking and jumping technique, approximately 250,000 base pairs of genomic DNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride ion channel, was identified (Riordan *et al.*, 1989; Rommens *et al.*, 1989). Expression of the *cftr* gene in recombinant baculovirus led to the confirmation of the role of CFTR in anion channel regulation (Kartner *et al.*, 1991).

The lack of functional CFTR at the apical membrane of a secretory epithelial cell results in defective Cl^- and bicarbonate secretion, increase Na^+ absorption and mucus secretion that leads to dehydration and acidification of the airway surface liquid (Chen *et al.*, 2010; Darch *et al.*, 2015; Tang *et al.*, 2012). These impaired mucociliary clearance that provokes recurrent infection and uncontrolled inflammation resulting in lung damage, the primary cause of morbidity and mortality of people with CF (Boucher, 2007; Stoltz *et al.*, 2015).

The CFTR protein is composed of 1,480 amino acids (aa) and 2026 different *cftr* mutations are classified (Riordan *et al.*, 1989). Classification of *cftr* mutation on the basis of therapeutic outcome is recently proposed (De Boeck and Amaral, 2016; Marson *et al.*, 2016). Based on clinical features classes I, II, and III are classified as more-severe disease whereas classes IV, V, and VI are grouped as less-severe (Marson *et al.*, 2016). But for simplicity, this dissertation adopts the *cftr* mutation based on the biological defect (Figure 1.2).(Veit *et al.*, 2016).

1. The Class I defect results from frameshift, splicing, or non-sense mutations that introduce premature termination codons which cause the expression of CFTR to be severely reduced or absent.

2. Class II defects encompass mutations that lead to misfolding and/or premature degradation in the endoplasmic reticulum. The defective protein biogenesis causes a highly reduced number of CFTR molecules on the secretory epithelial cell surface (Bombieri *et al.*, 2015).

3. Class III mutants have abnormal gating that is characterized by reduced open probability (CFF, 2011; Marson *et al.*, 2016; Veit *et al.*, 2016).

4. Class IV mutant of CFTR has altered channel conductance because of impedance in the ion conduction pore ultimately leading to a reduced unitary conductance (CFF, 2011; Hammerle *et al.*, 2001; Marson *et al.*, 2016; Sheppard *et al.*, 1993; Veit *et al.*, 2016).
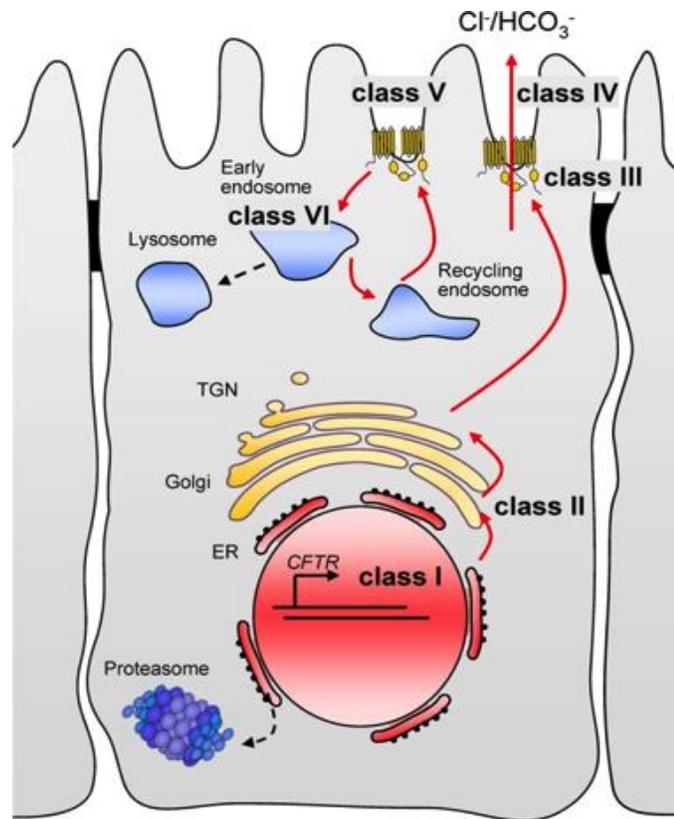
Figure 1.2. Classification of CF mutations based on their cellular phenotype. Class I: protein synthesis defect; class ll: maturation defect; class lll: gating defect; class IV: conductance defect; class V: reduced quantity; and class Vl: reduced stabitlity. ER, endoplasmic reticulum; TGN, trans-Golgi network. Courtesy (Veit *et al.*, 2016)

5. Class V type CFTR has altered promoter or splicing abnormalities resulting in protein abundance that do not change the conformation (CFF, 2011; Haardt *et al.*, 1999; Highsmith *et al.*, 1997; Marson *et al.*, 2016; Silvis *et al.*, 2003; Veit *et al.*, 2016; Zielenski and Tsui, 1995).

6. Class Vl a mutation, which reduces the conformational stability of the CFTR in post-endoplasmic reticulum and/or at the plasma membrane (Sheppard *et al.*, 1993:Hammerle, 2001 #592). In addition, class VI type mutation generates additional internalization signals. Because of defective *cftr* gene, dehydrated mucus layer builds up in an epithelial layer that impairs mucociliary clearance that is a characteristic feature of patients with CF (Cohen and Prince, 2012). The lack of mucociliary clearance causes the gradual thickening of mucus, which eventually leads to the mucus plugs formation. The mucus plugs not only
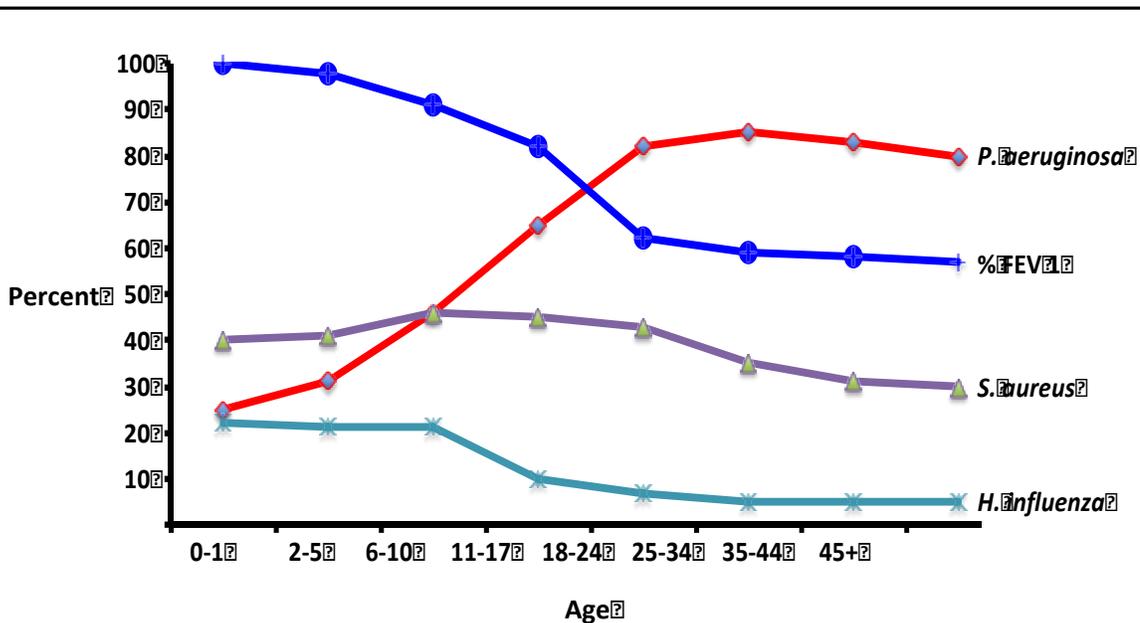
Figure 1.3. The most common microbes found in lung of CF. Relationship shows inverse relationship between mucoid *P. aeruginosa* and lung function predicted by forced expiratory volume 1 (FEV1). FEV1 indicates severity in lung function. Courtesy (Pier, 2000).

obstruct the airways but also create oxygen gradient from hypoxic to anoxic regions (Worlitzsch *et al.*, 2002). The environment is made further anoxic because of the growth of *P. aeruginosa* where the bacterium uses nitrates and nitrites as terminal oxygen accepter (Worlitzsch *et al.*, 2002). Furthermore, *P. aeruginosa* decreases the apical expression of *cftr* gene (MacEachran *et al.*, 2007; Rubino *et al.*, 2014; Trinh *et al.*, 2015). Oxygen availability, inflammation, and microbial communities are heterogeneous in CF lung that affects the host microbial interaction and eventually the disease progression.

Microbial communities in CF airways

The *Streptococcus*, *Veillonella*, and *Prevotella* are the dominant genera in a healthy lung (Li *et al.*, 2016). But, bacterial species like *Haemophilus influenzae*, *S. aureus*, *P. aeruginosa, Burkholderia cepacia, Alcaligenes xylosoxidans, Klebsiella spp*., some pathogenic virus like respiratory syncytial virus and adenoviruses, and the fungi *Aspergillus* and *Candida* species are commonly found in CF airways (Haase *et al.*, 1991; Lambiase *et al.*, 2006; Nixon *et al.*, 2001; Scoffone *et al.*, 2017; van Ewijk *et al.*, 2008; Willger *et al.*, 2014). In CF pediatric samples, *Streptococcus*, *Haemophilus*, *Staphylococcus,* and *Achromobacter* predominant whereas and in adult *Pseudomonas*, *Burkholderia*, *Streptococcus*, *Haemophilus,* and *Staphylococcus* are the dominant genus. *P. aeruginosa* becomes dominant pathogen as the increase in the age of people with CF (Figure 1.3). *Rothia*, *Preovtella*, *Gemella*, *Actinomyces,* and *Veillonella* are prevalent in both pediatric and adult samples (Coburn *et al.*, 2015). One of the methods that is used to assess the lung disease status of the individual with cystic fibrosis is forced expiratory

volume in one sec (FEV1). FEV1 is the quantity of air that can be forcibly exhaled from the lungs in first one second. The ratio of FEV1 of CF individual and FEV1 of reference population is %FEV1 that indicates the health status of a lung of people with CF (Wang *et al.*, 1993). It was observed that the FEV1 ratio of individual with CF is lower indicating the decline function of the lung (Pier, 2000)(Figure 1.3).
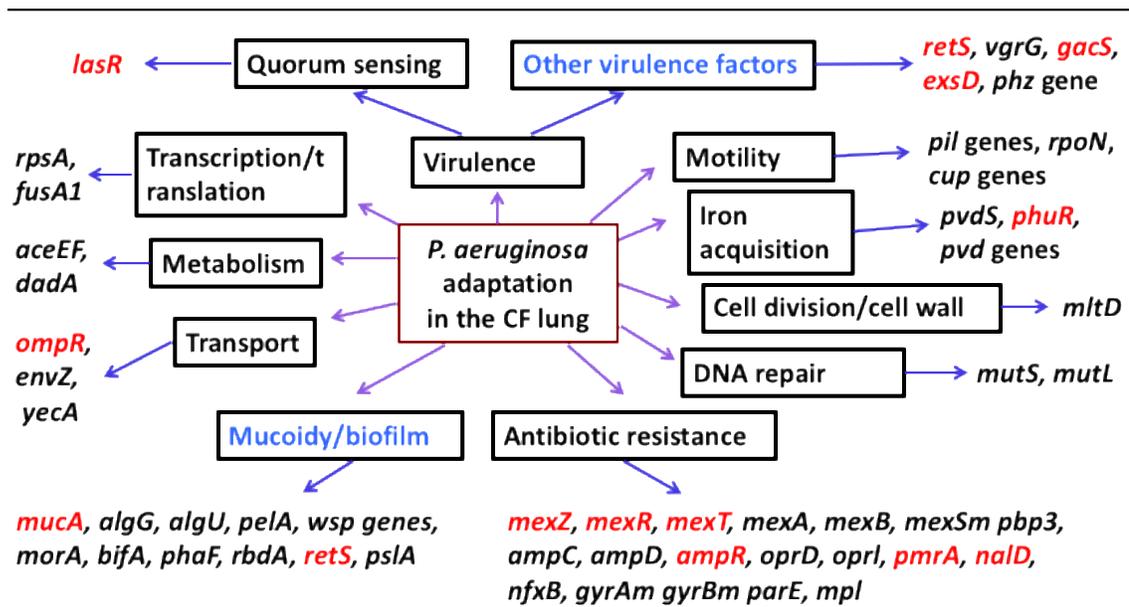
Figure 1.4. Common mutations in *P. aeruginosa* CF isolates. Genes encoding regulatory proteins are highlighted in red. The phenotype that is of interest of this dissertation is shown in box with blue color. Courtesy (Winstanley *et al.*, 2016)

Genotypic change in *P. aeruginosa* in CF lung

P. aeruginosa, during infection of CF airways, undergo evolution and adaptation (Figure 1.4) (Ciofu *et al.*, 2010). The adaptive phenotypes include increased alginate production, loss of quorum-sensing, motility, effector proteins of the type lll secretion system, and loss of the O-antigen component of the lipopolysaccharide, reduce virulence, reduced capacity of the non-mucoid phenotype for *in vitro* biofilm formation (Cigana *et*

al., 2016; Ciofu *et al.*, 2010; Cullen *et al.*, 2015; Oliver *et al.*, 2000; Smith *et al.*, 2006; Warren *et al.*, 2011; Williams *et al.*, 2018; Workentine *et al.*, 2013). It is important to understand that both environmentally-acquired strains and patient-to-patient transmissible strains of *P. aeruginosa* appear to have a similar genomic mutation that provides an adaptive advantage in the lung of CF patient (Fothergill *et al.*, 2012; Jelsbak *et al.*, 2007; Williams *et al.*, 2018). One study showed that the genotypic and phenotypic diversity could also be the result of recombination and not always the result of the spontaneous mutations occurs in the *P. aeruginosa* (Darch *et al.*, 2015).

One of the genotypic and phenotypic changes that is of interest of this dissertation is an emergence of mucoid colonies caused by increased production of alginate (Carlson and Matthews, 1966). Along with alginate two other polysaccharide (Pel and Psl) play a role in the development and structural maintenance of biofilm of *P. aeruginosa* (Colvin *et al.*, 2012; Stapper *et al.*, 2004).

β-D-ManUA-(1->4)-3-O-acetyl-β-D-ManUA-(1->4)-2-O-acetyl-β-D-ManUA-(1->4)-β-L-GulUA-(1->4)-2-O-acetyl-β-D-ManUA
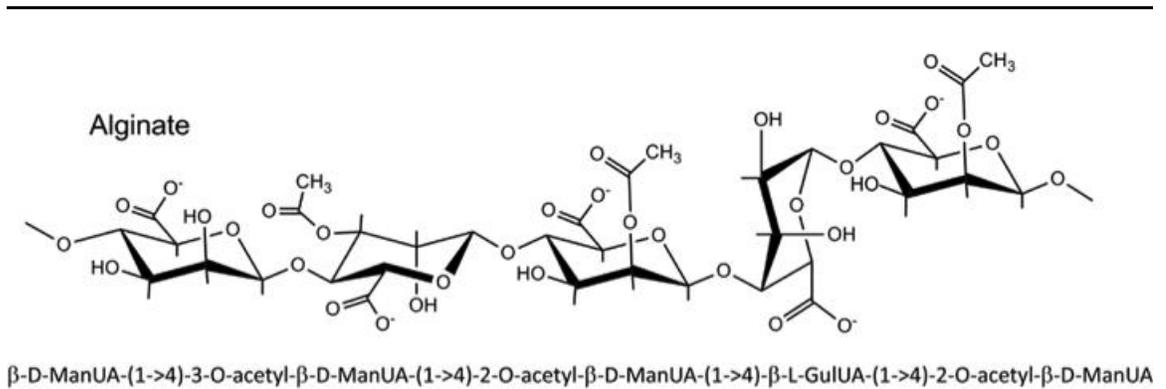
Figure 1.5. Alginate composition. The O-acetylation occurs in mannuronic acid. The ß-1,4-glycosidic bond link the guluronic and mannuronic acid. Courtesy (Franklin *et al.*, 2011)

Alginate

Alginate is an exopolysaccharide, meaning it is not covalently linked to the bacteria, composed of non-repeating unit of O-acetylated β-D-mannuronic (M) acid residues randomly interspersed with its C5' epimer α-L-guluronic acid (G) residues by 1,4-glycosidic bonds (Figure 1.5)(Chitnis and Ohman, 1990; Schurks *et al.*, 2002; Skjakbraek *et al.*, 1986). The alginate of *P. aeruginosa* is composed of random blocks of MM and MG residues. In *P. aeruginosa* FRD1, a sputum mucoid isolate from a CF patient, the molar ratio of mannuronic acid and guluronic acid was found to be 2:1 (Schurks *et al.*, 2002). Acetylation of alginate promotes the cell aggregation, confers elasticity, and provides resistance to the opsonic killing of *P. aeruginosa* (Moradali *et al.*, 2015; Pier *et al.*, 2001; Wloka *et al.*, 2004). Alginate is not required but it affects the formation of biofilm (Hentzer *et al.*, 2001; Stapper *et al.*, 2004). Antibiotics, high osmolarity, dehydration, and release of hydrogen peroxide by polymorphonuclear cells have all been shown to promote alginate production (Berry *et al.*, 1989; Devault *et al.*, 1990; Govan and Fyfe, 1978; Mathee *et al.*, 1999). Alginate protects *P. aeruginosa* from an oxidative burst from the immune system; provides an advantage for adherence to an extracellular material, antibiotic tobramycin, and protection from phagocytes (Hentzer *et al.*, 2001; Leid *et al.*, 2005; Tielen *et al.*, 2005). It has been shown that alginate-deficient *P. aeruginosa* develops biofilm but with a reduced number of viable cells compared to alginate-producing *P. aeruginosa* (Ghafoor *et al.*, 2011). Free radicals produced by macrophages and neutrophils cannot depolymerize nor is the alginate degraded during phagocytosis suggesting alginate is helpful for bacterial survival in CF lung (Simpson *et al.*, 1989). It is interesting that the mucoid *P. aeruginosa* isolated from chronic CF adults is less resistant than non-mucoid isolated against

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antibiotics colistin, tobramycin, meropenem, and ciprofloxacin suggesting the presence of a secondary mutation.

The high amount of immunoglobulin G (IgG) is mounted against the alginate in chronically affected CF patients but such IgG is ineffective to clear *P. aeruginosa* infection (Mauch *et al.*, 2017; Meluleni *et al.*, 1995).

Biosynthesis of alginate

Alginate biosynthesis requires multiple gene products (Figure 1.6) (Table 1.1). The *algD* operon (*algD8alg44algKalgEalgGalgXalgLalgIalgJalgFalgA*) and other gene, *algC*, which is not encoded in the *algD* operon, are required for alginate biosynthesis in *P. aeruginosa* (Table 1.1) (Chitnis and Ohman, 1993; Shortridge *et al.*, 1991). For alginate biosynthesis, the carbon is funneled from fructose 6-phosphate that is converted to mannose 6-phosphate by AlgA (Darzins *et al.*, 1986; Shinabarger *et al.*, 1991). Then, AlgC, a phosphomannomutase isomerizes mannose 6-phosphate to mannose 1-phosphate (Zielinski *et al.*, 1991). Other protein AlgA then converts mannose 1-phosphate to GDP-mannose by AlgA (May *et al.*, 1994). Then, AlgD oxidizes GDP-mannose to GDP-mannuronate (Roychoudhury *et al.*, 1989). For the synthesis of alginate, D-mannuronic acid is polymerized by the inner membrane protein Alg8, and copolymerase, Alg44 (Merighi *et al.*, 2007; Remminghorst *et al.*, 2009; Remminghorst and Rehm, 2006). Then AlgI, AlgJ, and AlgF play roles in the acetylation of the polymer (Franklin and Ohman, 1996; Shinabarger *et al.*, 1993). The O-acetylation of alginate occurs only on mannuronate residues (Davidson *et al.*, 1977).
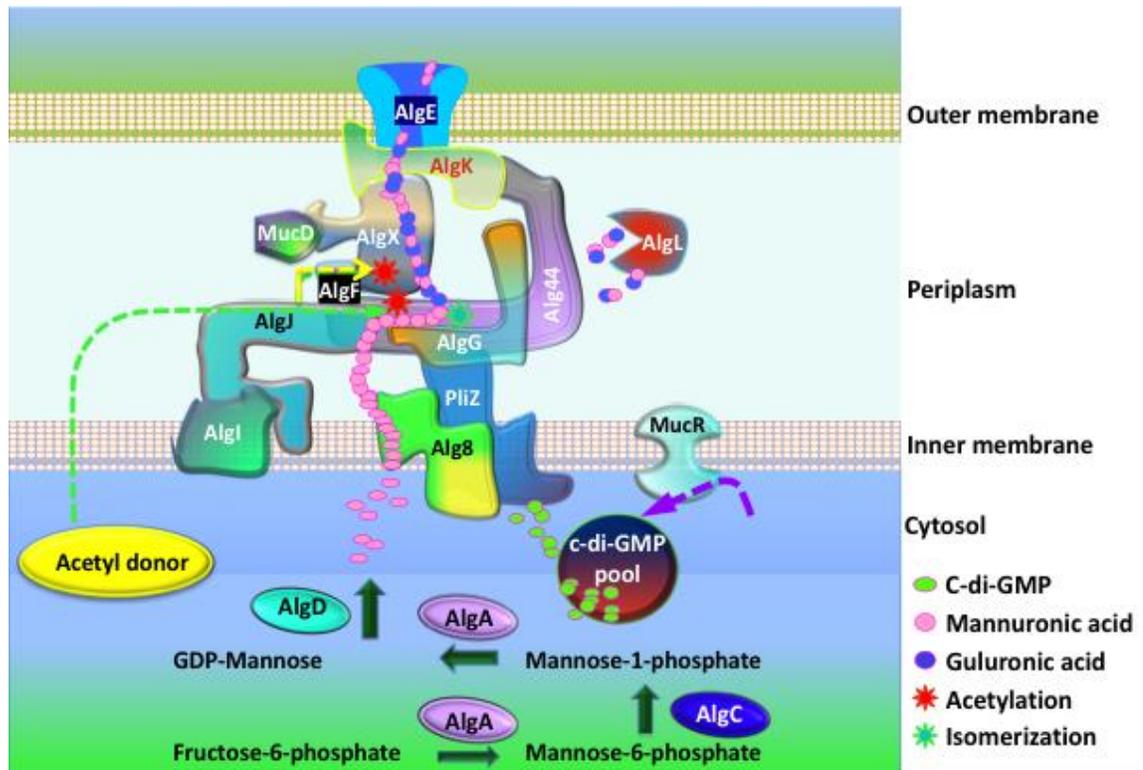
Figure 1.6. A proposed model of alginate biosynthesis. AlgA, AlgC, and AlgD construct the alginate monomers in the cytosol of the *P. aeruginosa*. Polymerization, acetylation, and epimerization occur in periplasm, and AlgE is responsible for exporting the polymer to the extracellular environment (Franklin *et al.*, 2011; Moradali *et al.*, 2015; Ramsey and Wozniak, 2005).

After acetylation of the mannuronate residues, the AlgG epimerizes the non-acetylated D-mannuronic acid residues into the L-guluronic acid (Franklin *et al.*, 1994). Still, it is not known whether epimerization occurs prior to or after acetylation. Physiologically the acetylation of alginate increases the overall gel volume that might protect *P. aeruginosa* from dehydration (Skjakbraek *et al.*, 1989). Immunologically O-acetylation of alginate protects the bacterium from neutrophils and lymphocytes (Mai *et al.*, 1993).

Regulation of alginate biosynthesis in *P. aeruginosa*

The 12 gene *algD* operon is positively regulated by an alternate sigma factor AlgT/U (Wozniak and Ohman, 1994). The *algD* promoter contains a large 367-bp leader region between the transcription start site and translation start codon (Wurtzel *et al.*, 2012). The direct SELEX enrichment approach showed AlgB binds 50 bp located at -274 to -224 upstream relative to *algD* transcription site, which is 367 bp upstream of *algD* translation start site (Leech *et al.*, 2008). It has been also shown that AlgT/U activates *algR* and *algB* both of which positively regulate *algD* transcription (Leech *et al.*, 2008; Wozniak and Ohman, 1994).

Alginate R (AlgR) is homologous to response regulator that has atypical sensor kinase FimS (formally known as AlgZ) whereas AlgB-KinB forms two-component system (Deretic *et al.*, 1989; Ma *et al.*, 1997a; Yu *et al.*, 1997). AlgR has two major structural domains, an N-terminal CheY-like receiver domain, and carboxyl DNA-binding domain of the LytR/YehT/AgrA family of a transcriptional regulator (Kato and Chakrabarty, 1991; Mohr *et al.*, 1991; Mohr *et al.*, 1992). Overexpression of AlgR represses *algD* transcription and alginate production (Deretic and Konyecsni, 1989). Both AlgB and AlgR do not require phosphorylation of the conserved aspartic acid residue in the regulatory domain to promote alginate production in mucoid *P. aeruginosa* (Ma *et al.*, 1998). Chromosome immunoprecipitation analysis showed AlgB directly binds to the *algD* promoter (Leech *et al.*, 2008).

Table 1.1 Genes associated with alginate biosynthesis

Gene	Function	References
	Alginate biosynthesis	
algA	Phosphomannose isomerase and GDP-mannose pyrophosphorylase activity	(Shinabarger *et al.*, 1991)
algC	Phosphomannomutase and phosphoglucomutase activities; involved in LPS biosynthesis	(Regni *et al.*, 2004; Zielinski *et al.*, 1991)
algD	GDP-mannose dehydrogenase	(Snook *et al.*, 2003; Tatnell *et al.*, 1993)
algE	Outer membrane, alginate-specific ion channel responsible for alginate export	(Chu *et al.*, 1991; Grabert *et al.*, 1990)
algF	Periplasmic protein involved in alginate O-acetylation	(Franklin and Ohman, 1993; Franklin and Ohman, 2002)
algG	Periplasmic associated, C5-mannuronan epimerase; protects alginate polymer from lyase activity	(Franklin *et al.*, 1994; Jain *et al.*, 2003)
algI	Inner membrane protein involved in alginate O-acetylation	(Franklin and Ohman, 2002)
algJ	Membrane-associated, periplasmic protein involved in alginate O-acetylation	(Franklin and Ohman, 2002)
algK	Periplasmic protein required for proper polymer formation	(Aarons *et al.*, 1997; Jain and Ohman, 1998)
algL	Periplasmic alginate lyase	(Monday and Schiller, 1996; Schiller *et al.*, 1993)
algX	Periplasmic protein required for proper polymer formation	(Monday and Schiller, 1996; Robles-Price *et al.*, 2004)
alg8	Membrane protein, alginate polymerase	(Maharaj *et al.*, 1993)
algB	Positive activator of *algD* transcription	(Goldberg and Dahnke, 1992; Wozniak and Ohman, 1991)
kinB	Inner membrane protein; cognate sensor kinase for AlgB	(Ma *et al.*, 1997b)
fimS	Hypothesized cognate sensor kinase for AlgR; positively activator of type IV-mediated twitching motility	(Whitchurch *et al.*, 1996; Yu *et al.*, 1997)

algR	Positively activates *algC* and *algD* transcription	(Deretic *et al.*, 1989; Nikolskaya and Galperin, 2002)
sspA	Hypothesized that it reduces transition of RpoD associated genes	(Yin *et al.*, 2013)
RpoN	Required for AlgT/U activity in *kinB* null mutant	(Damron *et al.*, 2009)
clpXP	Facilitated degradation of mutant MucA	(Qiu *et al.*, 2008b)

Other regulators of the *algD* operon is alginate and mobility regulator Z (AmrZ) (Baynham and Wozniak, 1996; Xu *et al.*, 2016). AmrZ has three segments a flexible N-terminus [1-11], a ribbon-helix-helix domain [12-66] and C-terminus [67-108]. Using Chip-Seq and electrophoretic mobility shift assay (EMSA), AmrZ-binding site (ZBS) in *algD* promoter was found around nucleotides -312(ZBS1), -289(ZBS2), -13(ZBS3) and, -75(ZBS4) upstream relative to the *algD* transcription start site of *algD* (Xu *et al.*, 2016). Transcriptional activation of *algD* requires at least 57 bp of DNA between ZBS1 and ZBS2 (Xu *et al.*, 2016). AmrZ binds 8 bp to a direct repeat [GCCATTAC], but a mutation within the repeat results in a four-fold decrease in AmrZ binding and 28-fold decrease in *algD* transcription (Baynham and Wozniak, 1996).

Another protein, the integration host factor (IHF) encoded by *himA* and *himD*, of *P. aeruginosa* is an αβ heterodimer, which through electrophoretic mobility shift assay (EMSA) it was shown that the IHF forms a stable complex with the *algD* promoter (Mohr and Deretic, 1992; Toussaint *et al.*, 1993). There are two IHF binding sites on the *algD* promoter, one between -80 to -68 and another between 79 to 105 relative to the transcription initiation site of *algD* mRNA. The amount of alginate in IHF mutant cell is half [200 μg/ mg dry cell wt] compared to wild-type *P. aeruginosa* [400 μg/ mg dry cell wt] (DelicAttree *et al.*, 1996).

Additionally, MucR an inner membrane-anchored protein positively regulates the alginate biosynthesis in *P. aeruginosa* by increasing the level of cyclic-di-GMP in the cytoplasm (Hay *et al.*, 2009).

AlgT/U regulates the expression of *algT*, *algD*, *algR*, *algB, algZ, algC* (Devries and Ohman, 1994b; Hershberger *et al.*, 1995; Martin *et al.*, 1994; Wozniak and Ohman, 1994; Zielinski *et al.*, 1991). Alginate production is a metabolically expensive process and to synthesize one set of all proteins for the alginate biosynthesis and production, it requires at least 348,984 GTPs. The required GTP number calculation is only for the protein synthesis. Additionally, the amount of GTP required to the synthesis of alginate molecule itself is yet to be calculated.

Alginate production is energetically expensive thus regulation in alginate production is very controlled transcriptionally by autoregulation of sigma factor AlgT/U and post transcriptionally by sequestration of AlgT/U by anti-sigma factor MucA. The release of AlgT/U from MucA is by the process known as regulated intramembrane proteolysis (RIP) that involves series of periplasmic and inner membrane proteases.

In this chapter, genes involved in regulation of alginate production in *P. aeruginosa* are discussed and compared with the genes in *E. coli* because of the similarity of the pathways between the two bacteria (Ades *et al.*, 1999; Qiu *et al.*, 2007).

Regulated intramembrane proteolysis cascade

The RpoE homologs function as extracytoplasmic sigma factors (Rowen and Deretic) (Lonetto *et al.*, 1994). The stress signals initiate a proteolytic cascade that eliminates an inner membrane negative-regulator i.e., an anti-sigma factor, freeing RpoE.

The RIP cascade is common to many bacteria, including *Bacillus subtilis* (Schobel *et al.*, 2004; Zellmeier *et al.*, 2006), *Mycobacterium tuberculosis* (Sklar *et al.*, 2010), and *Xanthomonas campestris* (Bordes *et al.*, 2011). The following section will investigate the role of the major players involved in the *P. aeruginosa* and *E. coli* RIP cascade (Table 1.1) and the signals involved. For simplicity, the nomenclature in column 2 and column 6 of the Table 1.2 will be adopted in this chapter. Although the members of the *E. coli* and *P. aeruginosa* RIP cascade are functionally conserved, there is a wide variation in sequence homology between gene orthologs (Table 1.3).

Extracytoplasmic stress sigma factor σ^E: AlgT/U and RpoE

     P. aeruginosa and *E. coli* RpoE share 66 % homology (Lonetto *et al.*, 1992). *E. coli* RpoE has been found to restore the mucoid (Alg+) phenotype in Δ*algT/U* mutant *P. aeruginosa* (Yu *et al.*, 1995). However, there is no in vivo experimental evidence as to whether *algT/U* complements the function of *rpoE* in *E. coli*; although AlgT/U has been shown to transcriptionally activate *E. coli* RpoE-dependent promoters *in vitro* (Hershberger *et al.*, 1995). Activation of alternative sigma factors in *P. aeruginosa* and *E. coli* is controlled both at the level of transcriptional initiation and post-translation. The *rpoE* operon contains four genes, which either positively or negatively regulate its activity in *E. coli* (Missiakas *et al.*, 1997). The three open reading frames downstream of *rpoE* encode regulators of sigma E and are thus named *rseA*, *rseB*, and *rseC* (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). In the case of *P. aeruginosa*, *algT/U* is a part of a five-gene operon containing *algT/U*, *mucA*, *mucB*, *mucC*, and *mucD* (Boucher *et al.*, 1996; Franklin and Ohman, 1996). These Muc proteins regulate AlgT/U activity.

E. coli RseA and *P. aeruginosa* MucA – the anti-sigma factors

Both regulator of sigma E (Rse) A (RseA) and Mucoid A (MucA) are negative regulators of the alternative sigma factors RpoE and AlgT/U, respectively, and are referred to as anti-sigma factors. For more details, analyses of other anti-sigma factors are reviewed previously (Hughes and Mathee, 1998). Though these two proteins are functional equivalents, they share very little sequence homology (Table 1.3). Transposon insertions in *rseA* resulted in a 12-fold increase whereas deletion mutants exhibited a 25-fold induction of RpoE activity (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). The large up-regulation upon the loss of *rseA* suggested that the major role of RseA is to act as a negative regulator of RpoE activity. Inactivation of *mucA* in *P. aeruginosa* resulted in a mucoid phenotype suggesting that MucA is a negative regulator of alginate production (Fyfe and Govan, 1980; Govan and Fyfe, 1978; Martin *et al.*, 1993a; Mathee *et al.*, 1999). Mapping and sequence analysis showed that *mucA* was located directly downstream of *algT/U* (Martin *et al.*, 1993a).

Both RseA and MucA are localized to the inner membrane with the N-terminus in the cytoplasm and C-terminus in the periplasm (De Las Penas *et al.*, 1997; Lewenza *et al.*, 2005; Mathee *et al.*, 1997; Missiakas *et al.*, 1997). Membrane preparations of RseA-overproducing bacteria showed RpoE co-eluted with RseA (Missiakas *et al.*, 1997).

Pull-down experiments using RpoE-specific antibodies showed RseA and RpoE co-immunoprecipitating at a molar ratio close to 1:1 (Missiakas *et al.*, 1997). In case of *P. aeruginosa*, co-expression of MucA and AlgT/U followed by cross-linking (Boucher *et al.*, 1996) suggested MucA and AlgT/U interact directly. The interaction of MucA and AlgT/U

is was supported by the co-fractionation of MucA and AlgT/U in glycerol sedimentation experiments (Xie *et al.*, 1996).

E. coli **RseB and** *P. aeruginosa* **MucB – Anti-anti sigma factors**

RseB and MucB share only 27.6% homology (Table 1.3), however, they are functional equivalents that interact with the anti-sigma factors and subsequently affect alternative sigma factor activity. Deletion of *rseB* in *E. coli* resulted in an increase in *rpoE* expression; whereas loss of *mucB* resulted in alginate production (Goldberg *et al.*, 1993; Martin *et al.*, 1993a). These findings clearly argued that RseB and MucB are negative regulators of RpoE and AlgT/U, respectively. Both RseB and MucB were localized to the periplasm (Boucher *et al.*, 1996; Mathee *et al.*, 1997; Missiakas *et al.*, 1997). Direct interaction between RseB and MucB with their cognate anti-sigma factors RseA and MucA, respectively, has been demonstrate   (Missiakas *et al.*, 1997; Rowen and Deretic, 2000).

Specifically, RseB and MucB were found to interact with the periplasmic C-terminal domain of their cognate anti-sigma factors (Cezairliyan and Sauer, 2007; Wood and Ohman, 2009). RseB is present as a dimer that protects RseA from cleavage by DegS (Wollman and Zeth, 2007; Cezairliyan and Sauer, 2007). In the case of MucB, it exists in both monomeric and dimeric forms, however; only the dimer binds to MucA (Cezairliyan and Sauer, 2009). The RseA-RseB interaction appears to stabilize RseA and strengthens RseA's bond with RpoE (Ades *et al.*, 1999; Collinet *et al.*, 2000).

Table 1.2. List of proteins involved in RpoE regulation

P. aeruginosa				*E. coli*				Product Name
Locus Tag	Protein	Alternative Name	Size (aa)	Locus Tag	Protein	Alternative Name	Size (aa)	
PA0762	AlgT/U	RpoE/σ^{22}	193	ECK2571	RpoE	σ^{24}/ σ^{E}/SigE	191	Sigma factor
PA0763	MucA		194	ECK2570	RseA	MclA/YfiJ	216	Anti-sigma factor
PA0764	MucB	AlgN	316	ECK1569	RseB	MclB	318	Negative regulator
PA0765	MucC	AlgM	151	ECK2568	RseC	MclC	159	
PA0766	MucD	AlgY/HtrA	474	ECK0160	DegP	HtrA/Ptd/Do	475	Serine endoprotease
PA4446	AlgW		389	ECK3224	DegS	HhoB/HtrH	355	Site 1 protease; HtrA family
PA3649	MucP		450	ECK0175	RseP	YaeL/EcfE	450	Site 2 protease; Zn dependent
PA4033	MucE		89	No homologs				
PA1802	ClpX	ClpX	426	ECK0432	ClpX		424	ATPase subunit of the protease
PA1801	ClpP	Clp1	213	ECK0431	ClpP	LopP/WseA	201	Proteolytic subunit of the protease
PA3326	Clp2		201					
PA4427	SspB		135	ECK3217	SspB		165	Stringent starvation protein

Table 1.3. Comparison of *E. coli* and *P. aeruginosa* homologs involved in the anti-sigma factor (RseA and MucA, respectively) proteolysis

		E. coli K12										
		RpoE	**RseA**	**RseB**	**RseC**	**DegP**	**DegS**	**RseP**	**ClpX**	**ClpP**	**SspA**	**SspB**
P. aeruginosa PA01	**AlgT/U**	66.0	7.8	18.7	15.2	20.7	18.8	21.2	25.4	16.2	9.3	15.7
	MucA	14.4	28.3	22.2	18.2	16.1	15.0	18.6	17.0	13.3	14.9	13.3
	MucB	19.4	16.2	27.6	29.9	17.2	11.4	19.5	18.4	17.0	19.3	18.1
	MucC	15.9	17.2	21.2	30.0	23.2	26.5	24.5	22.5	10.7	17.3	13.3
	MucD	22.0	19.4	17.9	15.0	39.2	33.4	15.4	15.2	23.2	16.9	19.3
	AlgW	17.4	17.6	15.8	23.9	36.8	46.3	17.2	14.3	20.3	18.4	18.7
	MucP	17.3	17.6	19.8	23.2	10.2	16.7	46.9	11.9	17.9	23.5	22.4
	ClpX	23.0	15.3	15.1	19.5	16.2	17.9	12.6	76.6	19.3	21.2	17.5
	ClpP	12.0	11.3	16.1	18.9	18.8	17.4	19.7	17.4	69.6	13.2	14.5
	ClpP2	16.2	12.6	19.4	19.6	14.9	19.4	16.4	22.6	41.5	14.2	15.7
	SspA	13.2	11.5	20.0	20.1	18.6	17.6	19.5	21.2	12.6	53.9	15.2
	SspB	16.3	19.2	20.0	18.0	21.5	20.8	22.2	18.5	16.3	20.0	53.3

The numbers refer to percentage homology as determined using ClustalW (Table 1.3). The shaded boxes compare the functional homologs. The protein sequences of *E. coli* and *P. aeruginosa* were retrieved from ecogene.com and pseudomonas.com.

Coaggregation of RseB with misfolded proteins suggests that it may act as a stress sensor, which may allow RseB to release from RseA to initiate the RIP cascade leading to RpoE-dependent activity (Collinet *et al.*, 2000).

E. coli RseC and *P. aeruginosa* MucC

Though both *E. coli rseC* and *P. aeruginosa mucC* are the third gene in their respective *rpoE* operons, they share only 30% homology. The role of RseC and MucC is ambiguous and little is currently known about the exact cellular function of these members of the *rpoE* and *algT/U* operons (Boucher *et al.*, 1997a; De Las Penas *et al.*, 1997; Franklin and Ohman, 1996; Missiakas *et al.*, 1997).

E. coli DegP and *P. aeruginosa* MucD

E. coli <u>d</u>egradation protein <u>P</u> (DegP) that is required for growing the bacterium above 42 °C and *P. aeruginosa* <u>Muc</u>oid <u>D</u> (MucD) share 39% homology (Table 1.3)(Gottesman and Zipser, 1978; Strauch *et al.*, 1989b). The presence of *mucD* in the *algT/U* operon suggests that MucD should play a role in the post-translational regulation of AlgT/U. In the case of *E. coli*, *degP* is not present in the *rpoE* operon (Strauch and Beckwith, 1988). Expression of both *degP* and *mucD* are under the control of RpoE and AlgT/U, respectively (Boucher *et al.*, 1996; Dartigalongue *et al.*, 2001; Erickson and Gross, 1989; Franklin and Ohman, 1996; Lipinska *et al.*, 1988; Wang and Kaguni, 1989).

Though mutations in *degP* had no effect on *E. coli rpoE* transcription, DegP is required for its growth at elevated temperatures (Lipinska *et al.*, 1989; Lipinska *et al.*, 1990). Both null and point mutations of *mucD* in non-mucoid PAO1 resulted in a mucoid phenotype conversion (Boucher *et al.*, 1996; Wood *et al.*, 2006). Alkaline-phosphatase

fusions of DegP and MucD showed them to be localized to the periplasm (Lewenza *et al.*, 2005; Strauch *et al.*, 1989a). The major role of both DegP and MucD is to degrade misfolded proteins from the periplasm (Strauch and Beckwith, 1988; Wood and Ohman, 2009) that may indirectly regulate RseB and MucB allowing it to re-interact with their cognate anti-sigma factors.

E. coli **DegS and** *P. aeruginosa* **AlgW**

The degradation protein S (DegS) and Alginate W (AlgW) share substantial homology (Table 1.3). They are also members of the HtrA family of serine proteases (Pallen and Wren, 1997). DegS and AlgW are also known as Site-1 proteases (S1P), which cleave anti-sigma factors on their periplasmic sides (Dalbey *et al.*, 2012; Qiu *et al.*, 2007).

DegS is an inner membrane protein with a large periplasmic domain (Alba *et al.*, 2002); (Waller and Sauer, 1996). Based on sequence homology, AlgW is also presumed to be an inner membrane protein with a single transmembrane domain. DegS initiates the first cleavage of RseA leading to an eventual release of RpoE to initiate transcription (Ades *et al.*, 1999; Alba *et al.*, 2002). DegS cleaves between amino acids Val148 and Ser149 in the transmembrane domain of RseA adjacent to the periplasmic side (Walsh *et al.*, 2003). AlgW primarily cleaves MucA between amino acids Ala136 and Gly137 (Cezairliyan and Sauer, 2009). DegS binding of the C-terminal -YXF motif of misfolded OMPs activates it to cleave RseA (Walsh *et al.*, 2003). But, in *P. aeruginosa*, *mucE* that encodes an outer membrane associated small protein bind with a specific C-terminal -WVF motif and initiates RIP cascade (Qiu *et al.*, 2007).

E. coli RseP and *P. aeruginosa* MucP

Both RseP and MucP are Site-2 Proteases (S2P) which are membrane-bound zinc metalloproteinases involved in the proteolytic activation of regulatory factors for sterol biosynthesis and for stress responses (Kanehara *et al.*, 2001; Kinch *et al.*, 2006; Qiu *et al.*, 2007). RseP and MucP were both identified based on sequence homology to other site-2-proteases (Kanehara *et al.*, 2001; Qiu *et al.*, 2007). RseP is essential in *E. coli* as depletion causes a rapid loss of viability, cell elongation, and growth cessation (Kanehara *et al.*, 2001).

RseP is an inner membrane protein with four transmembrane domains with both the N- and C- termini lying in the periplasm (Kanehara *et al.*, 2001). RseP also contains two periplasmic PDZ domains involved in its regulation (Inaba *et al.*, 2008); (Kanehara *et al.*, 2003). Our analysis using TMHMM shows MucP possessing a similar domain structure to RseP. Cleavage of RseA by RseP was confirmed using *in vitro* assays (Akiyama *et al.*, 2004). The regulated proteolysis of RseA requires a functional PDZ domain of RseP (Kanehara *et al.*, 2003). Indirect genetic evidence for MucP cleavage of MucA leading to AlgT/U expression has been shown using mutant strains (Damron *et al.*, 2011; Qiu *et al.*, 2007). No direct interaction with MucP and MucA has been demonstrated.

E. coli and *P. aeruginosa* ClpXP

After RseP-cleavage of RseA, a small fragment of RseA remains attached to RpoE (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). The removal of this RseA cytoplasmic fragment requires SspB and ClpXP (Flynn *et al.*, 2004). SspB was found to bind to both

the amino-terminus of RseA as well as ClpX thereby bringing the fragment close enough to the ClpXP protein complex to allow for RseA fragment degradation (Flynn *et al.*, 2004).

In *P. aeruginosa*, there are two ClpP homologs, ClpP (PA1801) and ClpP2 (PA3326). Mutagenesis of *clpX*, *clpP*, and *clpP2* individually resulted in the loss of a mucoid phenotype (Qiu *et al.*, 2008a). *P. aeruginosa* also harbors the *sspAB* (PA4426-PA4427) operon (Yin *et al.*, 2013). Unlike *E. coli*, SspA appears to play a role in alginate production (Yin *et al.*, 2013). A direct interaction between the MucA fragment and ClpXP or SspA has yet to be demonstrated.

RIP Models for *E. coli* and *P. aeruginosa*

Many questions remain unanswered. However, on the basis of the above findings, the following model is proposed for *E. coli* (Figure 1.7A). and *P. aeruginosa* (Figure 1.7B).

***E. coli* RIP cascade**

Misfolded OMP peptides with exposed C-terminal -YQF motifs bind to the PDZ domain of DegS allowing cleavage of RseA at the periplasm. It is also presumed that misfolded peptides compete for binding with RseB from RseA allowing cleavage of RseA by DegS. Alternatively, it has been postulated that RseB hinders RseP-recognition of RseA. Following cleavage by DegS, RseP cleaves RseA at the cytoplasm releasing RpoE with an N-terminal fragment of RseA. This N-terminal fragment of RseA remains attached until removed by SspB-ClpXP releasing the free RpoE to initiate transcription of the RpoE regulon.

P. aeruginosa **RIP cascade**

Similar to the *E. coli* system, MucA is sequentially degraded by AlgW, and MucP. The C-terminal -WVF motif of MucE has been shown to prime the AlgW protease to initiate the cleavage of MucA. The direct cleavage of MucA by MucP, or interaction of ClpXP or SspA/B has not been demonstrated but inferred from genetic data and by comparison to the *E. coli* system. In *P. aeruginosa* SspB appears to play a role in this pathway. Free AlgT/U activates all the genes in the *alg* regulon, leading to alginate production.

Inducers of alginate production

Chemical

Ammonium metavanadate

Pseudomonas isolation agar supplemented with ammonium metavanadate [0.27 mM] induces alginate production in *P. aeruginosa* (Damron *et al.*, 2011). Growing *P. aeruginosa* in ammonium metavanadate increases the expression of periplasmic stress chaperones MucD and well as cytoplasmic chaperones GroEL [PA4385], DnaK [PA4761], GrpE [PA4762]; and outer membrane proteins [OMP] OprF [PA1777], and OprO [PA3280] but exactly how the chemical induces the mucoid phenotype is not clear yet (Damron *et al.*, 2011). The mucoid *P. aeruginosa* grown on pseudomonas isolation agar (Imperi *et al.*) supplemented with ammonium metavanadate when streaked back to PIA, the mucoid *P. aeruginosa* turned non-mucoid suggesting ammonium metavanadate based mucoid phenotype is not due to the genetic change in the bacterium (Damron *et al.*, 2011).
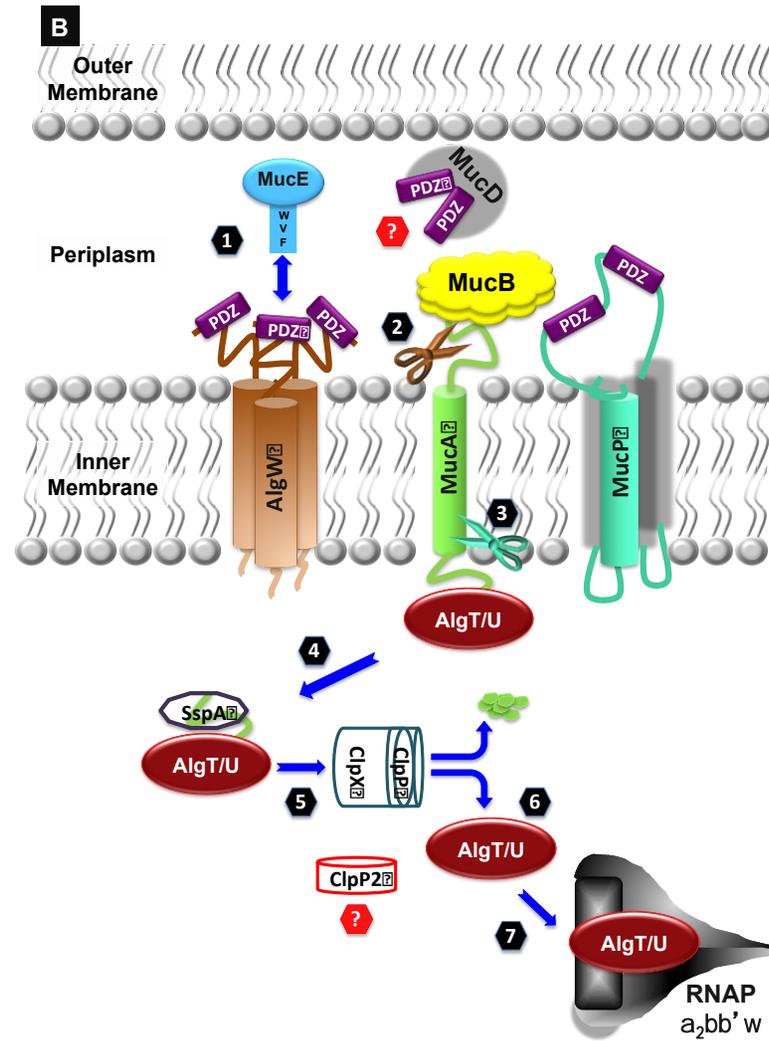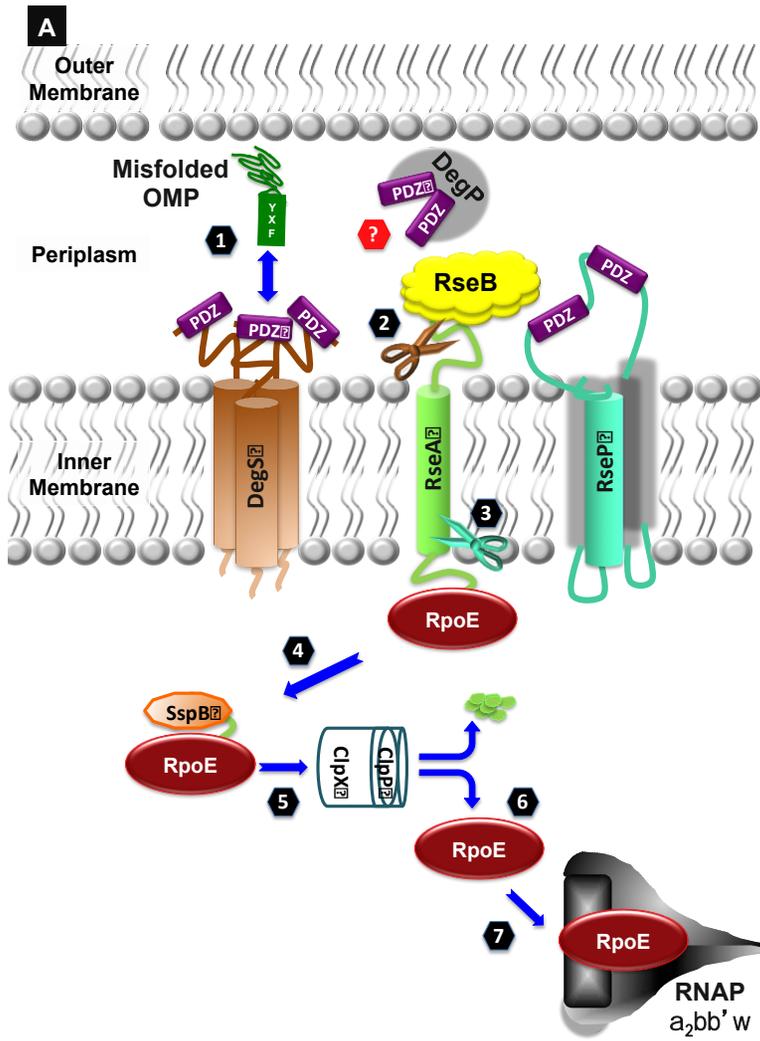
Figure 1.7. Proposed model for the anti-sigma factor proteolysis in *E. coli* (A) and *P. aeruginosa* (B). The model is based on currently available data. (A) The steps for *E. coli* are outlined as follows: (i) –YXF C-terminal motif exposed in misfolded OMPs binds to the PDZ domain of DegS activating its proteolytic domain. DegP may degrade misfolded OMP proteins prior to their interaction with DegS, perhaps preventing DegS activation; Based on *P. aeruginosa* system, RseB dissociates to allow site-1 proteolysis of RseA at its periplasmic side by DegS; (iii) The removal of C-terminal RseA allows for site-2 proteolysis at its cytoplasmic side by RseP. It has been postulated that RseP access to RseA can also be prevented by RseB; (iv) RseP activity releases RpoE with a fragment of RseA bound to it; (v) SspB binds to and brings the RseA fragment to the ClpXP proteasome; (vi) ClpXP degradation of the RseA fragment releases free RpoE; (vii) Free RpoE directs RNA polymerase that is made of five subunits ($\alpha_2\beta\beta'\omega$) to initiate transcription of the RpoE regulon. (B) The model is based on the available data and is, in part, inferred from the *E. coli* system. The steps involved are as follows: (i) –WVF motif on MucE binds to the AlgW PDZ domain activating its proteolytic activity. MucD is hypothesized to inhibit the interaction of MucE with AlgW, as well as binding misfolded proteins; (ii) Activated AlgW cleaves the periplasmic domain of MucA releasing MucB along with a C-terminal fragment of MucA; (iii) MucP induces site-2 proteolysis of MucA at the cytoplasmic side releasing AlgT/U with a small N-terminal fragment of MucA attached; (iv) Based on the *E. coli* system, SspA is proposed to be the adaptor molecule that brings AlgT/U with the MucA fragment to ClpXP; (V) The MucA fragment is removed from AlgT/U by the ClpXP proteasome. The role of ClpP2 homolog is not clear; (vii) Free AlgT/U directs RNA polymerase to initiate transcription of the AlgT/U regulon.

Imipenem

Growing *P. aeruginosa* in biofilms upon exposure to the antibiotic imipenem [1.0 µg/ml] for 24 hours cause the appearance of a mucoid phenotype (Bagge *et al.*, 2004). However, sub-culturing the mucoid *P. aeruginosa* from the imipenem-exposed biofilm on nonselective LB agar plates did not show the mucoid phenotype suggesting that the observed mucoidy was unstable (Bagge *et al.*, 2004).

Ethanol

Mucoid *P. aeruginosa* were obtained when the bacterium was grown for 6 days at room temperature (22 ºC) on (Yeast extract, tryptone, and glucose) YTG medium

supplemented with 3% - 5% ethanol (Devault *et al.*, 1990). The frequency of mucoid variant formation was one colony per 106 cells but the mucoid phenotype lasted for 14 days when grown in the same medium without ethanol before it reverted back to nonmucoid phenotype (Devault *et al.*, 1990).

Phage

Phage was isolated from the supernatant of phage-induced-mucoid variants of *P. aeruginosa* and applied to the non-mucoid strain of *P. aeruginosa*, which caused the non-mucoid strain to turn mucoid suggesting phage can also induce mucoidy in *P. aeruginosa* (Martin, 1973).

Estradiol

It has been shown in both human and mouse model that estradiol enhances the alginate production in *P. aeruginosa* may be responsible for a survival disadvantage of female patients with CF (Chotirmall *et al.*, 2012).

Mucoid phenotype and antibiotic resistance of *P. aeruginosa*

It has been proposed that alginate production contributes to antibiotic resistance in *P. aeruginosa,* but several results show that there is no correlation between mucoidy and antibiotic resistance. There is no significant effect of the antibiotics tobramycin and tigecycline to the wild-type PAO1 and mucoid *P. aeruginosa* (Oglesby-Sherrouse *et al.*, 2014).

Stability

It is commonly known that mucoid clinical *P. aeruginosa* reverts to the non-mucoid phenotype (Govan, 1975; Zierdt and Schmidt, 1964). For the clinical *P. aeruginosa* FRD strain, incubation of the strain in L-broth at 40 °C without aeration results in mucoid to non-mucoid conversion at the rate of 50% within 24 – 48 hours (Ohman and Chakrabarty, 1981). Mucoid to non-mucoid conversion is faster when the bacteria are grown without aeration in the nutrient medium compared to the bacteria grown with aeration in deoxycholate citrate agar (Govan, 1975). In case of the PAO1-derivative PDO300, the rate of mucoid to non-mucoid conversion was found to be 90 % within 48 hours (Sautter *et al.*, 2012). The mucoid to non-mucoid conversion has been mapped to mutations in the *algT/U* operon, *algO*, *algW*, and *mucP* (Boucher *et al.*, 1996; Qiu *et al.*, 2007; Reiling *et al.*, 2005; Sautter *et al.*, 2012; Schurr *et al.*, 1994). The switch from non-mucoid to a mucoid phenotype is associated with a poor prognosis for CF patients (Pillarisetti *et al.*). The alginate over-production enables *P. aeruginosa* to cause persistent infections in the airways of CF lungs that results in decreased lung function, increases the risk of respiratory failure, and ultimately the demise of the CF patients. The change from non-mucoid to mucoid phenotype is the result of the mutation of anti-sigma factor MucA resulting in the loss of regulation of the alternate sigma factor AlgU. The mutation allows AlgU to become free to interact with core RNA polymerase and direct transcription of the alginate biosynthetic genes located in the *algD* operon, in addition to its own operon *algU-mucA-mucB-mucC-mucD*. The alternate sigma factor AlgU belongs to the extra-cytoplasmic function (ECF) family. AlgU (also called AlgT) is 22 kDa protein that shares 66% identity with the *E. coli*

sigma factor RpoE. In case of AlgT/U the mutation was mapped to codon 18, where a GAC in (Alg[+]) strains was changed to GGC that encodes glycine (De Las Penas *et al.*, 1997). Another change was at codon 29, where TAC [Tyr] was changed to TGC [Cys]. Both of mutation in codon 18 or codon 29 makes AlgT/U unable to direct the transcription resulting in a non-mucoid phenotype of *P. aeruginosa*.

Strategy to map suppressor of alginate production mutations

Cosmid-based technique

A minimum tiling cosmid library that covers 93.7% of the *P. aeruginosa* genome with 334 colonies is commonly used to map mutation in the genome (Huang *et al.*, 2000).

Localized mutagenesis

The localized mutagenesis technique is similar to the one used to obtain temperature-sensitive mutant *Salmonella typhimurium* where transducing phage is *in vitro* mutagenized prior to transduction. To find the novel gene involved in acetylation of alginate, small fragment of bacterial DNA encoding the alginate biosynthetic operon was placed in a cosmid. Lambda lysate was prepared on *E. coli* HB101 to package these cosmids into phage particles. The packaged cosmids were mutagenized *in vitro* by treatment with hydroxylamine. Hydroxylamine is a highly specific mutagen that reacts with cytosine and causes the specific replacement of nucleotide G by an A. Hydroxylamine is a highly specific mutagen that reacts with cytosine and causes the specific replacement of nucleotide G by an A. Then the mutagenized cosmid is packed in the phage and transduced into *E. coli*.

Then the mutagenized cosmid is packed in the phage and transduced into *E. coli*. Then triparental mating is done to transfer the mutagenized cosmid from *E. coli* to *P. aeruginosa* and finally, *P. aeruginosa* carrying the mutagenized cosmid is screened for the particular phenotype (Franklin and Ohman, 1996).

Sequencing-based technique

In whole genome sequencing-based approach purified DNA from the suppressor strain can be used for sequencing using high throughput sequencing platform. Several sequencing platforms such as Illumina Hiseq200 can be used.

Purpose of my study

Our lab isolated nonmucoid revertants termed as a suppressor of alginate production strains (*sap*) derived from the constitutively mucoid (Alg$^+$) PDO300. One of the previously uncharacterized *sap* mutants, *sap27* (non- mucoid), was used in the present study. Complementation of the *sap27* by a cosmid pMO012217 from the *P. aeruginosa* cosmid library restored the mucoid phenotype of *sap27* suggesting the at least one the genes in the cosmid is required for the mucoid phenotype. Preliminary transposon mutagenesis suggests a three-gene operon (*lptD-surA-pdxA*; *PA0595-PA0594-PA0593*), is involved in alginate overproduction. To map the specific gene responsible for this complementation in the cosmid pMO02217, and to understand its role in alginate production/regulation, the project has following three aims:

Specific Aim 1: To map the genes on pMO012217 involved in alginate production. The working hypothesis is that one of the 18 open reading frames (ORF) present in the cosmid is responsible for restoring the mucoid phenotype in *sap27*. The

preliminary result suggests a three-gene operon (*lptD-surA-pdxA*), is involved in alginate overproduction. The genes were individually expressed in *sap27* to identify the gene involved in alginate production. In addition, the gene in *sap27* was sequenced. Subsequently, the role of the gene in alginate production was determined.

Specific Aim 2: To determine the mechanism of action of LptD. The working hypothesis is that LptD bypasses the proteins that are involved in alginate production in *sap27*. To test this, LptD was overexpressed in the strains whose previously known alginate related gene was deleted and scored for the revertant mutant to understand the gene hierarchy of LptD in RIP cascade.

Specific Aim 3: To identify the role of PLP in virulence of *P. aeruginosa*. Preliminary data show an exogenous addition of pyridoxal 5'-phosphate (PLP) can affect
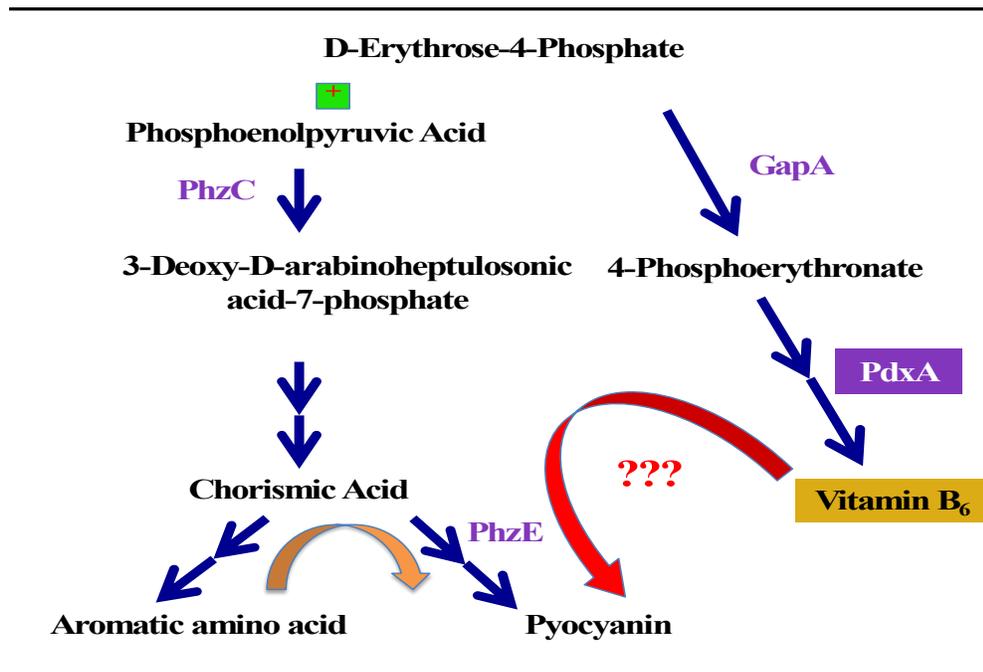
Figure 1.8. Pyocyanin production pathway in *P. aeruginosa*. Erythose-4-phosphate and phosphoenol pyruvate are precursors for synthesis of both vitamin B_6 and shikimiate. Then shikimate is converted into chorismic acid that eventually converted into pyocyanin. Culturing *P. aruginosa* in a media with vitamin B_6 or in presence of aromatic amino acid directs the pathway to pyocyanin production.

pyocyanin production. To understand the pathway for pyocyanin production we used quantitative PCR of the genes shown in purple color (Figure 1.8).

CHAPTER 2

Outer membrane protein LptD (PA0595) plays a role in the regulation of alginate

synthesis in *Pseudomonas aeruginosa*

Abstract

The emergence of increased alginate producing mucoid strains is linked to a poor prognosis in people with cystic fibrosis. The mucoid phenotype is very unstable outside of the CF lung. In this study, a set of nonmucoid revertant strains was used to identify novel genes involved in alginate production. Complementation analysis of these mutants using a minimal tilling path cosmid library identified a cosmid pMO012217 that restored the mucoid phenotype of *sap27*. The cosmid contains 18 open reading frames. Transposon mutagenesis of the cosmid mapped the mutation to LptD (*PA0595*) encoding a Lipopolysaccharide transport protein D (LptD), a protein associated with lipopolysaccharide transport to the outer membrane of the bacterium. We established LptD is part of three gene operon *lptD-surA-pdxA*. Our result indicates LptD alone restored the mucoid phenotype of *sap27*. We took the advantage of PAO1, and its isogenic *mucA22* mutant PDO300; and *algO,* and *algW* mutant of both of these mutants as well of the plasmids harboring *algO* and *algW* to identify the pathways that restored the mucoid phenotype of the bacterium due to LptD.

Introduction

Pseudomonas aeruginosa, a Gram-negative, ubiquitous bacterium is pathogenic to plants, animals, and humans (Bayes *et al.*, 2016; McAvoy *et al.*, 1989; Rahme *et al.*, 1995). The most severe *P. aeruginosa* infections in humans include endocarditis, malignant otitis externa, septicemia, endophthalmitis, eye keratitis, pneumonia, and meningitis (Bodey *et al.*, 1983). Annually, there are 51,000 *P. aeruginosa* infections of which 6,700 are multidrug-resistant isolates causing 440 deaths in the USA (2015). The Centers for Disease

Control and Prevention has ranked *P. aeruginosa* as a pathogen of serious threat level (CDC, 2015).

P. aeruginosa is the leading cause of morbidity and mortality in patients with CF (2016). Colonization of the lungs of CF patients starts a few years after birth and remains for life (Hoiby *et al.*, 1977) An early acute infection transitions to a chronic phase when isolated *P. aeruginosa* colonies exhibit a mucoid phenotype due to constitutive production of alginate (Alg$^+$) (Doggett *et al.*, 1971; Hoiby *et al.*, 1977). Though alginate-producing *P. aeruginosa* is primarily associated with CF individuals, occasionally it has been isolated from patients with bronchiectasis, urinary tract, and middle-ear infections (McAvoy *et al.*, 1989). Mucoid *P. aeruginosa* strains have also been isolated from wastewater systems, and the guttural pouch of an equine with chronic mucopurulent nasal discharge (Govan *et al.*, 1992; Grobe *et al.*, 1995).

The presence of alginate-producing strains in the lungs of CF patients is associated with poor prognosis (Hoiby, 1975; Pedersen *et al.*, 1992). Alginate protects *P. aeruginosa* from phagocytosis, antibiotics, oxygen radicals, and the host immune response (Govan and Fyfe, 1978; Hodges and Gordon, 1991; Kulczycki *et al.*, 1978; Mathee *et al.*, 1999; Oliver and Weir, 1985; Schwarzmann and Boring, 1971). The importance of alginate in the virulence of *P. aeruginosa* has also been demonstrated in mouse models (Boucher *et al.*, 1997a; Bragonzi *et al.*, 2006; Pier *et al.*, 2001; Song *et al.*, 2003; Yu *et al.*, 1998). Compared to wild-type *P. aeruginosa,* an alginate-overproducing strain causes aggressive polymorphonuclear (PMN) leukocyte infiltration (similar to human infection) and causes inefficient pulmonary clearance (Song *et al.*, 2003). A protracted lung infection has the

potential to spread to other organs, such as the spleen has observed in the mouse model (Song *et al.*, 2003). These properties suggest that alginate is an important virulence factor.

Exposing the bacteria to stress can induce alginate production. Mucoid conversion of *P. aeruginosa in vitro* can be triggered by growth in high osmolarity media, with limited nutrients (like nitrogen, phosphate, and carbon), static culture in acetamide broth, continued culture in the presence of antibiotics, Pseudomonas Isolation Agar supplemented with ammonium metavanadate, ethanol dehydration and extended exposure to oxygen radicals such as hydrogen peroxide (Damron *et al.*, 2011; Devault *et al.*, 1990; Evans and Linker, 1973; Govan and Fyfe, 1978; Mathee *et al.*, 1999; Speert *et al.*, 1990; Terry *et al.*, 1991). Alginate production can also be stimulated by anaerobiosis (Bragonzi *et al.*, 2006; Hassett, 1996; Worlitzsch *et al.*, 2002). Though it is difficult to ascertain every factor involved, it appears that in CF patients, continuous exposure to antibiotics and PMN-derived hydrogen peroxide may be the major contributors of mucoid conversion in *P. aeruginosa* (Mathee *et al.*, 1999).

Alginate production is tightly controlled by a myriad of regulatory factors. AlgR, AmrZ (previously called as AlgZ), AlgB, and AlgT/U regulate transcription of the *algD* operon (Baynham and Wozniak, 1996; Goldberg *et al.*, 1993; Kato and Chakrabarty, 1991; Leech *et al.*, 2008; Mohr *et al.*, 1992; Worlitzsch *et al.*, 2002; Wozniak and Ohman, 1991). The AlgR and AlgB proteins are response regulators of different two-component systems, FimS-AlgR, and KinB-AlgB that are essential for high levels of alginate synthesis (Goldberg and Dahnke, 1992; Goldberg and Ohman, 1987; Ma *et al.*, 1997a; Whitchurch *et al.*, 1996; Wozniak and Ohman, 1991; Yu *et al.*, 1997). However, the conserved phosphorylation domains of both AlgB and AlgR are not required for alginate production

(Damron *et al.*, 2009; Ma *et al.*, 1998; Worlitzsch *et al.*, 2002). Phosphorylated AlgR is not needed for alginate production but plays a role in twitching motility (Whitchurch *et al.*, 1996). Another protein that increases alginate production is AmrZ, a ribbon-helix-helix DNA binding protein (Baynham and Wozniak, 1996). The membrane-associated cyclic-dimeric-GMP synthesizing protein MucR is also required for the synthesis of alginate in *P. aeruginosa* (Hay *et al.*, 2009).

The master regulator of alginate synthesis is AlgT/U (σ^{22}), which belongs to the family of the extracytoplasmic sigma factors (ECF) and has high homology to SigE (σ^E) found in *E. coli, Streptomyces coelicolor, Bacillus subtilis* and *Salmonella* Typhimurium (Devries and Ohman, 1994a; Hershberger *et al.*, 1995; Lonetto *et al.*, 1992; Martin *et al.*, 1993; Worlitzsch *et al.*, 2002; Wozniak and Ohman, 1994). Expression of *algT/U* is autoregulated, and AlgT/U, in turn, regulates expression of the *algD*, *algB, algR, amrZ* and *mucE* operons (Baynham and Wozniak, 1996; Devries and Ohman, 1994b; Wozniak and Ohman, 1994; Yin *et al.*, 2013). The *algT/U* gene is a part of the *algT/U-mucA-mucB-mucC-mucD* operon that plays an essential role in converting nonmucoid *P. aeruginosa* into its mucoid phenotype (Devries and Ohman, 1994a; Goldberg *et al.*, 1993; Martin *et al.*, 1993). MucA is an inner membrane protein, and its N-terminal domain in the cytoplasm sequesters AlgT/U and prevents its activity (Mathee *et al.*, 1997; Xie *et al.*, 1996). Most mucoid CF isolates acquire mutations in *mucA,* and 84% of these harbor a common allele known as *mucA22* that has a deletion of one nucleotide in a stretch of five guanine nucleotides located at position 426-430 of the open reading frame (ORF) resulting in premature translation termination (Boucher *et al.*, 1997b; Bragonzi *et al.*, 2006; Martin *et al.*, 1993). MucA truncation leads to the constitutive production of alginate. In addition,

inactivation of *mucB* and *mucD* also leads to constitutive alginate production (Boucher *et al.*, 1996; Goldberg *et al.*, 1993; Martin *et al.*, 1993; Schurr *et al.*, 1996).

In *P. aeruginosa*, MucE accumulates in response to cell wall stress (Step 1, Figure. 1.7(B)) then the site-1 RIP serine-protease AlgW is activated to clip the C-terminus of MucA between amino acids Ala_{136} - Gly_{137} in the periplasm (Step 2, Figure. 1.7(B)) (Cezairliyan and Sauer, 2009; Qiu *et al.*, 2007). Then, the site-2 zinc-metalloprotease MucP cleaves MucA within the inner membrane (Step 3, Fig. 1.7(B)) but the exact cleavage site remains to be elucidated (Damron *et al.*, 2011; Wood and Ohman, 2009). The MucA fragment tethered to AlgT/U is further processed by a cytoplasmic protease protein complex ClpXP with the aid of the SspA protein, finally releasing the sigma factor AlgT/U (Qiu *et al.*, 2008a; Yin *et al.*, 2013). The MucA C-terminus that lies on the periplasmic side is presumed to bind MucB (Cezairliyan and Sauer, 2009; Mathee *et al.*, 1997). It has been shown *in vitro* that the presence of MucB in the reaction mixture prevents the cleavage of MucA by the periplasmic protease AlgW (Cezairliyan and Sauer, 2009). MucD is a chaperone/protease that controls periplasmic protein quality and can trigger the degradation of MucA upon detection of abnormal protein accumulation (Damron and Yu, 2011; Wood and Ohman, 2006). In the *mucD* mutants, an alternate RIP pathway appears to be activated in which MucP directly cleaves MucA without pre-cleavage by AlgW (Damron and Yu, 2011; Qiu *et al.*, 2007). The role of MucC is currently controversial: reportedly being a positive and a negative regulator of alginate production (Boucher *et al.*, 1997a; Ohman, 1996). AlgO is a serine periplasmic protease that positively affects alginate production in P. aeruginosa; however, its precise role in the RIP cascade remains to be elucidated (Reiling *et al.*, 2005; Sautter *et al.*, 2012). AlgO, AlgW, and MucD contain PDZ

(PSD-95/Discs-large/ZO1) domains that are involved in the protein-protein interaction (Ponting, 1997). In the wild-type background, the release of AlgT/U requires the sequential degradation of MucA through a process known as **r**egulated **i**ntramembrane **p**roteolysis (RIP) (Figure. 1.7), a process that is conserved from bacteria to humans (Ruiz *et al.*, 2008; Wood and Ohman, 2009).

Besides mutations in biosynthetic and regulatory genes, alginate production in *P. aeruginosa* can be suppressed by various means that includes increasing the copy number of genes such as *rpoD*, prolonged growth in the presence of sulfate ions or without aeration (Min *et al.*, 2014; Ohman and Chakrabarty, 1981; Yin *et al.*, 2013). The reversion phenotype has been exploited to study the regulation and to find novel genes associated with alginate production (Devries and Ohman, 1994b; Min *et al.*, 2014; Sautter *et al.*, 2012). The reversion from mucoid to nonmucoid, when grown without aeration, was mainly mapped to mutations in *algT/U* and *algO* (Devries and Ohman, 1994a; Sautter *et al.*, 2012). In this study, the reversion phenomenon was further exploited to map novel genes using a genetically well-defined constitutively mucoid strain, PDO300, with a *mucA22* allele derived from the prototypic nonmucoid PAO1 strain to isolate nonmucoid variants that are termed **s**uppressor of **a**lginate **p**roduction (*sap*) mutants (Mathee *et al.*, 1999; Sautter *et al.*, 2012). One of the previously uncharacterized *sap* mutants, *sap27*, was used in this study. Complementation and mutational analysis led to the identification of *lptD* (*PA0595*), encoding an outer membrane protein as responsible for restoring the mucoid phenotype. However, *sap27* had no mutation in *lptD*, but in *algO*. This study will explore the role of LptD and AlgO in alginate production.

METHODS

Bacterial strains, plasmids, media and primers

The *E. coli* and *P. aeruginosa* strains and plasmids used are listed in Table 2.1. *E. coli* was grown in Luria-Bertani (LB) medium supplemented with chloramphenicol (Cm, 10 μg ml^{-1}), gentamicin (Gm, 15 μg ml^{-1}), and kanamycin (Km, 25 μg ml^{-1}) as required. *P. aeruginosa* was grown in LB or LB-PIA (at 1:1 LB agar and Pseudomonas isolation agar (PIA) (Mathee *et al.*, 1997). The LB-PIA media was used to select for trans conjugants. Tetracycline (Tc, 100 μg ml^{-1}), and gentamicin (Gm, 100 μg ml^{-1}) were used for *P. aeruginosa*. Cultures were grown at 37°C unless otherwise stated.

DNA manipulations

All molecular techniques were performed according to standard protocols (Sambrook *et al.*, 1989). DNA sequencing was performed at Genewiz. Inc. (South Plainfield, NJ). All primers (Table 2.2) used in the experiments were synthesized and supplied by Integrated DNA Technologies (Coralville, IA).

Triparental mating

Plasmids and cosmids were conjugated into *P. aeruginosa* via triparental mating using mobilizer plasmids pRK2013, and pRK600 (Figurski and Helinski, 1979; Finan *et al.*, 1986). Trans conjugants were then selected for on LB-PIA (1:1) plates containing Tc (100 μg ml^{-1}) or Gm (100 μg ml^{-1}).

Isolation of *sap* mutants

Thirty-four nonmucoid *sap* mutants were isolated in a previous study, and a subset of those was characterized earlier (Sautter *et al.*, 2012). One of the uncharacterized mutants, *sap27,* is the subject of this study.

Complementation of *sap*27

En masse complementation was performed using the minimal tiling path (MTP) library (PAO1 library that contains 336 clones covering 93.7% of the genome) obtained from Paul Phibbs, East Carolina University (Huang *et al.*, 2000). The 334 clones were previously pooled into four donor pools, each of which originated from the four-microtiter plates (MTP1-MTP96, MTP97-192, MTP193-MTP288, and MTP289-MTP334) used for the library's storage (Sautter *et al.*, 2012). To score for mucoidy, LB:PIA plates with and without glycerol were used to reduce false positives. After one-two days of growth at 37°C, colonies were restreaked to confirm the mucoid phenotype. The identity of the cosmid was confirmed as described previously (Sautter *et al.*, 2012). The ability of the cosmid pMO012217 to complement was further confirmed by using the original MTP strain — MTP36 (Table 2.1).

Table 2.1. Bacterial strains and plasmids used in this study

Strains/plasmids	Genotype/description	References
Strains		
E. coli		
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44Φ80Δ(lacZ)M15 gyrA96 recA1relA1 endA1 thi-1 hsdr17*	New England Biolabs
MTP36	Tc^R; pMO012217 (PAO1 fragment 637674–658948)	(Huang *et al.*, 2000)

P. aeruginosa		
PAO1	Alg⁻; Prototypic strain, nonmucoid	(Holloway, 1955)
PDO300	Alg⁺; PAO*mucA22*, constitutively mucoid	(Mathee *et al.*, 1999)
PKM800	Alg⁻; *mucA22 algO107* (a derivative of PDO300 (*sap22*))	Previously called *algO96*; (Sautter *et al.*, 2012)
PKM817	Alg⁻; *mucA22 algO107* (a derivative of PDO300 (*sap27*))	This study
PKM832	Alg⁻; Δ*algO* (PAO1 derivative with a clean in-frame deletion of *algO*)	PAOΔ*algO*; HK403; This study
PKM833	Alg⁻; *mucA22* Δ*algO* (PDO300 derivative with a clean in-frame deletion of *algO*)	PDOΔ*lagO*; HK404; This study
PKM834	Alg⁻; Δ*algW* (PAO1 with clean in-frame deletion of *algW*)	PAOΔ*algW*; HK409; This study
PKM835	Alg⁻; *mucA22* Δ*algW* (PDO300 derivative with a clean in-frame deletion of *algW*)	PDOΔ*algW*; HK410; This study
Plasmids		
pAlgO	Tc^R; derivative of pVS1 with *algO*	pRTS6000; (Sautter *et al.*, 2012)
pLAFR3	Tc^R; Cloning vector	(Staskawicz *et al.*, 1987)
pRK600	Cm^R; *colE1 tra⁺ rk2 mob⁺*; Helper plasmid for conjugation	(Finan *et al.*, 1986)
pRK2013	Km^R; *colE1 tra⁺ rk2 mob⁺*; Hplasmid for conjugation	(Figurski and Helinski, 1979)
pMQ72	Gm^R; *colE1;* Expression vector containing arabinose promoter	(Shanks *et al.*, 2006)

pEXG2	Gm^R; *colE1*, *oriT mob$^+$ sacB$^+$*; *P. aeruginosa* suicide vector with sucrose counterselection	(Rietsch *et al.*, 2005)
pMQ30	Gm^R; *colE1*, *oriT sacB$^+$*; *P. aeruginosa* suicide with sucrose counter selection	(Shanks *et al.*, 2006)
pSP50	Gm^R; *colE1*; A pMQ72 derivative with a ~ 5.01-kb fragment containing *lptD* operon downstream of P$_{BAD}$	pLptD-operon; This study
pSP51	Gm^R; *colE1*; A pMQ72 derivative with a ~ 2.77-kb fragment containing *lptD* downstream of P$_{BAD}$	pLptD; This study
pSP155	Gm^R; *colE1*; A pMQ72 derivative with a ~ 1.29-kb fragment containing *surA* downstream of P$_{BAD}$	pSurA; This study
pSP156	Gm^R; *colE1*; A pMQ72 derivative with a ~ 0.98-kb fragment containing *pdxA* ORF downstream of P$_{BAD}$	pPdxA; This study
pSP157	Gm^R; *colE1*; A pMQ72 derivative with a ~ 4.04-kb fragment containing *lptD-surA* downstream of P$_{BAD}$	pLptD-SurA; This study
pSP158	Gm^R; *colE1*; A pMQ72 derivative with a ~ 2.37-kb fragment containing *optP* downstream of P$_{BAD}$	pOptP; This study
pSP160	Gm^R; *colE1*; A pMQ72 derivative with a ~ 0.62-kb containing *lptE* downstream of P$_{BAD}$	pLptE; This study
pSP161	Gm^R; *colE1*; A pSP157 derivative with *lptE* ORF from pSP160 subcloned downstream of *lptD-surA* creating an operon with *lptD-surA-lptE* downstream of P$_{BAD}$	pLptD-SurA-LptE; This study
pSP162	Gm^R; *colE1*; A pMQ72 derivative with a 2.77-kb containing *lptD* fused to six histidine residues at C-terminus and cloned downstream of P$_{BAD}$	pLptD-His$_6$; This study

Mutagenesis of pMO012217

The *sap*27 mutant strain with the pMO012217 cosmid has the mucoid phenotype. This phenotype in the trans conjugants would be lost if the gene responsible for the complementation in the cosmid is disrupted by transposon mutagenesis. To accomplish this, the pMO012217 cosmid was mutagenized *in vitro* using the EZ::TN transposon according to the manufacturer's protocol. A 200 nanogram aliquot of pMO012217 was subjected to the EZ::Tn5 <KAN-2> insertion reaction for six hours (Epicenter

Biotechniques, Madison, WI) followed by transformation into *E. coli* DH5α cells. The

transformants were cultured on a plate-containing Km (30 μg ml^{-1}). More than 500

transformants were pooled as the donors for triparental mating with *sap27*. The trans

conjugants were screened for the non-mucoid phenotype. Then, the mutagenized cosmid

was isolated from *sap27,* and the transposon junction was sequenced using the KAN-2 FP-

1 primer (Table 2.2) to identify the disrupted ORF.

Table 2.2. Primers used in this study

Primers Name	Sequences (5′ - 3′)
SP_OptP_(F)	**ACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCG GTACCCGGGGAAGGAGAT**ATACATATGAAGCAC CACTACCGCCT
SP_OptP_(R)	**AATCTTCTCTCATCCGCCAAAACAGCAAGCTTG CATGCCTGCAG**TCAGAAGTCGTAGCGCAGCGA
SP_Seq_OptP1	CTATAATCACGGCAG
SP_Seq_OptP2	ACAACGTCTACCTGG
SP_Seq_OptP3	CGCCAAGGTCGAGCG
SP_Seq_OptP4	CTGATTTAATCTGTA
SP_Seq_OptP5	CCGCAGGGCACGGGG
SP_Seq_OptP6	GACCTTCCTGCGCTG
SP_Seq_OptP7	TGCCCTGCGGACCGC
SP_Seq_pMQ72_(F)	CTAACCAAACCGGTAACCCC
SP_Seq_pMQ72_(R)	AATTGATGCCTGGCAGTTCC
SP_LptD_Operon_(F)	**ACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCG GTACCCGGGGAAGGAGAT**ATACATATGGCAGTG AAATCCCTCGTG
SP_LptD_Operon_(R)	**AATCTTCTCTCATCCGCCAAAACAGCCAAGCTT GCATGCCTGCAG**TCAGCAGCGGCTGGCCGCCATC TG
SP_LptD_(F)	ggctag*gaattc*ctaggagatataccATGGCAGTGAAATCCCTC GTGTTTCGAA
SP_LptD_(R)	acgttcat*cccggg*TTACATAGCTTGATCTTCACGTTGACG
SP_LptD-SurA_(F)	ggctag*gaattc*ctaggagatataccATGGCAGTGAAATCCCTC GTGTTTCGAA

SP_LptD-SurA_(R)	acgttcat*cccggg*TCACTGCTTGATTTCCACGTAGGCCTC
SP_SurA_(F)	**ACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCG GTACCCGGGGAAGGAGAT**ATACATATGAAGATC AAGCTATGTAAC
SP_SurA_(R)	**AATCTTCTCTCATCCGCCAAAACAGCCAAGCTT GCATGCCTGCAG**TCACTGCTTGATTTCCACGTAG GCCTC
SP_PdxA_(F)	**ACCCGTTTTTTTGGGCTAGCGAATTCGAGCTCG GTACCCGGGGAAGGAGAT**ATACATATGAGCCTG CGCTTCGCGCTC
SP_PdxA_(R)	**AATCTTCTCTCATCCGCCAAAACAGCCAAGCTT GCATGCCTGCAG**TCAGCAGCGGCTGGCCGCCATC TG
SP_JuntSurAPdxA_(R)	TTGATCACGCCCTTGTGCA
SP_LptD_Seq1	AAATCCCGCAGTGCGGACTA
SP_LptD_Seq2	ACAACGCCGAGTACGTGATCCA
SP_LptD_Seq3	AAGGCGAGTTCCGCTACCTGA
SP_LptD_Seq4	GGAATGCAATTCACCTATGGCA
SP_LptD_Seq5	CCATGTACTTGTACGTTCCAT
SP_LptD_Seq6	GGTAGCGCCATCTTCCACTA
SP_LptD_Seq7	GTAGGCAACAAGACGGAAATG
SP_LptD_Seq8	CGGTGGCAGGTTGGCATT
SP_LptD_Seq9	TAGTCCGCACTGGGGGATTT
SP_LptD_seq10	GGGAGCAATCCCAG
SP_SurA_Seq1	GTGCACCAGACGCTG
SP_SurA_Seq2	GGCCAATATCCTGATC
SP_SurA_Seq3	CTGCTCAAGCCCAGC
SP_SurA_Seq4	CCGTCGCGGGTGAGGG
SP_SurA_Seq5	GCCGAACTGCGAGCGG
SP_PdxA_Seq1	TTCCGGCCATACCGAA
SP_PdxA_Seq2	CAGACCAGGATGCGCGGAT
Cos-1	CGCCCTCTGGTAAGGTTG
pLA2	CCTGTCTCTTGATCAAGATC
KAN-2 FP-1	ACCTACAACAAAGCTCTCATGAACC
HK_AlgW_DR	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGT TCTGATTGGATGAAAGGTTCCGCA
HK_AlgW_DF	CCCCAAGGATTTTCCCGATGCTAGCTAACTAGTCC CCGTCATTGAAAACGGC

HK_AlgW_UR	GCCGTTTTCAATGACGGGGACTAGTTAGCTAGCAT CGGGAAATCCTTGGGG
HK_AlgW_UF	GGAATTGTGAGCGGATAACAATTTCACACAGGAA ACAGCTGTCTGCTCGGAAACCTCGCC
HK_AlgW_P1_FW_Se q	TAATGGACGGCTTCCCCCTA
HK_AlgW_P2_RE_Seq	CAGGGAGTCATCGTCCGCCA
HK_AlgW_P3_FW_Ex t	AGCGCCTGTACCCCGTAGC
HK_AlgW_P3_RE_Ext	GCTGTTTCCGGACGTGACCTAC
SP_AlgO _(F)	ATTAATAAGCTTGCTGCCTCCATAGTGGG
SP_AlgO_(R)	AATTATAAGCTTTTATGACGCTCCCGCTGAACT
CC_AlgO_P1_(F)	AAGCTTAGCAACTCCAGGTTCAACCCGG
CC_AlgO_P1_(R)	GCTAGCGCTGATTCGACTGATCGCGGAGT
CC_AlgO_P2 _(F)	GCTAGCGTTCAGCGGGAGCGTCATAAA
CC_AlgO_P2_(R)	GGATCCCGAGGTGCGAACGTCGACCGCG
CC_AlgO_Seq1	GGCGCTGCCGACGGAGAC
CC_AlgO_Seq2	GCAACAGGTTCGACGACT
CC_AlgO_Seq3	CGGACAACGCGGAAAACT
CC_AlgO_Seq4	CTACAAGCTCGGCGTGAT
CC_AlgO_Seq5	ACCGTGCAGACCATCCAGC
CC_AlgO_Seq6	CCAGTTGGCCATGGAGAACG
CC_AlgO_Seq7	CATCGACCAGGGCCCGACCG
CC_AlgO_Seq8	GTGTCCCAGGGCATCGAGTCG
CC_AlgO_Seq9	TGGCGATGACCTGCTCGCGG
CC_AlgO_Seq10	GTCCAGGTAGAAGGCGGGCA
CC_AlgO_Seq11	GAGCCGACCTTTCAGGCGG
SP_LptE_(F)	ggctag*cccggg*taggagatataccATGAAACGTATCCTGACCA GCGCCGCGC
SP_LptE_(R)	cagcc*aagctt*TCACGGGGTGGGGAACTCGATCGG
SP_LptD-HindII_6xHis (R)	GCA*aagctt*TTA**ATGATGATGATGATGATG**CATAGC TTGATCTTCACGTTGACG

Alginate assay

In brief, cells from a single plate were harvested after growth in LB for 36 hr and resuspended in 5 ml PBS (pH 7.4) and the OD600 was determined. Then, the cell suspension was centrifuged at 12,000 X *g* for 30 minutes. The supernatant was collected, and the alginate was precipitated using 5 ml of 2% (w/v) cetylpyridinium chloride. The mixture was centrifuged at 12,000 X *g* for 10 minutes and the pellet was resuspended in 5 ml of 1 M NaCl and 5 ml of cold (-20 °C) isopropanol. The suspension was again centrifuged at 12,000 X *g* for 10 minutes and the pellet was dried at 37 °C for 30 minutes to evaporate any residual isopropanol. Finally, the pellet was resuspended in 5 ml of 0.9% (w/v) NaCl. To determine the alginate concentration, a set of standards was made with sodium alginate (Sigma-Aldrich, St. Louis, MO). The amount of alginate was determined using a borate/carbazole method. A 70-µl-alginate sample was mixed with 600 µl of ice-cold borate solution then immediately placed on ice and was followed by the addition of 20 µl of 0.1% carbazole and mixed briefly. Then the mixture was heated for 30 minutes at 55 °C, and the absorbance was measured at 530 nm. The absorbance was compared with standards to determine the alginate concentration which was expressed in mg per ml supernatant and normalized to the bacterial $OD_{600} = 0.41$ (Qiu *et al.*, 2007). All the alginate assays were performed in triplicate.

Construction of plasmids containing the ORF *lptD*-operon, *surA*, *pdxA*, and *optP*

The ORFs *surA, pdxA* and *optP* were amplified from the genomic DNA of PAO1 using standard procedures (Sambrook *et al.*, 1989). The primer pairs SP_LptD_Operon_(F), and SP_LptD_Operon_(R), SP_SurA_(F) and SP_SurA_(R),

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SP_PdxA_(F)-SP_PdxA_(R), and SP_OptP_(F)-SP_OptP_(R), (Table 2.2) were used to amplify the *lptD*-operon (5.01 kb), *surA* (1.29 kb), *pdxA* (0.98 kb), and *optP* (2.373 kb), respectively. The forward primers used contained a ribosome-binding sequence upstream of the start codon of the respective ORFs as well as 45-bp tails (shown in bold, Table 2.2) that target recombination downstream of the P_{BAD} promoter on pMQ72 that has pRO1600 and *colE1* origins of replication (Shanks *et al.*, 2006). The respective reverse primers also have 44-bp tails (shown in bold, Table 2.2) that target recombination upstream of T1T2 transcriptional terminators present in the vector (Shanks *et al.*, 2006). The vector pMQ72 was digested with restriction enzyme *Sma*I. Then the digested vector pMQ72 along with PCR product were co-transformed into overnight cultures of *Saccharomyces cerevisiae* (Shanks *et al.*, 2006). The transformed cells were individually tested for the desired construct by colony PCR using the primer pair SP_Seq_pMQ72_(F)— SP_Seq_pMQ72_(R) for the presence of an insert (Shanks *et al.*, 2006; Shanks *et al.*, 2009). *E. coli* DH5α was then transformed with the recombinant pMQ72 plasmids containing their respective ORFs and screened for Gm resistance. The amplified ORFs from the recombinant plasmids were sequenced to ensure fidelity (Table 2.2). The plasmids with P_{BAD} driving the expression of *lptD*-operon, *surA, pdxA,* and *optP* are pSP50, pSP155, pSP156, and pSP158, respectively (Table 2.1). These plasmids henceforth will be referred to as pLptD-operon, pSurA, pPdxA, and pOptP, respectively.

Construction of plasmids containing the *lptD, surA* and *lptE*

The ORFs, *lptD* (2.77 kb), *lptD-surA* (4.048 kb), and *lptE* (0.62 kb), were amplified from PAO1 genomic DNA using the primer pairs SP_LptD_(F)—SP_LptD_(R),

SP_LptD-SurA_(F)—SP_LptD-SurA_(R), and SP_LptE_(F)—SP_LptE_(R), respectively (Table 2.2). The amplified product *lptD* and *lptD-surA* were digested with the enzymes *Eco*RI and *Xma*I then ligated downstream of P$_{BAD}$ in pMQ72 (Shanks *et al.*, 2006) cut with same restriction enzymes. Then, the resulting plasmids harboring *lptD* and *lptD-surA* genes were named as pSP51 and pSP157, respectively.

The PCR-amplified product of *lptE* was restriction digested by enzymes *Xma*I and *Hin*dlll then ligated downstream of P$_{BAD}$ in pMQ72 digested with same restriction enzymes. Then the *lptE* harboring plasmid was named as pSP160. The *lptE* fragment of pSP160 was subcloned into plasmid pSP157 downstream of *lptD-surA* using restriction enzymes *Xma*I and *Hin*dIII to create pSP161. The plasmid pSP161 contains *lptD-surA-lptE* under the control of P$_{BAD}$.

All the constructs were sequenced to ensure fidelity using the appropriate primers shown in Table 2.2. The constructs, pSP51, pSP157, pSP160 and pSP161, henceforth will be referred to as pLptD, pLptD-SurA, pLptE and pLptD-SurA-LptE, respectively.

His-tagging of LptD

A 2.77-kb fragment with *lptD* was amplified from PAO1 genomic DNA using the primer pairs SP_LptD (F) and SP_LptD-6xHis (R) (Table 2.2). The latter primer has six histidine codons added in frame to the 3' end of the coding sequence of *lptD*. The amplified product with *lptD*-His$_6$ was then ligated downstream of P$_{BAD}$ in pMQ72 to construct pSP162 (pLptD-His$_6$). The cloned fragment was sequenced for fidelity. The plasmid pLptD-His$_6$ was introduced into the *sap27* strain by standard electroporation (Choi *et al.*, 2006).

Western blotting

Cells were grown to an OD_{600} of 0.4 and induced with 2 % arabinose for 2 hours at 25 °C. Then the cells were harvested by centrifugation at the speed of 5,000 rpm at 4°C for 10 minutes. The pellets were resuspended in 10 ml lysis buffer (20 mM Tris [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 1X Roche complete protease inhibitor cocktail, lysozyme [0.5 mg/ml]). After lysis by sonication (15 cycles of 10-s pulse on and 30-s pulse off; amplitude, 40%), the cell lysates were centrifuged 5,000 rpm for 10 minutes at 4 °C. Two ml of the cell lysate from each sample was saved as the total cell fraction, and remaining were further fractioned by ultracentrifugation at 36,000 rpm (SW 41 Ti rotor) for one hour at 4°C. The supernatants were saved as soluble (cytoplasmic and periplasmic) fraction whereas pellets were resuspended with 1 ml of membrane buffer (20 mM Tris-Cl [pH7.5], 0.2 % Sodium lauroyl sarcosinate [Sarkosyal; Fluka], 0.1 % Sodium dodecyl sulfate [SDS]). One hundred ml of the whole cell, periplasmic/cytoplasmic and membrane fractions were aliquoted and stored at -80°C. The concentration of protein was determined by the bicinchoninic acid method (Smith *et al.*, 1985). Ten micrograms of total protein were separated on a 12% SDS-polyacrylamide gel. The presence of LptD and σ^{70} (loading control) was detected using anti-His$_6$ (Thermo Scientific, Rockford, IL) and anti-σ^{70} (Neoclone Biotechnologies International, Madison, WI) antibodies. The fluorescent secondary antibody (Anti-mouse IgG (H+L) (DyLight™ 680 Conjugate) was purchased from Cell Signaling Technology, Inc (Danvers, MA). The fluorescence was detected using Odyssey CLx near Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Construction of Δ*algO* and Δ*algW* strains

An in-frame deletion of *algO* was constructed using overlap extension PCR and homologous recombination as previously described (Horton *et al.*, 1990). The upstream and downstream flanking regions of *algO* using the primer pairs CC_AlgO_P1_(F)—CC_AlgO_P1_(R) and CC_AlgO_P2_(F)—CC_AlgO_P2_(R), respectively were amplified and fused to construct the deletion (Table 2.2). The PCR products were then ligated through another round of PCR and cloned into the suicide vector pEXG2 (Rietsch *et al.*, 2005). The recombinant plasmid was introduced into PAO1 and PDO300, where the deletion replaced the wild-type *algO* on the chromosome via homologous recombination using a two-step process, involving screening by Gm sensitivity followed by sucrose resistance. PCR amplification of the region spanning the deletion and sequencing confirmed the absence of *algO*. These deletion mutants were called PAOΔ*algO* and PDOΔ*algO*.

A gene-splicing method using the yeast system was used to generate a precise in-frame deletion of *algW* in *P. aeruginosa* (Shanks *et al.*, 2006). Upstream (P1) and downstream (P2) flanking regions of *algW* were amplified from PAO1 genomic DNA by PCR using the primer pairs HK_AlgW_UF—HK_AlgW_UR and HK_AlgW_DF—HK_AlgW_DR (Table 2). The primers HK_AlgW_UF and HK_AlgW_DR had regions of homology to target recombination of P1 and P2 with pMQ30 vector (Shanks *et al.*, 2006). The primer pairs also had complementary sequences at the 3′ end to facilitate joining to create the P3 fragment, as well as stop codons (5′-CTAGTTAGCTAG-3′) to prevent translational read through. The pMQ30 vector has double selection markers *URA3* for yeast and Gm^R for *E. coli* (Shanks *et al.*, 2006). Yeast cells were transformed with the P1,

P2 and *Bam*Hl-digested linearized pMQ30 using standard protocols and colonies were selected on sucrose-uracil plates. The yeast colonies were checked for the presence of the P3 construct for *algW* deletions by amplifications using primer pairs HK_AlgW_UF—HK_AlgW_DR. Yeast DNA was isolated from the positive colonies as previously described (Shanks *et al.*, 2009). *E. coli* DH5α was transformed with the recombinant pMQ30 plasmids containing P3 and screened for Gm resistance. The amplified P3 from the recombinant plasmids were sequenced to ensure fidelity. The constructs were then conjugated into PAO1 using triparental mating and screened for single and double crossovers using counter selection with sucrose and Gm, as previously described (Shanks *et al.*, 2006; Shanks *et al.*, 2009). The presence of the gene deletion in all the mutants was confirmed using standard molecular methods. The strain is henceforth referred to as PAOΔ*algW*. Using similar strategies Δ*algW* in mucoid PDO300 was constructed and named PDOΔ*algW*.

RNA isolation and cDNA synthesis

RNA was isolated from *P. aeruginosa* wild-type PAO1 as described previously (Balasubramanian *et al.*, 2014). Briefly, 10-ml overnight cultures grown in LB broth at 37°C were washed with 0.85% saline solution to remove spent media. Then the cells were sub-cultured in 15 ml by inoculating the culture in LB media at an OD_{600} of 0.01 at 37°C at 200 rpm. Subsequently, the cells were harvested when the culture reached an OD_{600} of 0.5-0.6. The RNA was stabilized by addition of phenol-ethanol mixture as described previously (Brencic *et al.*, 2009). Stabilized RNA was then isolated using RNeasy Mini Kit (Qiagen Inc, Germantown, MD, USA) as per the manufacturer's protocol. Residual

genomic DNA contamination was removed using RQ1 RNase-free DNase (Promega, Madison, WI, USA) and RNA was repurified using RNeasy Mini Kit (Qiagen Inc, Germantown, MD, USA) The quality of purified RNA was assessed on a denaturing agarose gel (NorthernMax Gly, Ambion, Life Technologies, Carlsbad, CA) and quantified at OD_{260} (BioTEK®, Synergy™ HT, Winooski, VT, USA). cDNA was then synthesized by annealing NS5 random primers to total purified RNA, and subsequent extension was carried out using SuperScript III reverse transcriptase (Invitrogen™, Life Technologies, Carlsbad, CA, USA).

Results

Identification of the *sap27* complementing cosmid

The *sap27* mutant is a nonmucoid revertant isolated by growing mucoid PDO300 without aeration (Sautter *et al.*, 2012). The mucoid phenotype in *sap27* was restored via *en masse* complementation with the PAO1 minimum tiling path (MTP) genomic cosmid library (Huang *et al.*, 2000). The complementing cosmid was extracted and the junction where the *P. aeruginosa* genome fragment is integrated was sequenced to identify the *P. aeruginosa* genes contained in pMO012217 (PAO1 coordinates: 637674 to 658948; Figure 2.1) (Huang *et al.*, 2000). To ensure that was pMO012217 was the complementing cosmid, *sap27* was re-transformed by conjugation with *E. coli* containing the original cosmid clone pMO012217 from the MTP library (MTP36) (Figure 2.3(a)). As negative controls, PAO1 and *sap27* were transformed with vector pLAFR3. As expected, PAO1 with the vector remained nonmucoid. The positive controls, the Alg⁺ parent PDO300 with the cosmid and plasmid vectors, remained mucoid when transformed individually. The

cosmid pMO012217 restored the mucoid phenotype in *sap27* (Figure 2.3(a)). The pMO012217 cosmid contained 18 ORFs (Figure 2.1) and did not harbor any known alginate genes indicating that a novel ORF was involved in the restoration of alginate production.

Mapping the gene in the complementing cosmid pMO012217 using transposon (Tn) mutagenesis
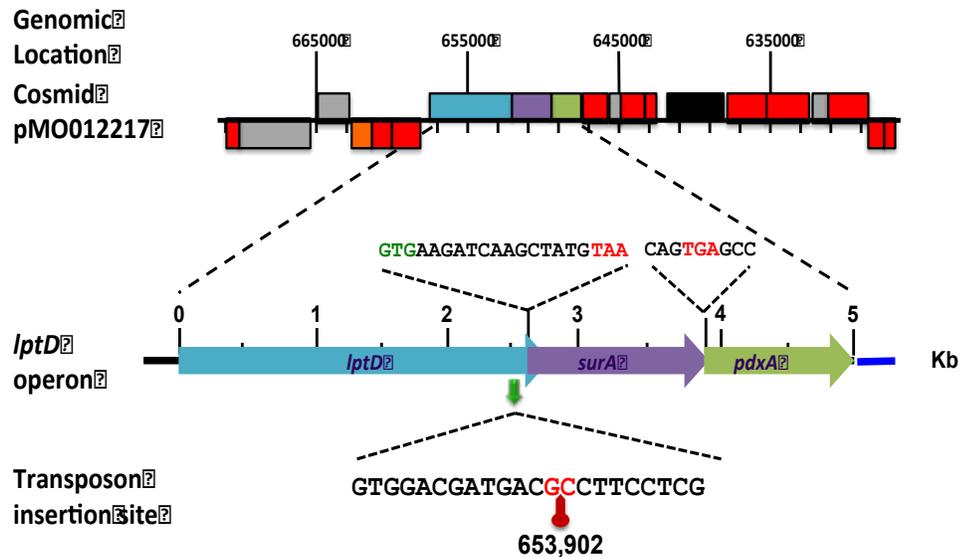
Figure 2.1. Genetic map of cosmid pMO012217 and *lptD*-operon. The cosmid was identified in the *en masse* complementation of *sap27* with the MTP library, which restored the mucoid phenotype. The cosmid contains 18 ORF. The figure of the cosmid was retrieved from pseudomonas.com and reversed to make it easier for observation because the *lptD* operon lies in negative strand of the chromosome of *P. aeruginosa*. Through sequencing, we identified the transposon was inserted at the end of *lptD*, which is marked by an orange arrow. The *in silico* sequence analysis shows that the 3' of the *lptD* overlaps with 5' end of *surA*, which is shown by dotted line [start codon [GTG] of *surA* begins before stop codon [TAA] of *lptD*] and stop codon of *surA* [TGA] and start codon [GTG] of *pdxA* overlaps [GTGA] also shown by dotted line that originate from intersection of *surA* and *pdxA*. The operon was cloned into an expression vector pMQ72 and named as pLptD-operon. Plasmids pLptD-operon, pLptD, pSurA, and pPdxA are pMQ72-derived plasmids with *lptD*-operon, *lptD*, *surA*, and *pdxA* insertions, respectively. The plasmids were constructed as described in the materials and methods. The red arrow shows the transposon insertion site in *lptD* gene and the genomic position.

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The rationale for the Tn mutagenesis is that the loss of mucoidy in the trans conjugants would be due to the disruption of the complementing ORF present in the cosmid.

To identify the particular ORF responsible for the mucoid conversion, pMO012217 was mutagenized *in vitro* using the EZ::TN transposon (Epicenter Biotechniques, Madison, WI). The transposon mutant library was introduced into *sap27* and screened for the loss of the mucoid phenotype in the trans conjugants. Sequencing of the Tn junction in the constructs that failed to restore the mucoid phenotype using the cos-1 primer (Table 2.2) revealed the insertion to be at the $3'$ end of *lptD* (PA0595) at position 653,902 of *P. aeruginosa* PAO1 genome (Figure 2.1). This insertion disrupts the alanine codon at the position 876 of LptD, truncating the protein.

Analysis of the *lptD* locus

Analysis of the locus suggests that *lptD* might be a part of the three-gene operon (Winsor *et al.*, 2016). Thus, the Tn insertion could have a polar effect on the downstream genes, namely *surA* and *pdxA*. In *E. coli,* it has been shown that SurA is an essential protein for survival in starvation and a major chaperone protein for all outer membrane protein. In *E. coli,* PdxA is a NAD-dependent dehydrogenase and is involved in the vitamin B_6 biosynthetic pathway (Banks and Cane, 2004; Laber *et al.*, 1999). To verify if *lptD-surA pdxA* forms an operon in *P. aeruginosa*, total RNA was isolated from PAO1 in late log-phase, and non-specific primers were used to synthesize cDNA for RT-PCR. The presence of cDNA across the pair of ORFs *lptD-surA* and *surA-pdxA* would suggest that these genes form an operon. The expected PCR products of sizes 1000 bp (Lanes 2, 3 and 5) and 900 bp (Lanes 8, 9 and 11) for *lptD-surA* and *surA-pdxA,* respectively, were observed (Figure

2.2). As expected, there was no product in negative controls when amplification was done without the DNA polymerase (Lanes 7 and 13); or using RNA prepared after DNAse I treatment (Lanes 4 and 10) or without reverse transcriptase (Lanes 6 and 12).

The presence of expected PCR products in the cDNA (Lanes 5 and 11) confirms the existence of mRNA spanning the junctions. These data suggest *lptD, surA* and *pdxA* form an operon. Since the Tn is inserted at the 3′ end of the first gene, *lptD*, at 653,902 in the three-gene operon, it is possible that all three genes may be involved in alginate production (Figure 2.2). Thus, the three ORFs in the *lptD*-operon alone or combination might be responsible for restoring the mucoid phenotype in *sap27*. To determine the ORF
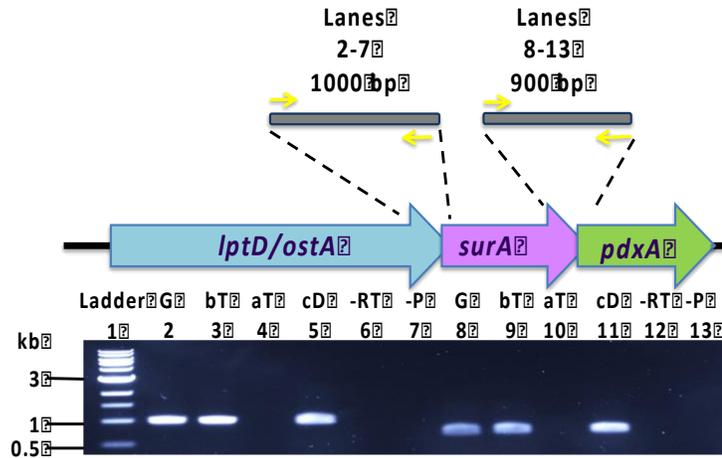
Figure 2.2. Verification of *lptD* is part of a three-gene operon. The PCR amplification of the junctions between the genes, *lptD-surA* and, *surA-pdxA* with specific primer pairs shown in Table 2. The amplified product was run in 1% agarose gel. Lane 1, 1 Kb Ladder [M] as a marker; Lanes 2-7 and Lanes 8-13 is the PCR products of the junction between *lptD-surA* and *surA-pdxA*, respectively. Lanes 2 & 8, PCR products from genomic DNA [G] used as positive control; Lanes 3,4,9, and 10, PCR products before treatment and after treatments (aT) of DNA polymerase; Lanes 5 & 11, PCR products from cDNA [cD]; Lanes 6 & 12, PCR amplification without the reverse transcriptase [-RT] used as negative control; Lanes 7 & 13, PCR amplification in the absence of DNA polymerase [-P] used as negative control.

responsible, *lptD, surA,* and *pdxA* were individually cloned downstream of the P$_{BAD}$

promoter and the plasmids were named pLptD, pSurA, and pPdxA, respectively (Table

2.1). In addition, the entire operon was also subcloned into the same vector (pMQ72). All
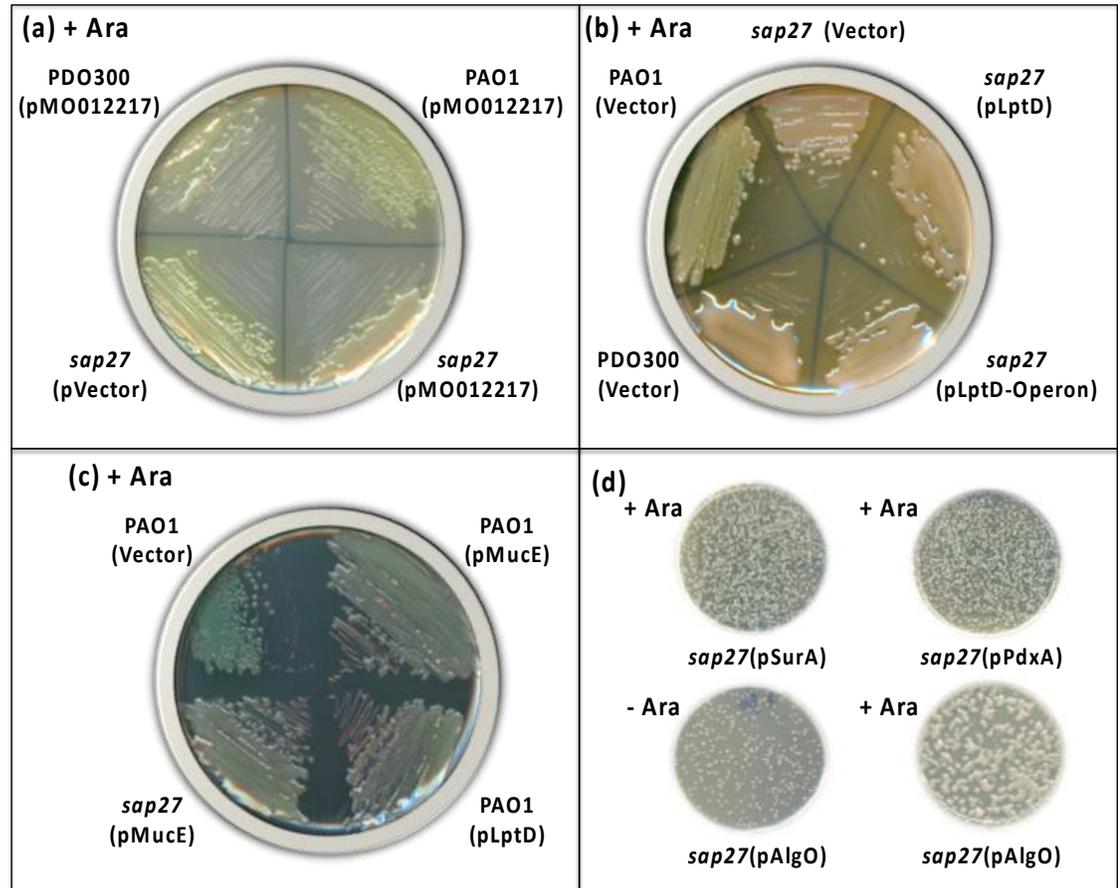
Figure 2.3. Identification and mapping of the gene responsible for restoring mucoid phenotype. Identification of genes responsible for restoring the mucoid phenotype. (a) The cosmid pMO012217 introduced into *sap27* restoring the mucoid phenotype. PAO1 and PDO300 were used as negative and positive controls, respectively. The plasmid pLAFR3 was used as a control vector. (b) The plasmids pLptD and pLptD-operon which harbor *lptD* and *lptD-surA-pdxA* were separately introduced into *sap27* restoring the mucoid phenotype. (c) The plasmids, pMucE and pLptD were introduced into PAO1 and *sap27* inducing a mucoid phenotype. (d) The plasmids, pSurA, pPdxA and pAlgO were introduced into *sap27*. Mucoid phenotype was restored only when *sap27(*pAlgO) was induced with arabinose. The strains were plated on LB containing 2% arabinose (Ara) where indicated and incubated at 37°C for 36 hours.

the constructs were introduced into *sap27* and PDO300. The vector (pMQ72) was also

introduced into *sap27* and PDO300 as negative controls. The plasmid harboring *mucE*

(pMucE) that is known to induce alginate production was introduced into *sap27* and PAO1

as positive controls (Figure 2.3(c))(Qiu *et al.*, 2007). After 36 hr, the mucoid phenotype in

sap27 was restored by the pLptD-operon and pLptD in the presence of arabinose (2%)

(Figure 2.3(b)) but not by pSurA and pPdxA (Fig. 2.3(d)). As expected, the amount of

alginate produced by *sap27* in the presence of *lptD* and *lptD*-operon was comparable to

PDO300 (Figure. 2.4). These findings suggest that *lptD* alone is sufficient to restore the

mucoid phenotype in *sap27*.

Co-expression of *lptD*, *surA*, and *lptE* in *sap27*

The 20 kDa proper folding of LptD in the outer membrane (Chimalakonda *et al.*,

2011; Chng *et al.*, 2010; Grabowicz *et al.*, 2013). In addition, LptD folding also requires

the chaperone protein SurA (Vertommen *et al.*, 2009).One could argue that alginate

production in *sap27* is due to stress induced by the accumulation of misfolded LptD as a

result of increased expression of *lptD* alone without *surA* and/or *lptE*. To rule out the

potential for LptD misfolding, plasmids with *lptD-surA* (pLptD-SurA), and *lptD-surA-lptE*

(pLptD-SurA-LptE) under the control of P_{BAD} were constructed (Table 2.1). These

constructs were introduced individually into *sap27* and the genes were induced with

arabinose. Alginate production was restored in *sap27* in the presence of both of the

plasmids individually (Figure 2.4). This suggests that the mucoid conversion of *sap27* upon

lptD overexpression is not due to misfolding of LptD, instead, it is the function of

increasing the copy number of *lptD*.
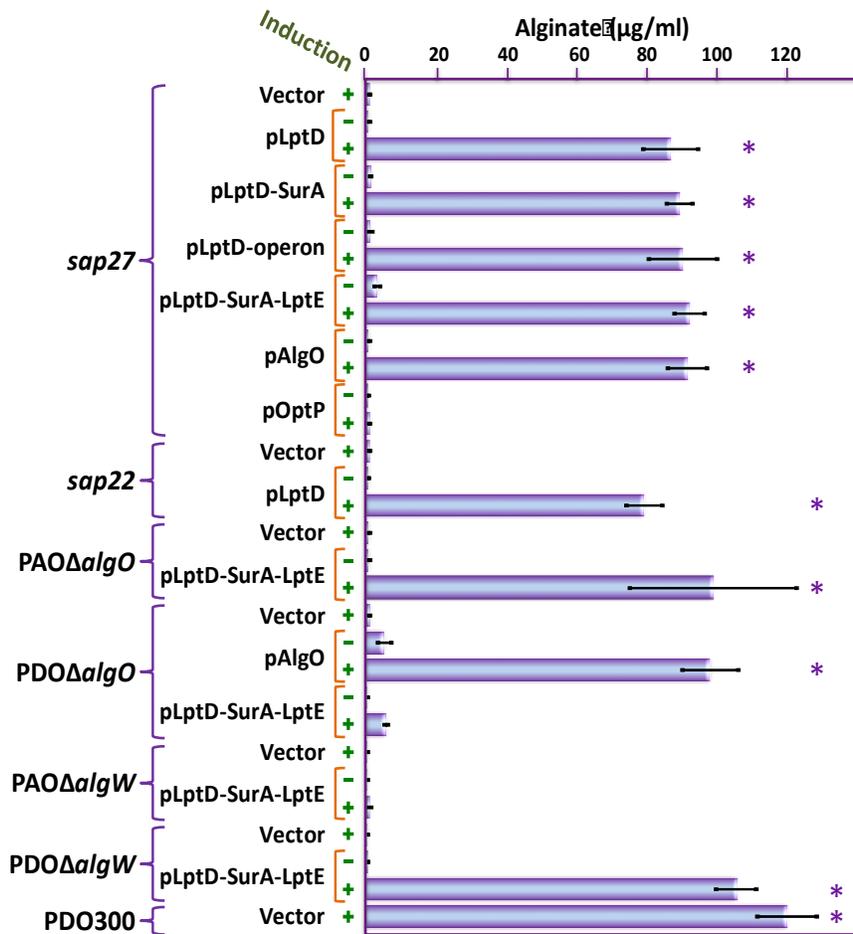
Figure 2.4. Alginate levels in the presence of increased expression of *lptD*. Plasmids containing the *lptD, lptD-surA, lptD-surA-lptE, optP, oprP,* and *algO* were introduced separately into *sap27,* and strains with clean in-frame deletion of *algO* [PAOΔ*algO* and PDOΔ*algO*] and *algW* [PAOΔ*algW* and PDOΔ*algW*]. Cells were grown for 36 hours on LB plates supplemented with gentamycin [100 μg/ml for selection and 2% arabinose as an inducer. The alginate level was quantified and normalized to an $OD_{600} = 0.41$ as described in materials and methods. The strains *sap27* and PDO300 with vectors were used as the negative and the positive control, respectively. The level of significance [p – value] is less than 0.05 for each set of experiment. The experiments were done in triplicates.

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The latter would then imply that the concentration of LptD will be higher in *sap27* if the expression of *lptD* is induced. We confirmed this by His-tagging LptD and Western-blotting using anti-His$_6$ antibody (Figure 2.5). In addition, we also demonstrate that the LptD is only found in the insoluble fraction suggesting membrane localization (Figure 2.5).
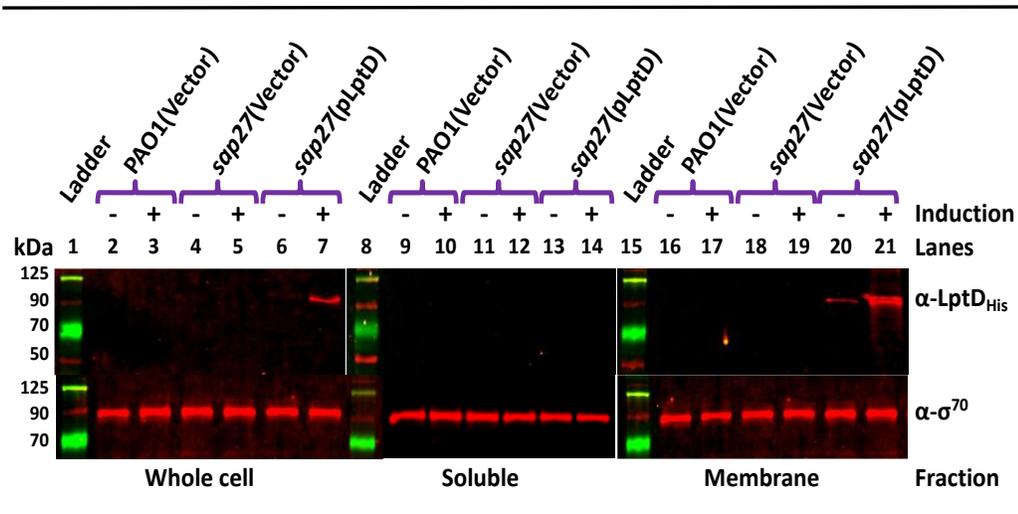
Figure 2.5. Cellular localization of LptD. Equal amounts of total proteins were loaded per well. His-tagged LptD was introduced into *sap27* as described in materials and methods. Whole cell fractions were extracted from cells that were uninduced (-) or induced (+) with 2% arabinose for 3 hours at 25˚C. The induced whole cell extract was further separated into soluble and membrane fraction. The presence of LptD was shown using anti-His$_6$ antibody. Sigma 70 was used as the loading controls. The whole cell and membrane fractions were probed with unconjugated purified anti-*E. coli* RNA sigma 70 antibodies. The soluble fraction was probed with conjugated Direct-Blot™ HRP anti-*E. coli* RNA sigma 70 antibody, thus the ladder that was run parallel was superimposed in this image.

Expression of *optP* encoding a large OMP in *sap27*

To determine if the mucoid restoration of *sap27* is exclusive to *lptD*, *optP* (*PAO192*) was expressed under the P$_{BAD}$ promoter in *sap27*. The protein OptP was chosen

as it is predicted to be a large outer membrane protein (790 aa) comparable in size to the 924-aa LptD (Winsor *et al.*, 2016). Overexpression of *optP* did not turn the *sap27* strain into the mucoid phenotype and consequently, no alginate was detected (Figure 2.4), suggesting that it is not a general function of large OMPs. This suggests that LptD might have potentially a specific role for alginate production in *sap27*.

Mapping of the mutation in *sap27*

The above finding suggested that *sap27* harbors a mutation in *lptD*. To rule out any promoter mutation that may influence *lptD* expression, the entire operon including the promoter region was sequenced in *sap27* and no mutation was found. This suggested that

Amino acid	106	107	108			318	319	320	321
PAO1	GCC	GAG	TTC	.	.	CTG	GAT	GAA	.
	Ala	Glu	Phe	.	.	Leu	Asp	Glu	.
sap27	GCC	GA**T**	GTT	.	.	CCT	GGA	TGA	
	Ala	Asp	Val	.	.	Pro	Gly	*	

Figure 2.6. Mapping the *algo* mutation in *sap27*. The codon numbers of the wild-type *algO* are shown on top. Sequencing of *algO* from *sap27* revealed an insertion of a nucleotide thymine (T) (shown in red) causing a frame-shift at codon 107 leading to premature translational termination of AlgO at codon 320 resulting in a truncated protein. The amino acid sequences from codon 107 to 319 share no similarity to any other *P. aeruginosa* proteins.

the increased expression of *lptD* must be bypassing some other mutation. Our preliminary studies showed that the mucoid phenotype in *sap22* with a mutation in *algO* (*PA3257*) can be rescued by another cosmid (Reiling *et al.*, 2005; Sautter *et al.*, 2012). This led to our hypothesis that *sap27* may harbor a mutation in *algO* (*PA3257*). The introduction of a

plasmid carrying *algO* (pAlgO) into *sap27* rescued the mucoid phenotype (Figure 2.3(d) and Figure 2.4). Ideally, the cosmid containing *algO* should have complemented *sap27* in the original *en masse* screening. However, it was missed in-part due to the subjective nature of the procedure. The sequence analysis of *algO,* encoding a 709-amino acid long protein in *sap27* genome, revealed an insertion of a single nucleotide, thymine (T), at position 3,643,250 on the genome (Figure 2.6). This insertion results in a frame-shift mutation leading to premature termination of *algO* at codon 320 (Fig. 2.6), which is converted from glutamic acid to UGA, an opal stop codon. The truncated protein has an identity to AlgO at the first 106 amino acids of the N-terminus and the remaining 213 amino acids share no similarity to any other *P. aeruginosa* proteins. In fact, the same mutation was identified in *sap22* (Reiling *et al.*, 2005; Sautter *et al.*, 2012). However, the codon number was stated differently as the start codon for *algO* is off by 11 codons that were recently corrected on pseudomonas.com (Winsor *et al.*, 2016).

To ensure the phenotype seen in *sap27* is not strain-specific and can be reproduced in *sap22,* the plasmid pLptD was introduced in the latter rescuing the mucoid phenotype (Figure 2.3(c)). To further confirm that *lptD in trans* can overcome the *algO* mutation, a clean in-frame deletion of *algO* was constructed in PAO1 and PDO300 and the strains were named as PAOΔ*algO* (Alg⁻) and PDOΔ*algO* (Alg⁻), respectively. The plasmid pLptD-SurA-LptE was introduced in these strains and induced with arabinose. The increase in *lptD* copy number (expected to be between 1-7 copies) (Jahn *et al.*, 2016). leading to higher protein levels in PAO1 and PAOΔ*algO* results in the strains turning mucoid with significant production of alginate ((Figure 2.3(c), Figure 2.4). Thus, the LptD-dependent alginate-production does not require *algO.* The induction of *lptD* in PDOΔ*algO* (Alg⁻)

failed to rescue the alginate phenotype (Figure 2.4). However, the alginate phenotype can be rescued by *lptD* in the *sap27* mutant strain (Figure 2.4). Both of these strains express truncated MucA (MucA22). The difference between PDO∆*algO* and *sap27* (PDO*algO107*) is that one has no AlgO whereas the latter expresses a truncated AlgO. This suggests that LptD-dependent alginate production in *sap27* requires the truncated AlgO. One cannot rule out the possibility that the truncated AlgO in *sap27* might be contributing to the observed phenotype.

Increased expression of *lptD-surA-lptE* in ∆*algW* mutant of PAO1 and PDO300

To determine if LptD-dependent induction of alginate production has a role in the RIP cascade, attempts were made to construct deletion mutants of *algW* and *mucP*. Repeated efforts to make *mucP* deletion mutants failed. However, PAO1 and PDO300 derivatives with clean in-frame *algW* deletions were constructed and are referred to as PAO∆*algW* and PDO∆*algW,* respectively. As expected, the loss of *algW* resulted in the loss of alginate production in PDO300 (Figure 2.4). Multiple efforts to complement PDO∆*algW* with *algW* also failed. The plasmids pLptD and the pLptD-SurA-LptE were introduced into PAO∆*algW* and PDO∆*algW.* The mucoid phenotype of PDO∆*algW* was rescued by increasing the copy number of *lptD* (Figure 2.4). Inducing the expression of *lptD* in PAO∆*algW* did not result in alginate production (Figure 2.4). This suggests that the LptD-dependent alginate production in PAO1 requires AlgW. Thus, in a *mucA22* background, LptD can bypass AlgW.

Discussion

The emergence of alginate-producing strains of *P. aeruginosa* in patients with CF is an indication of chronic infection that signals a deterioration of lung function and ultimately pulmonary failure. The importance of alginate in CF patients was corroborated in the early 1970s. However, the genetic determinant responsible for the conversion was mapped to a single locus in the 1980s, and the genes responsible were identified in the 1990s. Almost 35 years later, with the identification of over 30 alginate genes, a solution to prevent mucoid conversion continues to elude us. This study was undertaken to identify novel genes by mapping second site-suppressor mutations of alginate production. The gain-of-phenotype in a non-mucoid *sap27* was mapped to *lptD* (PA0595) encoding an outer membrane protein. To date, all the genes involved in the regulation of alginate production encode for products that reside in the periplasm, the inner membrane, and the cytoplasm. This report demonstrates the potential role of a *P. aeruginosa* OMP, LptD (PA0595), in alginate production.

LptD transports lipopolysaccharide to the outer leaflet of the outer membrane and is an essential OMP in *E. coli* and *P. aeruginosa* but not in *Neisseria meningitidis* (Bos *et al.*, 2004; Braun and Silhavy, 2002; Werneburg *et al.*, 2012). In *E. coli*, LptD is a member of the σ^E regulon with multiple designations: LptD, OstA, and Imp owing to its different roles (Abe *et al.*, 2003; Aono *et al.*, 1994; Braun and Silhavy, 2002; Dartigalongue *et al.*, 2001; Sampson *et al.*, 1989). Actually, LptD designation comes from **L**ipo**P**olysaccharide **T**ransport **P**rotein because of its involvement in outer membrane biogenesis (Braun and Silhavy, 2002; Sperandeo *et al.*, 2009), the OstA from *o*rganic *s*olvent *t*olerance **A** because of its importance in the growth of *E. coli* K12 in xylene and n-hexane (Abe *et al.*, 2003;

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Aono *et al.*, 1994); and finally, Imp for *i*ncreased *m*embrane *p*ermeability that contributes to resistance to antibiotics, detergents and dyes (Charlson *et al.*, 2006; Sampson *et al.*, 1989). *P. aeruginosa* LptD is also involved in LPS transport and controlling membrane permeability and confers resistance to antibiotics such as rifampin, ciprofloxacin, tetracycline, novobiocin, azithromycin and imipenem (Balibar and Grabowicz, 2016; Werneburg *et al.*, 2012). Similar to *E. coli, P. aeruginosa* LptD is involved in LPS palmitoylation (Balibar and Grabowicz, 2016; Ruiz *et al.*, 2008; Wu *et al.*, 2006). It is not uncommon to find proteins with multiple roles in bacterial physiology, for an example, AlgC is involved in LPS, Pel, Psl, alginate, and rhamnolipid production (Ma *et al.*, 2012; Olvera *et al.*, 1999; Zielinski *et al.*, 1991).

Outer membrane protein LptD regulates alginate production

One can argue that increasing expression will lead to misfolding of LptD that may cause alginate production in *sap27*, since it requires LptE and the SurA as chaperone (Chimalakonda *et al.*, 2011; Lazar *et al.*, 1998). However, the co-expression of *lptD* with *surA* and *lptE* continued to restore the mucoid phenotype of *sap27* ruling out misfolding of LptD (Figure 2.4). Further expression of *optP* encoding an OMP of comparable size in *sap27* did not result in rescuing the mucoid phenotype (Figure 2.4). These results certainly argue that LptD, an OMP, may have a specific role in the regulation of alginate production.

LptD bypasses AlgO107

It is interesting that the *sap27* strain did not have any mutations in the *lptD* locus but in *algO*. A role for LptD in alginate production is further augmented by its ability to rescue the loss of *algO* in *sap22* that was previously identified with the same mutation

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(Figure 2.4). In a previous publication, the allele was named as *algO96* (Sautter *et al.*, 2012). However, with correction of the ATG start codon location upstream, we renamed the allele as *algO107*. Increasing the expression of *lptD* bypassed *algO107* but failed to rescue the alginate phenotype when *algO* was completely deleted in PDO300. This suggests that the presence of truncated AlgO contributes to the observed phenotype. This phenotype is independent of the proteolytic activity of AlgO as the truncated protein (AlgO107) is missing that catalytic triad Ser490, Asp501, and Lys515. It is not possible to rule out that the last 213-amino acid in the AlgO107 is contributing to the observed phenotype without further experimentation. Previously, it was argued that MucA22 is the substrate for AlgO, a putative serine protease (Reiling *et al.*, 2005). However, no concrete evidence has surfaced to support this argument.

LptD influences members of RIP cascade

To determine if LptD played a role in the RIP cascade, *algW* was deleted in PDO300 and PAO1. The loss of alginate production in PDOΔ*algW* was rescued by increasing *lptD* expression (Figure 2.4). This observation suggests that the release of AlgT/U by MucA22 (Figure 1.7 (B)) does not require site-1 cleavage by AlgW, despite the fact that the major AlgW cleavage site on MucA (A_{136} and G_{137}) is still present in MucA22 (Damron and Yu, 2011). However, in the wild-type *P. aeruginosa* PAO1, mucoid conversion by overexpression of *lptD* requires AlgW. This finding argues that LptD directly or indirectly influences the members of RIP cascade.

Two pathways for MucA proteolysis

As previously shown, our results also suggest a difference in the regulation of alginate production in wild-type versus isolates with the *mucA22* allele (Qiu *et al.*, 2007). In the wild-type background, alginate production is very tightly controlled since MucA is intact and protected by MucB against proteolytic cleavage (Cezairliyan and Sauer, 2009). We propose that, upon increased expression of *lptD*, AlgW is activated to initiate MucA
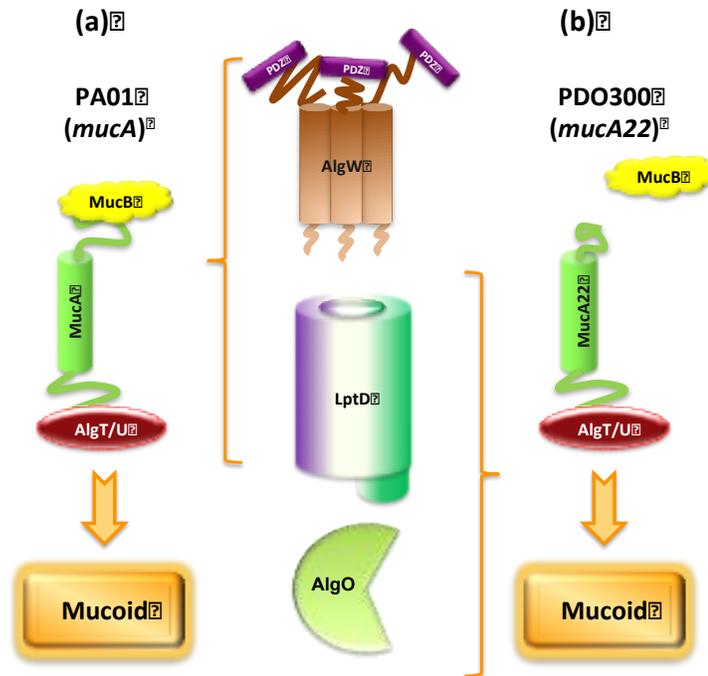
Figure 2.7. Model for mucoid conversion due to increase expression of *lptD* in *mucA⁺* (PAO1) and *mucA22* (PDO300) backgrounds. We proposed that (a) in PAO1, increased expression of *lptD* displaces MucB and induces AlgW to cleave MucA resulting in proteolysis of MucA as described previously in figure 1.7. There might not be any role for AlgO in PAO1 under the tested conditions. (b) in the *mucA22* background, MucB cannot bind to MucA, thus the cleavage site of MucA22 is exposed. The overexpression of LptD might activate the truncated AlgO leading to MucA22 proteolysis via an unknown mechanism.

cleavage followed by MucP to release AlgT/U for alginate synthesis (Figure 2.7(a)). In the mucoid *P. aeruginosa* isolates with the *mucA22* allele, AlgO seems to be playing an important role by activating cleavage of the truncated MucA22 (Figure 2.7(b)) either directly or indirectly by means of some unknown protein. Mechanistically, the specific role of LptD in the activation of AlgW or AlgO is not clear at the moment. The regulation of alginate production due to LptD seems depend on strain backgrounds and requires further investigation.

The X-ray crystal structure of *E. coli* LptD shows the protein has C-terminal β-barrel domain while its N-terminal periplasmic domain has a jelly-roll like structure (Dong *et al.*, 2014; Qiao *et al.*, 2014). Bioinformatic analysis shows LptD of *P. aeruginosa* is structurally similar to that of *E. coli* (Werneburg *et al.*, 2012). It is not known whether the N-terminal domain of LtpD could interact with other periplasmic proteins to induce alginate production. Moreover, it is possible that the N-terminal domain of LptD or LPS might displace MucB, leaving MucA vulnerable to proteolysis (Figure 2.7(b)) as seen in the *E. coli* counterpart (Lima *et al.*, 2013; Wilken *et al.*, 2004; Wollmann and Zeth, 2007). To understand the mechanistic detail standard protein-protein interaction studies (pull-down, cross-linking, competition assays) can be performed to explore if there is any direct interaction between LptD and MucB and/or MucA.

Implication

To date, *P. aeruginosa* LptD has not been associated with alginate production. *P. aeruginosa* LptD is a target of peptidomimetic antibiotics based on the antimicrobial peptide protegrin I that appears to be species-specific (Srinivas *et al.*, 2010; Werneburg *et*

al., 2012). It is also shown that depletion of LptD results in antibiotic susceptibility in *P. aeruginosa* and *Acinetobacter baumannii* (Balibar and Grabowicz, 2016; Bojkovic *et al.*, 2015). Furthermore, LptD is a subject of study for a candidate vaccine in Gram-negative bacteria such as *Neisseria gonorrhea* and *Vibrio parahaemolyticus* (Zha *et al.*, 2016; Zielke *et al.*, 2016) Multidimensional protein identification technology showed that in *P. aeruginosa* 63 proteins including LptD are exposed to the extracellular environment (Srinivas *et al.*, 2010; Vecchietti *et al.*, 2012). Thus, it would be interesting to determine if the peptidomimetic antibiotics, as well as a vaccine against LptD, can prevent alginate production in *P. aeruginosa*.

CHAPTER 3

Pyridoxal 5'-phosphate (PLP) enhances pyocyanin biosynthesis in *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa is a metabolically versatile pathogen. In previous chapter (Chapter 2), we identified an operon that contains three genes *lptD*, *surA* and *pdxA*. The *pdxA* encoding an outer membrane protein, chaperone protein, and an enzyme involved in pyridoxal 5'-phosphate (PLP) biosynthesis, respectively. PLP is also known as vitamin B_6. PLP biosynthetic pathway has a common precursor, D-erythrose-4-phosphate, that converted to chorismic acid that can either synthesize pyocyanin and aromatic amino acids. We hypothesized that PLP is involved in *P. aeruginosa* virulence. Culturing bacteria in medium supplemented with PLP resulted in increased pyocyanin production. Among the aromatic amino acids, tryptophan, phenylalanine and tyrosine the latter two shunted the pathway to produce pyocyanin. The finding in this chapter show that PLP, and aromatic amino acids phenylalanine and tyrosine indeed causes *P. aeruginosa* to increase in pyocyanin production suggesting a possible role for *pdxA* in virulence.

Introduction

P. aeruginosa, a Gram-negative bacterium, is a leading cause of infection in cystic fibrosis (CF) patients (CFF, 2016). *P. aeruginosa* is a metabolically versatile organism which in part is due to pyridoxal 5'-phosphate (PLP) which is only the active B_6 vitamers. PLP serves as a cofactor of various essential enzymes that are involved in the metabolism of amino acids, fatty acids, and carbohydrates (Percudani and Peracchi, 2003; Percudani and Peracchi, 2009). Bioinformatic analyses show 23 proteins encoded by *P. aeruginosa* require PLP as a co-factor (Table 3.1) (Percudani and Peracchi, 2009).

There are two biochemical pathways for PLP biosynthesis, and these pathways are mutually exclusive (Fitzpatrick *et al.*, 2007). In *P. aeruginosa*, the PLP is synthesized by a deoxyxylulose-5-phosphate (DXP) dependent pathway (Fitzpatrick *et al.*, 2007). The canonical PLP binding sequence is Val-Ile-Tyr-Glu-Thr-Glu-Ser-Thr-His-Lys-Leu-Leu-Ala-Ala-Phe where either histidine or lysine residue may play a direct role in catalysis

Table 3.1. Predicted ORFs that requires PLP as a co-factor

S/N	Gene	Locus	Protein
1	*cysM*	PA0932	Cysteine synthase
2		PA3659	Probable aminotransferase
3	*bauA*	PA1232	Beta-alanine-pyruvate transaminase
4	*phnW*	PA1310	2-aminoethylphosphonate:Pyruvate transferase
5	*phhC*	PA0870	Aromatic amino acid aminotransferase
6	*pvdH*	PA2413	Diaminobutyric acid aminotransferase
7	*aruC*	PA0895	N2-Succinylornithine 5-aminotransferase
8	*aruH*	PA4976	Arginine:Pyruvate transaminase
9	*selA*	PA4808	L-seryl-tRNA(Ser) selenium transferase
10	*ldcA*	PA1818	Probable Orn/Arg/Lys decarboxylase
11	*lysA*	PA5277	Diaminopimelate decarboxylase
12	*itaA*	PA5413	Low-specificity L-threonine aldolase
13	*pabC*	PA2964	4-amino-4-deoxychorismate lyase
14	*trpB*	PA0036	Tryptophan synthase beta chain
15	*thrC*	PA3735	Threonine synthase
16		PA2683	Probable serine/threonine dehydratase
17		PA3357	D-serine dehydratase
18		PA0400	probable cystathionine gamma-lyase
19	*alr*	PA4930	Alanine racemase, biosynthetic
20	*hemL*	PA3977	Glutamate-1-semialdeyde 2,1-aminomutase
21	*arnB*	PA3552	UDS-4-deoxy-L-arabinose synthase
22	*cobC*	PA1276	Cobalamin biosynthetic protein CobC
23	*metZ*	PA3107	O-succinylhomoserine sulfhydrylase

(Sabo and Fischer, 1974; Wolosker *et al.*, 1999). The role of the PLP in the production of one of the virulence factors, pyocyanin, has not been established yet in *P. aeruginosa*.
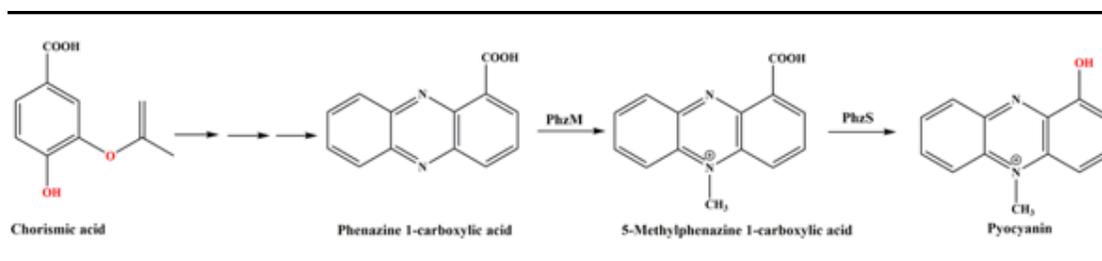
Figure 3.1. Synthesis of pyocyanin from chorismic acid. Chroismic acid undergoes biosynthetic reaction to synthesize phenazine-1-carboxylic which is then by enzyme PhzM converted to 5-Methylphenazine-1-carboxylic to synthesize pyocyanin by PhzS. Courtesy (Rada and Leto, 2013)

Pyocyanin is a water-soluble, blue-green phenazine compound, and functions as an important virulence factor, as it affects *P. aeruginosa* biofilm development as well as the release of extracellular DNA (Baron and Rowe, 1981; Das *et al.*, 2015; Das and Manefield, 2012; Frank and Demoss, 1959; Grossowicz *et al.*, 1957). In *P. aeruginosa,* one of the precursor molecules of pyocyanin is chorismic acid (Figure 3.1). Chorismic acid is also involved in the synthesis of aromatic acid (Palmer *et al.*, 2013; Tamir and Srinivasan, 1971). The function of pyocyanin is to balance the NADH/NAD$^+$ ratio in the *P. aeruginosa* cytoplasm when the microbes are in stationary phase (Price-Whelan *et al.*, 2007).

Pyocyanin has been shown to have antibacterial activity against Gram-positive bacteria *Micrococcus luteus*, Gram-negative bacteria *E. coli,* and *Shewanella oneidensis* (Costa *et al.*, 2015; Kanthakumar *et al.*, 1993; Mazzola *et al.*, 1992). Pyocyanin biosynthesis is increased when *P. aeruginosa* is co-cultured with *Candida albicans* and was shown to be toxic to the fungus (Gibson *et al.*, 2009; Morales *et al.*, 2010; Trejo-

Hernandez *et al.*, 2014). By using *Saccharomyces cerevisiae* and the human lung carcinoma alveolar type II cell line A549, it was shown that pyocyanin generated reactive oxygen species that inactivated human vacuolar ATPase, negatively impacting the lung function of people with CF (Ran *et al.*, 2003). Additionally, pyocyanin has been shown to cause erythoptosis-inducing effects in erythrocytes suggesting a systemic effect on patients suffering from CF (Qadri *et al.*, 2016). In a mouse model, it has been shown that pyocyanin caused tissue damage and necrosis that contributed to the development of pneumonia (Lau *et al.*, 2004).

P. aeruginosa harbors two homologous core loci (*phzA1-G1* and *phzA2-G2*) and three additional genes (*phzM*, *phzS*, and *phzH*) that are involved in the pyocyanin biosynthesis (Figure 3.1)(Mavrodi *et al.*, 2001). None of these genes appear to have a canonical PLP-binding site. However, both PLP and pyocyanin synthesis use the same starting material, D-erythrose-4-phosphate (Figure 3.2). What drives the reaction toward pyocyanin production is unclear. Growing *P. aeruginosa* in a synthetic CF sputum medium supplemented with the aromatic amino acids also enhanced the production of pyocyanin (Palmer *et al.*, 2007). It was shown that *Pseudomonas* quinolone signal (PQS), a cell-cell signaling system, positively affects pyocyanin production.

Recently, we demonstrated that the expression of *lptD* restores the mucoid phenotype in a nonmucoid strain (Pandey *et al.*, 2018). The *lptD* gene is part of a three-gene-operon *lptD-surA-pdxA*. The *lptD* gene encodes an outer membrane protein (OMP) involved in lipopolysaccharide transport (Braun and Silhavy, 2002; Sperandeo *et al.*, 2009; Wu *et al.*, 2006). SurA influences the biogenesis of LptD whereas PdxA is involved in pyridoxal 5'-phosphate synthesis (PLP) (Figure 3.2)(Denoncin *et al.*, 2010; Vertommen *et*

al., 2009). The PLP is one of the six B_6 vitamers that includes pyridoxal, pyridoxine, and pyridoxamine and their phosphorylated derivatives PLP, pyridoxine-5-phosphate, and pyrdidoxamine-5-phosphate (Figure 3.3). PLP is the only active form of vitamin B_6 that is serves as cofactor for more than 160 vitamin B_6-dependent enzymes (Percudani and Peracchi, 2003). The vitamin B_6 dependent enzymes are involved in amino acid metabolism, amine biosynthesis, carbohydrate breakdown, heme synthesis, nucleic acid synthesis and, neurotransmitter biosynthesis (Percudani and Peracchi, 2009). Animals cannot synthesize PLP but have to depend on microorganisms and plants (Fitzpatrick *et al.*, 2007). The focus of this chapter is PdxA, as it has not been previously characterized. Quorum sensing genes also regulate pyocyanin biosynthesis in *P. aeruginosa* (Lee and Zhang, 2015). Many of these genes are involved in amino acid syntheses. The role of these genes in pyocyanin production, however, has never been explored. One of the intermediates in the pathway, chorismic acid, can either be funneled into the synthesis of aromatic amino acids or pyocyanin (Figure 3.2).

It is known that the presence of exogenous aromatic amino acids also results in pyocyanin production (Palmer *et al.*, 2007). The present study initiated the work needed to tease apart the intricate mechanism that drives pyocyanin production and possible relationship with PLP.
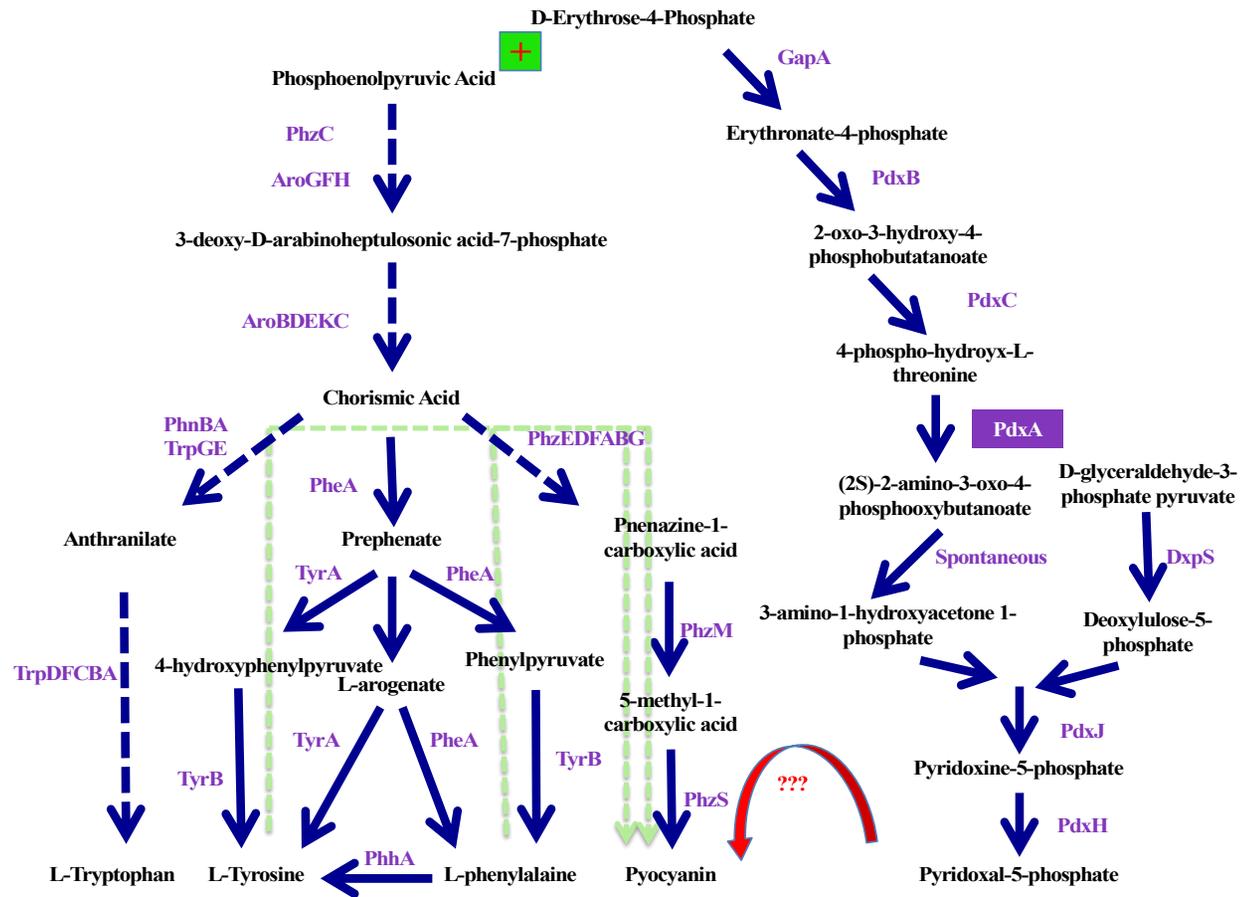
Figure 3.2. Detail pathway for the synthesis of pyocyanin. The pathway to synthesize aromatic amino acid, pyocyanin and pyridoxal 5'-phosphate is shown. The solid line and dotted line indicate single and multi-step pathways, respectively.

Methods

Bacterial strains, media and growth conditions

The bacterial strain used in this study is *P. aeruginosa* PAO1 strain. It was cultured overnight at 37 °C. Then, the next day it was sub-cultured in a flask until an $OD_{600} = 1$. Then, the culture was diluted to an $OD_{600} = 0.5$ in fresh LB. Then, a 2.5-ml of the diluted culture was taken and supplemented with aromatic amino acids or PLP to the desired concentration to a final volume of 5-ml and the mixture was cultured was incubated for 15 hours.

Pyocyanin assay

The pyocyanin assay was carried out by extracting the pigment from culture supernatant using the chloroform-HCL technique as previously described (Essar *et al.*, 1990).
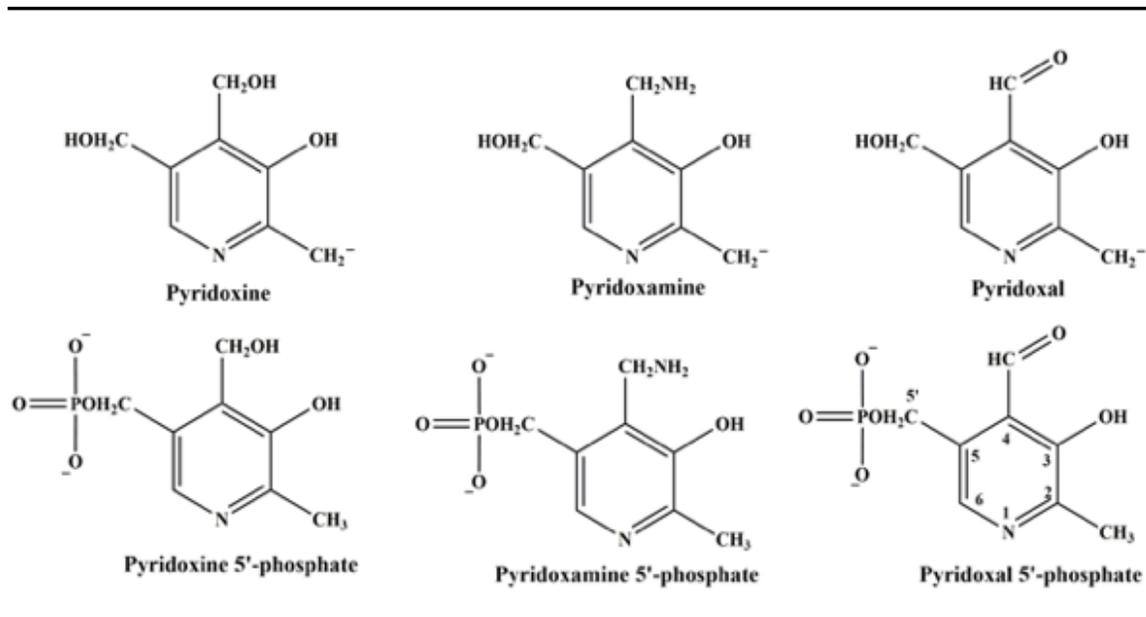
Figure 3.3. Different forms of B$_6$ vitamers. B$_6$ vitamers are pyridoxine, pyridoxamine, pyridoxal and their phosphate forms. Pyridoxal 5'-phosphate is active B$_6$ vitamers. Courtesy (Vrolijk *et al.*, 2017).

A 5-ml culture was centrifuged at 8500 rpm for 10 min at 18 ℃, then 4-ml of the supernatant was mixed with 3-ml of chloroform, then the mixture was vortexed for 2 minutes and the phases were allowed to separate settled down for 20 min. Then, a 2-ml of the lower phase was aspirated and mixed with 1-ml of 0.2 N HCl, followed by vortexing for 2 min and let it stand for 20 min. Then, the top layer was used to quantify the pyocyanin by measuring at the absorbance at 520 nm (A_{520}).

RNA isolation, cDNA synthesis, and qPCR

All primers used in the experiments were synthesized and supplied by Integrated DNA Technologies (Coralville, IA) (Table 3.2).

Table 3.2. Primers used for qPCR

S/N	Primer name	Primers
1	SP_qRT_phzC1 (F)	GGCCGGTGATCCGCGTCG
2	SP_qRT_phzC1(R)	CCGCCGCATAGCCCTTGAG
3	SP_qRT_phzE1(F)	ATGACCTGCTGGCGCTGAT
4	SP_qRT_phzE1(R)	GCGACCGATCTCGTCGGCG
5	DBS_qRT_clpX(F)	TGCGATTACGATGTGGAGA
6	DBS_qRT_clpX(R)	CCCTCGATGAGCTTCAGCA

The Ribonucleic acid (RNA) was isolated from the prototypic *P. aeruginosa* grown in LB broth. Briefly, the overnight culture of *P. aeruginosa* grown in LB broth at 37 ℃ was washed with sterile 0.85 % saline solution to remove spent media and was sub-cultured at 37 ℃, 200 rpm in LB media. When the cells reached an optical density of 600 nm (OD_{600}) of 0.6 - 0.7 the bacterial culture was treated with 5.92 mM PLP for 1 hour. Post-treatment, RNA was stabilized by addition of phenol-ethanol mixture. The stabilized RNA was then isolated using RNeasy Mini Kit (Qiagen, Inc Venio, Limburg, Netherland) as per

the manufacturer's protocol. Residual genomic DNA contamination was removed using RQ1 RNase-free DNase (Promega, Madison, Wl, USA) and RNA was re-purified using Rneasy kit (Qiagen, Inc Venio, Limburg, Netherlands). The quality of purified RNA was assessed on a denaturing agarose gel (NorthernMax Gly, Ambion, Life Technologies, Carlsbad, CA, USA) and quantified at 260 nm (BioTEK, Synergy HT, Winooski, VT, USA). The complementary DNA (cDNA) was then synthesized by annealing NS5 random primers to total purified RNA and subsequent extension was carried out using SuperScript lll reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA). The quantitative polymerase chain reaction (qPCR) to study expression levels of *phzC1* and *phzE1* under PLP induction was performed using an Applied Biosystem Step One cycler and detection system with PowerSYBR Green PCR MasterMix with ROX (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The cycling conditions used were 95 °C (2 minutes, holding); 40 cycles of 95 °C (15 sec), 60 °C (60 seconds, cycling); 95 °C (15 second), 60 °C (1 minute), 95 °C (15 second) (0.6 °C ramp) (melt curve). Expression was normalized to the housekeeping gene *clpX* (*PA1802*), whose expression remained unchanged under the tested conditions.

Results

PLP up-regulates the pyocyanin production

To investigate the role of PLP in pyocyanin biosynthesis, *P. aeruginosa* was cultured for 15 hours in LB with and without PLP. The assay showed that *P. aeruginosa* produced a significantly higher quantity of pyocyanin in the presence of PLP suggesting that PLP influences a role in pyocyanin biosynthesis *P. aeruginosa* (Figure. 3.4).

Pyocyanin production in the presence of aromatic amino acids

Common precursor molecules, chorismite and anthranilate are used to synthesize pyocyanin, pseudomonas quinolone signal, and aromatic amino acids (Figure 3.3) (Lee and Zhang, 2015). So, we hypothesized that supplementation of aromatic amino acids in LB medium would divert intracellular chorismate to pyocyanin synthesis. To test this hypothesis, *P. aeruginosa* was grown in LB medium supplemented with different concentrations of phenylalanine, tyrosine, and tryptophan. There was no significant change in pyocyanin production when *P. aeruginosa* was grown in the LB medium supplemented with tryptophan (Figure 3.4). Pyocyanin was significantly overproduced when *P. aeruginosa* was grown in LB supplemented with phenylalanine and tyrosine (Figure 3.4).
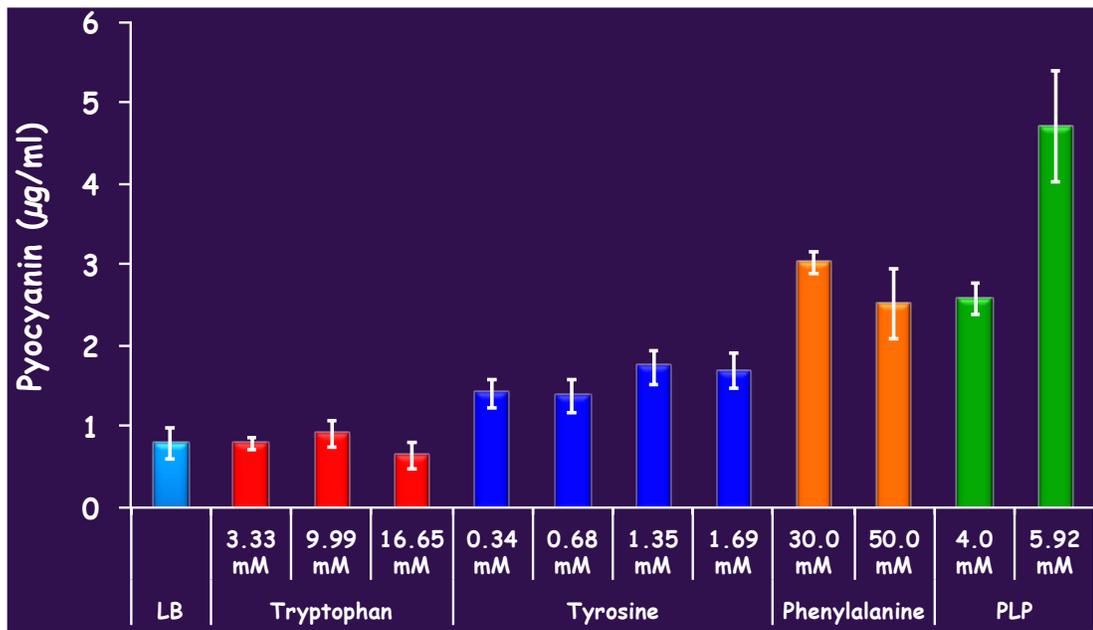
Figure 3.4. Pyocyanin production in the presence of PLP and aromatic amino acids. *P. aeruginosa* PAO1 was grown in aromatic amino acids or PLP and levels of pyocyanin were quantified.

PLP increases the transcription of the pyocyanin biosynthesis operon

In *P. aeruginosa,* two operons are involved in pyocyanin biosynthesis. The pyocyanin biosynthesis operon *phz1* (*phzA1B1C1D1E1G1)* is located at positions 4,713,795 to 4,720,062 bp in the genome, while the other operon, *phz2* (*phzA2B2C2D2E2G2),* is located approximately 2.6 Mb from *phz1* at the position 2,070,685 to 2,076,985 bp (Winsor *et al.*, 2016). One operon is required for pyocyanin biosynthesis (Cui *et al.*, 2016; Recinos *et al.*, 2012).
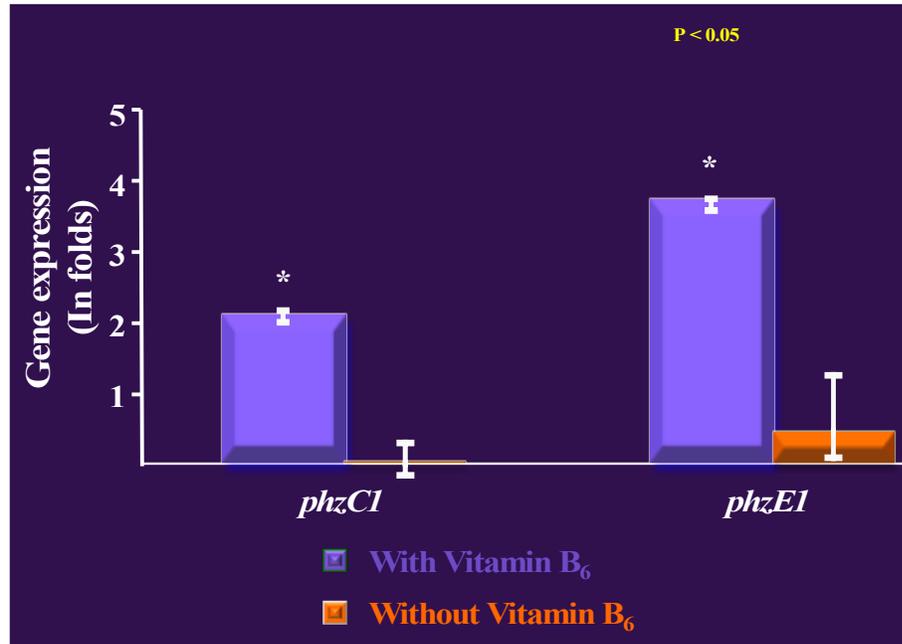
Figure 3.5. Expression of genes in *phz*-operon due to PLP. *P. aruginosa* PAO1 was grown in LB media supplemented with or without vitamin B_6. Then RNA was isolated, and cDNA was synthesized. The qPCR was done to quantify the expression level *phzC1* and *phzE1*.

To identify whether PLP affects phenazine genes at the operons at the transcriptional level, we determined the relative levels of *phzC1* (PA4212) and *phzE1*

(PA4214) transcripts by qPCR. *P. aeruginosa* PAO1 was grown in LB media supplemented with/without PLP. Complementary DNA (cDNA) was synthesized and qPCR was performed using the primers shown in (Table 3.2). Our result showed that *phzC1* and *phzE1* transcripts increased three and five-fold, respectively, when *P. aeruginosa* was grown in the PLP-treated versus untreated media (Figure 3.5). Our results indicate that PLP increases the transcription of the *phz1* operon. However, whether PLP directly or indirectly plays a role in the increased transcription of *phz1* operon remain to be explored.

Discussion

Little is known about the role of PLP biosynthesis and its role in the virulence of bacteria. Neither the biosynthesis pathway in *P. aeruginosa* nor the importance of PLP in virulence has been explored. In this chapter, the role of PLP, as well as preliminary mechanism of pyocyanin biosynthesis when the *P. aeruginosa* is grown in medium supplemented with PLP, is explored.

PLP is synthesized in bacteria via two *de novo* pathways known as deoxyxylulose-5-phosphate (DXP)-dependent and DXP-independent pathway (Fitzpatrick *et al.*, 2007). The DXP-dependent pathway has been mostly present in γ proteobacteria such as *P. aeruginosa* where *pdxA* is involved in synthesis of 2-amino-3-oxo-4-phosphoxoybutanoate from 4-phospho-hydroxy-L-threonine (Figure 3.2). Our results showed that the growth of *P. aeruginosa* in the presence of PLP increased the production of pyocyanin production, one of the virulence factors of *P. aeruginosa.* The pyocyanin and PLP are synthesized from a common precursor D-erythrose -4-phosphate (Figure 3.2). From D-erythrose-4-phosphate either PLP or chorismic acid is synthesized via two independent biosynthetic

pathways. The chorismic acid further can be used to synthesize either aromatic amino acids or pyocyanin (Figure 3.2).   We hypothesized that culturing of *P. aeruginosa* in presence of PLP shunted D-erythrose-4-phosphate to a pathway in favor of pyocyanin production. Furthermore, since chorismic acid is used synthesis of either aromatic amino acid or pyocyanin production, we hypothesized that in excess of aromatic amino acids, chorismic acid would push the pathway to the production of pyocyanin. Our results suggest indeed growth of *P. aeruginosa* in a media supplemented with aromatic amino acids tyrosine and phenylalanine increased the production of pyocyanin.

Our data show that among the three aromatic amino acids the highest amount of pyocyanin was produced when *P. aeruginosa* was cultured in LB medium supplemented with excess phenylalanine whereas lowest in the presence of tryptophan. The higher amount of pyocyanin biosynthesis when *P. aeruginosa* was grown in presence of phenylalanine may be the result of the existence of the aromatic amino acid permease transport systems I and II (Kay and Gronlund, 1971). The aromatic amino acid permease transport system transport systems have different affinities for the three aromatic amino acids, system I recognizes and transports phenylalanine, tyrosine, and tryptophan respectively in this order, whereas the aromatic transport system II recognizes tryptophan, phenylalanine, and tyrosine in this order (Kay and Gronlund, 1971). In addition, it has been shown that phenylalanine and tyrosine accumulates inside *P. aeruginosa* to a concentration approximately 10 times greater than that found for tryptophan (Kay and Gronlund, 1971).

Future direction

The results still leave the role of *pdxA* unanswered. The only way to show *pdxA* role in virulence and vitamin B_6 synthesis is by constructing knockout strain. However, attempts to delete the gene failed suggesting that it is an essential gene, as such a conditional mutant need to be constructed.

Construction of a conditional *pdxA* mutant

A strain with the *pdxA* gene under the control of the *araBAD* promoter (P_{BAD}) should be constructed. A 300-bp spanning the 5' region of *pdxA* was PCR amplified from the genome of PAO1 using the primers SP_pdxA$_{300}$_ *EcoRI*_(F) and SP_pdxA$_{300}$_
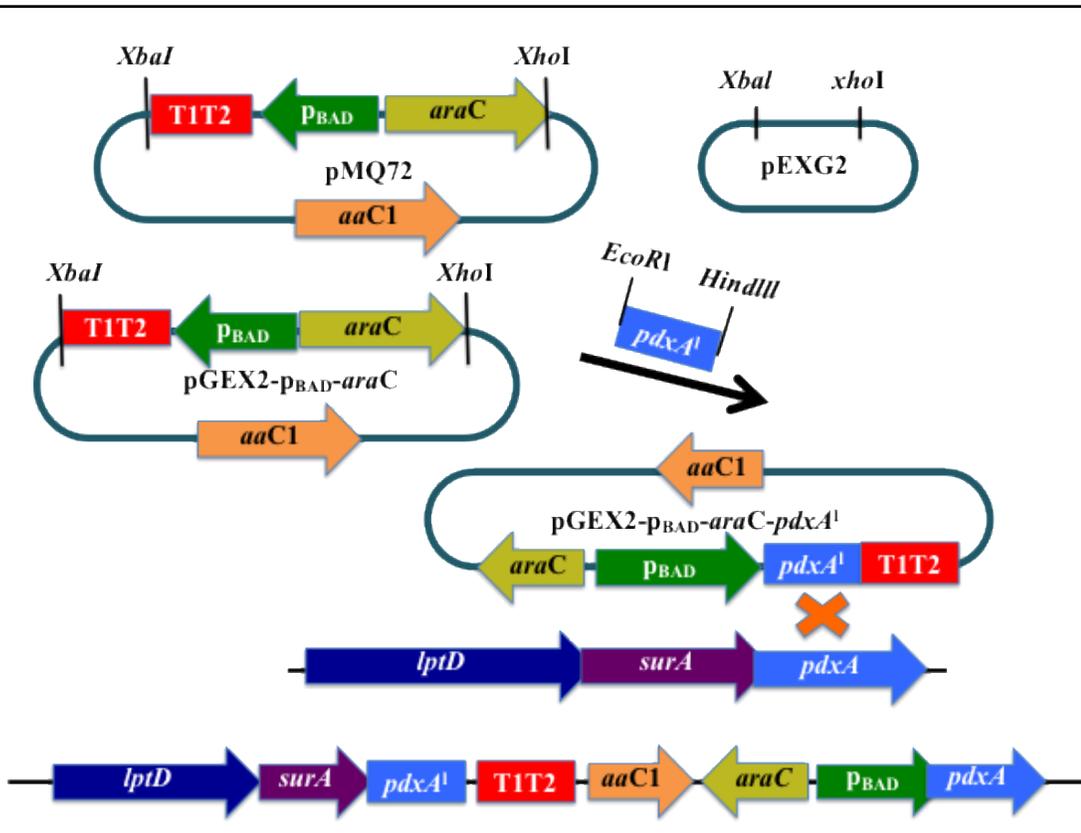
Figure 3.6. Strategy to construct *pdxA* conditional mutant of *P. aeruginosa*. The methods of construction of *pdxA* conditional mutation PAO1.

*Hind*III_(R). The PCR product was digested with *EcoR*I and *Hind*III and ligated downstream of P$_{\text{BAD}}$ promoter into pMQ72 digested with *EcoR*I and *Hind*III and the plasmid was called as pMQ72-pdxA$_{300}$. Resulting plasmid pMQ72-pdxA$_{300}$ was used to amplify a 2,239-bp fragment containing *araC-pdxA$_{300}$-T1T2* using the primer pairs pBAD (F) and pBAD (R). The fragment was then digested with *Xho*I and *Xba*I and ligated into integration proficient vector pEXG2 digested by the same restriction enzymes resulting in the plasmid pEXG2P$_{\text{BAD}}$:*pdxA$_{300}$*. The plasmid pEXG2P$_{\text{BAD}}$:*pdxA$_{300}$* was used to transform *E. coli*. The plasmid was then transferred into *P. aeruginosa* PAO1 by triparental mating, with the selection of the conditional mutant on PIA (Difco) medium supplemented with arabinose (0.5%) and Gm (100 µg ml^{-1}). The integration was confirmed by sequencing and PCR, resulting in the strain PAO1-pEXG2:*pdxA$_{300}$* (Figure 3.6).

qPCR analysis of the first genes in the pathways, namely *gapA, phzC*, and *phzE*

We postulate that the presence of PLP will result in the decreased expression of *gapA* and increased expression *phzC* and *phzE* (Figure 3.3). Briefly, wild type and *pdxA* mutant strain should be grown in M9 broth at 37 °C in the presence of arabinose and glucose (to deplete PdxA) for 3 hours, and RNA should be isolated using RNeasy Mini Kit (Qiagen) as per the manufacturer's protocol. The RNA should be purified, and cDNA should be synthesized. The qPCR of the respective genes (Figure 3.2) should be performed to explore the possible pathway for pyocyanin production when *P. aeruginosa* is grown in the presence of PLP.

CHAPTER 4

Discussion

Living cells have a sophisticated regulatory mechanism that controls how they transduce the environmental clues downstream to the cell. One of the mechanisms that are evolutionarily conserved from humans to bacteria is termed regulated intramembrane proteolysis (RIP). The RIP process is predominantly involved in a progressive two-step proteolytic degradation initiated by the cleavage of a single-span transmembrane protein ultimately releasing extracellular and/or intracellular fragments (Brown *et al.*, 2000; Lichtenthaler and Steiner, 2007; Qiu *et al.*, 2007). The RIP system in cells functions in various ways, including proliferation, differentiation, protein degradation, cell adhesion, lipid metabolism, mitophagy, and transcriptional regulation (Damron and Yu, 2011; Qiu *et al.*, 2007; Shanbhag *et al.*, 2012; Yoshida *et al.*, 2013). In this dissertation, we explored the activation of the RIP system of *P. aeruginosa* by an outer membrane protein referred as lipopolysaccharide transport protein D (LptD). The *lptD* is part of three gene operon *lptD-surA-pdxA*. The PdxA is involved in synthesis of pyridoxal 5'-phosphate (PLP). Culturing *P. aeruginosa* in the LB supplemented with the of PLP shows the increase in biosynthesis of pyocyanin. The pyocyanin and aromatic amino acid are synthesized from chorismic acid. Culturing *P. aeruginosa* in LB media supplemented with aromatic amino acids; phenylalanine and tyrosine also increase the pyocyanin production.

A variety of organisms, including bacteria, fungi, parasites, viruses and other agents are the cause of nosocomial infections. Recently, using data from hospital-based surveillance studies and Infectious Diseases Society of America, a certain group of nosocomial pathogens has been referred to as ESKAPE pathogens (Peters *et al.*, 2008; Pogue *et al.*, 2015; Rice, 2010). The ESKAPE pathogens includes a group of both Gram-negative and Gram-positive bacteria made up of *Enterococcus faecium*, *Staphylococcus*

aureus, *Klebsiella pneumonia*e, *Acinetobacter baumannii*, *P. aeruginosa,* and *Enterobacter* species. Among ESKAPE pathogens, *P. aeruginosa* is a formidable pathogen because of its metabolic versatility, intrinsic and acquired antibiotic resistance, biofilm formation, and production of multiple virulence factors. One of the virulence factors of *P. aeruginosa* is the exopolysaccharide alginate (Hoiby, 1975). Alginate production in the lung of the CF patients infected by *P. aeruginosa* indicates a chronic infection that leads to a poor prognosis (Hoiby *et al.*, 1977; Pedersen *et al.*, 1992). Thus, it is important to understand the genes involved and their mechanism of action in the production of alginate in *P. aeruginosa.* One part of this study focused on identifying novel genes associated with the production of alginate and pathways of alginate production while in the other section, we investigated the production of another virulence factor pyocyanin.

CF individuals are thought to be initially colonized by *P. aeruginosa* from diverse environmental sources as well as from CF clinics (Denton *et al.*, 2002; Jensen *et al.*, 1997; Romling *et al.*, 1994; Scott and Pitt, 2004). Initial colonizing *P. aeruginosa* strains have non-mucoid phenotype with a wild-type *mucA*, however, mucoid *mucA* mutants eventually emerge and dominate the population of *P. aeruginosa* in the CF lung (Martin *et al.*, 1993). In wild-type PAO1 MucA sequesters AlgT/U at the inner membrane, preventing the sigma factor from directing RNA polymerase to transcribe the alginate transcriptome; however, when *mucA* is mutated, AlgT/U is free to guide RNA polymerase (Schurr *et al.*, 1996). In the clinical setting, it has been found that 25% of mucoid *P. aeruginosa* isolated from CF individuals has a deletion of a single guanine nucleotide (G) in a string of five G nucleotides located at 426-430 bp in *mucA* ORF (Martin *et al.*, 1993; Schurr *et al.*, 1996). In this study, we used a constitutively mucoid laboratory strain PDO300 (PAO*mucA22*) that was derived

from non-mucoid PAO1 (Mathee *et al.*, 1999). The mucoid PDO300 was grown under oxygen-limited conditions, a stressful situation that reverts the metabolically demanding mucoid PDO300 to non-mucoid phenotype which are referred to as the suppressor of alginate production (*sap*) mutants (Mathee *et al.*, 1999). One of the *sap* mutants that is of interest in this dissertation is *sap27*. To identify mutations in *sap27* mutants, the strains were complemented with the PAO1 minimum tiling path (MTP) library that was previously divided into four pools (Sautter *et al.*, 2012). The four pools of cosmids were introduced into the *sap27* and mucoid phenotype of the strains was screened. The junctional sequencing revealed that cosmid pMO012217 restored the mucoidy of the *sap27*. Finally, by transposon mutagenizing the cosmid pMO012217 that restored the mucoid phenotype, we mapped *lptD* (*PA0595*) that can restore the mucoid phenotype of one of *sap27* and *lptD-surA-pdxA* forms an operon (chapter 2). The *pdxA* is involved in pyridoxal 5'-phosphate (PLP) synthesis in Gram-negative bacteria. Culturing *P. aeruginosa* in LB media supplemented with PLP as well as aromatic amino acids phenylalanine and tyrosine increases the production of pyocyanin (chapter 3). The pyocyanin is one of the virulent factors of *P. aeruginosa* (Gibson *et al.*, 2009; Kanthakumar *et al.*, 1993; Morales *et al.*, 2010).

Outer membrane protein LptD

Previously, LptD was known as Imp and OstA (Abe *et al.*, 2003; Aono *et al.*, 1994; Sampson *et al.*, 1989). The protein has been extensively studied and deemed as a promising vaccine antigen and potential immunotherapeutic target in several Gram-negative bacteria, such as *Neisseria gonorrhoeae*, *A. baumannii*, *Neisseria meningitidis*, *Vibrio*

parahaemolyticus and *P. aeruginosa* (Balibar and Grabowicz, 2016; Bojkovic *et al.*, 2015; Bos *et al.*, 2004; Zha *et al.*, 2016). The role of LptD, along with other seven essential proteins (LptA-LptG), is to transport lipopolysaccharide (LPS) to the cell surface, thus involved in outer membrane biogenesis (Braun and Silhavy, 2002). LptD is an outer membrane protein (OMP) with a C-terminal β-barrel and an N-terminal jellyroll domain containing three nonconsecutive disulfide bonds (Srinivas *et al.*, 2010; Werneburg *et al.*, 2012). Survival protein during stationary phase (SurA), 17-Kilodalton Protein (Skp), and FK506-binding protein A (FkpA) maintain the folding-competent state while LptD is translocating from the periplasm to the outer membrane (Bitto and McKay, 2002; Rouviere and Gross, 1996; Schwalm *et al.*, 2013; Tormo *et al.*, 1990). In the outer membrane, the Bam complex assembles LptD in coordination with LptE (Lee *et al.*, 2016). The three disulfide bonds are rearranged to become a mature LptD-LptE complex (Moehle *et al.*, 2016).

LPS is the major endotoxin that stimulates the host inflammatory response (Janda, 1972; Raetz and Whitfield, 2002; Rosenfeld *et al.*, 2006). In mice, LptD induces a strong host immune response (Zha *et al.*, 2016). LptD is an essential outer membrane protein of most of the Gram-negative bacteria and has the capacity to generate an immune response in humans, making the protein an ideal universal vaccine candidate (Srinivas *et al.*, 2010; Zha *et al.*, 2016). Photoaffinity labeling, liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) and a polyclonal antibody raised against a C-terminus domain of LptD show that the LptD is an outer membrane protein with a mass of approximately 100 kDa (Srinivas *et al.*, 2010). We also confirmed the finding that LptD localizes to the membrane fraction of *P. aeruginosa* (Figure 2.5).

The role of the second gene of the *lptD*-operon, *surA*, in LptD translocation

In all organisms, proteins are translated primarily by the cytosolic ribosome and such proteins have to be transported to various cellular locations. In bacterial cells, three known translocation processes that transport the proteins across the cell membrane have been reported: (a) Sec-mediated translocation across the cytoplasmic membrane (Manting and Driessen, 2000); (b) twin-arginine transport-mediated translocation across the cytoplasmic membrane (Palmer and Berks, 2012); and (c) more specialized mechanisms for delivery of specific proteins to the outermost surface of the cell (Thanassi and Hultgren, 2000). Of these mechanisms, one of the major pathways of protein translocation from the cytosol across the inner membrane in bacteria is provided by the Sec machinery (Corey *et al.*, 2016; Duong and Wickner, 1997; Nouwen and Driessen, 2002). Transportation of outer membrane proteins initiates with the translocation of nascent OMPs through the Sec machinery located in the inner membrane (Huber *et al.*, 2017). Once in the periplasm, nascent OMPs interact with periplasmic chaperones such as SurA (Hennecke *et al.*, 2005; Xu *et al.*, 2007). SurA has been shown to interact directly with the A2 helix of β-barrel assembly machinery A (BamA) polypeptide transport-associated protein (POTRA 1) (Bennion *et al.*, 2010). No data exists that shows the interactions with other periplasmic chaperones, including the 17- kilodalton protein (Skp) and degradation protein P (DegP). Chaperone-bound OMPs are then thought to be offloaded to the BamA POTRA domain by a mechanism known as β-augmentation, in which the β-strands of substrate OMPs align with the β-strand of POTRA in a sequence and orientation-independent manner (Workman *et al.*, 2012). It has been shown in *surA* mutant of an *E. coli* that the transcription of *lptD* is not significantly affected but the LptD protein levels are decreased suggesting that in the

periplasm SurA is the true chaperon of LptD (Vertommen *et al.*, 2009). The LptD is a true substrate of SurA is further reinforced by the fact that in *γ*-proteobacteria, the *lptD-surA* operon is conserved even though nearby genes are not (Braun and Silhavy, 2002; Winsor *et al.*, 2016). This further suggests that the LptD is a preferred substrate of SurA. Furthermore, it has been shown that though LptD is highly dependent on SurA as its preferential chaperone protein, other periplasmic chaperone proteins such as Skp and FkpA are also required for the assembly of LptD (Schwalm *et al.*, 2013).

In *E. coli*, the domains of SurA consists of the N-terminal domain [aa 21 -162], P1 domains [aa 172-274], P2 domain [aa 282-385], and C-terminal domain [aa 388-428](Bitto and McKay, 2002). The N and C-terminals of SurA including P2 act as the core chaperone module whereas the P1 domain is known as an inactive peptidyl-prolyl-cis/trans isomerase (PPIase) domain. The P1 form makes noncovalent interactions with the core chaperone module that occludes the function of SurA. Both P1 and P2 domains are dispensable as mutants lacking these domains are sufficient for the chaperone function of SurA *in vitro*. SurA has been shown to bind to peptides that are rich in aromatic amino acids (Hennecke *et al.*, 2005).

Role of LptE in the proper folding of LptD in the outer membrane

In *E. coli*, His-tagged LptD was used initially to show that LptE and LptD interact and later it was found that the proteins interact in 1:1 ratio (Chng *et al.*, 2010; Wu *et al.*, 2006). Through pull-down experiments, it was shown that the both the full length LptD as well as only C-termini LptD tagged with histidine (C-LptD$_{His}$) could pull down similar amounts of LptE, suggesting that the LptE interacts with the C-terminus of LptD (Chng *et*

al., 2010). In *E. coli*, LptE helps in the proper rearrangement of disulfide bonds present in LptD but the exact mechanism is not known (Ruiz *et al.*, 2010). LptD of *P. aeruginosa* is composed of 924 amino acids. The periplasmic domain of the LptD (34- 323) is 100 amino acids longer than LptD of *E. coli* and contains a pair of cysteine residues that could form a disulfide bond. In SDS-PAGE, heat denatured LptD with and without reduction with dithiothreitol (DTT) showed denatured and non-reduced LptD with an apparent size of 130 kDa and the DTT-reduced form with an apparent size of 100 kDa suggesting the presence of a putative interdomain di-sulfide bond (Andolina *et al.*, 2018). Additionally, by sequential replacement of all six cysteine residues to serine and examining the gel mobility of such modified LptD in SDS-PAGE showed that the protein is of 100 kDa, suggesting the presence of internal disulfide bonds of LptD (Andolina *et al.*, 2018). The Ni-affinity pull down showed LptD was co-purified along with LptE$_{His}$, indicating both proteins interact with each other but how LptE helps in the rearrangement of disulfide bond present in LptD remains to be explored (Andolina *et al.*, 2018).

Alginate production

In the lungs of patients with cystic fibrosis, *P. aeruginosa* starts with a non-mucoid phenotype and converts to an alginate overproducing mucoid phenotype, indicating of a chronic infection (Evans and Linker, 1973; Govan and Fyfe, 1978; Pressler *et al.*, 2011a). It has been shown that motile *P. aeruginosa* eventually deposited into the hypoxic mucus zone in the lung of CF airway responds to such oxygen-limiting conditions by increasing alginate production (Worlitzsch *et al.*, 2002). Alginate is a linear polymer of β-D-mannuronic acid and the α-L-guluronic acid that has an ability to stimulate the production

of antibodies IgA and IgG (Aanaes *et al.*, 2013; Evans and Linker, 1973; Pedersen *et al.*, 1990).

Alginate production in *P. aeruginosa* involves a very complex regulatory process where the key player is an alternative sigma factor AlgT/U (Devries and Ohman, 1994a; Martin *et al.*, 1993a). The activity of AlgT/U is post-translationally inhibited by the anti-sigma factors MucA and MucB (Goldberg *et al.*, 1993; Martin *et al.*, 1993b; Martin *et al.*, 1993; Mathee *et al.*, 1997). The release of AlgT/U from MucA is regulated by a mechanism known as the regulated intramembrane proteolytic pathway (RIP)(Qiu *et al.*, 2007). In the RIP pathway, MucA undergoes sequential degradation and such degradation process is stimulated by the action of the periplasmic/outer membrane protein MucE due to the accumulation of unfolded proteins in the periplasm or the stress in the outer membrane activates MucE (Qiu *et al.*, 2007). The activated-MucE interacts with AlgO to activate the inner membrane proteolytic protein AlgW to cleave MucA (Cezairliyan and Sauer, 2009; Qiu *et al.*, 2007). The AlgW-cleaved MucA undergoes further cleavage by MucP, eventually releasing AlgT/U with a small portion of MucA still bound to it (Damron and Yu, 2011; Qiu *et al.*, 2007). A cytoplasmic protein, SspA, guides the complex of cleaved MucA-AlgT/U to ClpXP where the remaining portion of MucA is degraded and eventually AlgT/U is free to bind to RNA polymerase (Qiu *et al.*, 2008b; Yin *et al.*, 2013). The AlgT/U bound RNA polymerase directs the transcription of its own *algT/U*-operon and other transcription factors AlgR, AlgB, and AmrZ (Devries and Ohman, 1994a; Martin *et al.*, 1994; Wozniak and Ohman, 1994; Wozniak *et al.*, 2003). These transcription factors AlgR, AlgB*,* and AmrZ along with RNA polymerase-AlgT/U complex direct the transcription of *algD* promoter for the biosynthesis of alginate (Baynham and Wozniak, 1996; Kato and

Chakrabarty, 1991; Leech *et al.*, 2008; Mohr *et al.*, 1991; Mohr *et al.*, 1992). AlgR not only regulates the *algD*-operon but also activates the transcription of *algC* that encodes a phosphomannomutase or phosphoglucomutase, which is essential for alginate, Psl, and rhamnolipid production (Lizewski *et al.*, 2004; Wozniak and Ohman, 1994; Zielinski *et al.*, 1991). Another important regulator of alginate production in *P. aeruginosa* is diguanylate cyclase/phosphatase, which by regulating the local pool of bis- (3'-5')-cyclic dimeric GMP (c-di-GMP) in the vicinity of the pili Z (PilZ) domain of the inner membrane protein Alg44, positively influencing the alginate production in *P. aeruginosa* (Hay *et al.*, 2009; Merighi *et al.*, 2007; Remminghorst and Rehm, 2006).

Role of LptD the alginate production: a potential model

Our results show that an outer membrane protein, LptD, acts as a positive regulator of alginate production (Chapter 2)(Pandey *et al.*, 2018). We showed that in a *mucA* mutant background, LptD is capable of restoring the mucoid phenotype. To understand the hierarchy of LptD in the RIP cascade, *algW* was deleted in both PAO1 and PDO300. LptD rescued the mucoid phenotype in PDOΔ*algW* suggesting that in a *mucA* mutant background, LptD acts upstream of AlgW in the RIP cascade whereas, in the case of PDOΔ*algO*, LptD failed to restore the mucoid phenotype. But, increasing the production of LptD in *sap27* restores the mucoid phenotype suggesting LptD might interact with AlgO to produce the mucoid phenotype in a *mucA* mutant background. However, in wild type PAO1, increased expression of LptD, AlgO is dispensable for rescuing mucoid phenotype. Based on this evidence, we concluded that LptD dependent alginate production is strain specific (Figure 2.8).

Role of PLP in pyocyanin production in *P. aeruginosa*

The *lptD* gene is part of a three-gene-operon *lptD-surA-pdxA*. The *lptD* gene encodes an outer membrane protein (OMP) involved in lipopolysaccharide transport. Additionally, it has been shown that *lptD* positively influences alginate production (Pandey *et al.*, 2018). SurA is the cognate chaperone protein of LptD, and PdxA plays an important role in vitamin B_6 synthesis (Figure 3.2) (Denoncin *et al.*, 2010; Fitzpatrick *et al.*, 2007; Laber *et al.*, 1999; Rouviere and Gross, 1996). One of the aims of this dissertation was also to explore the role of vitamin B_6 in the virulence of *P. aeruginosa*.

In *E. coli*, PdxA acts as an enzyme for the synthesis of vitamin B_6 (Laber *et al.*, 1999). The importance of vitamin B_6 for bacterial pathogenesis has been demonstrated in *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Campylobacter jejuni*, and *Helicobacter pylori* (Asakura *et al.*, 2013; Dick *et al.*, 2010; Grubman *et al.*, 2010; Raschle *et al.*, 2005). Our data suggest that vitamin B_6, and the aromatic amino acids tyrosine and phenylalanine play a role in increased pyocyanin production in *P. aeruginosa* (Figure 3.3). But, the roles of PLP, tyrosine, and phenylalanine in pyocyanin production in *P. aeruginosa* remain unknown.

Future directions

This is the first report of an OMP component that plays a role in regulating alginate production. This study also opened many unanswered questions. Delineating the role of the LptD in the RIP cascade can help in identifying stress signals that trigger upstream or downstream proteolysis of MucA that releases the AlgT/U for mucoid conversion of *P. aeruginosa*. By targeting LptD as a drug target, a tool can be developed to prevent alginate

production. Secondly, MucD, AlgO, AlgW, and MucP are protease proteins. To achieve

better treatment outcomes in chronic *P. aeruginosa* infections identifying the inhibitors of

the proteases that can prevent alginate production can be a significant strategy. Finally, it

is well established that microbes and plants can only synthesize pyridoxal 5'-phosphate

(PLP) and humans have to depend on them for metabolic needs. By targeting the *P. aeruginosa* PLP biosynthesis pathway, growth or virulence factors of pathogenic microbes

can be specifically inhibited that could result in the better prognosis for CF patients and

prevent other infections by the microbe.

Identify the role of LptD/OstA in alginate regulatory cascade

To determine the role and point at which LptD/OstA is involved in the alginate regulatory cascade, *mucD*, *mucE*, *algW*, *algO* and *mucP* can be expressed in the *lptD* mutant strain. Next, *lptD/ostA* can be expressed in *mucD*, *algW*, *algO* and *mucP* deletion strains. Then, the presence and absence of a mucoid phenotype can be scored. Based on the results, the location and role of the LptD/OstA in the alginate pathway can be deduced.

Identify proteins interacting with LptD/OstA

LptD might interact with a periplasmic protein to exert its effect on alginate production. To date, known periplasmic proteins associated with alginate production are MucD, MucE, AlgO, and MucB. To determine the mechanism of action of LptD for alginate synthesis, it is imperative to identify the protein/s that interact with LptD. A co-immunoprecipitation assay can be done with His-tagged LptD to find its interacting partners.

Conversely, the periplasmic proteins (MucB, AlgO, MucD, and MucE) can be His-tagged for the pull-down assay. The interacting partners can be identified using antibodies against the known proteins. In *E. coli,* SurA acts as a chaperone for LptD and LptE binds in the lumen of LptD (Chimalakonda *et al.*, 2011; Chng *et al.*, 2010; Denoncin *et al.*, 2010; Moehle *et al.*, 2016). This suggests that LptD might interact with other non-alginate periplasmic proteins. To test LptD-SurA or LptD-LptE interaction in *P. aeruginosa*, His-tagged SurA or LptE can be used in co-immunoprecipitation assays.

LptD as a drug target

LptD and the lipoprotein LptE are essential for the transport of lipopolysaccharide molecules from the periplasm to the outer leaflet of the outer membrane for the outer membrane biogenesis of in Gram-negative bacteria (Chng *et al.*, 2010). It has been shown that macrocyclic peptidomimetics and the related molecule Murepavadin specifically target LptD inhibiting the LPS translocation in *Pseudomonas* spp. (Andolina *et al.*, 2018; Srinivas *et al.*, 2010; Werneburg *et al.*, 2012). Our result showed LptD in *P. aeruginosa* is also involved in alginate production (Chapter 2). Thus, it is possible that the peptidomimetic drugs could also inhibit alginate production by *P. aeruginosa*, which has not been explored yet.

Role of truncated AlgO in sap27

LptD restored the mucoid phenotype of *sap27* (Figure 2.3b) but could not restore the phenotype of PDOΔ*algO*. Both of the strains have

mucA22 allele but truncated AlgO is present in the *sap27* whereas, in the PDOΔ*algO,* the gene is deleted. This led to the hypothesis that the truncated AlgO could still retain the function in *sap27*. It would be interesting to test whether the truncated AlgO has retained all or part of its function.

Identification of small molecule inhibitors of alginate production

The chronic infection in CF patients is the appearance of an increase in alginate production causing a mucoid phenotype in *P. aeruginosa.* The mucoid phenotype of the bacterium makes antibiotic treatments ineffective. The trypsin-like serine proteases (MucD and AlgW), a serine protease (AlgO) and Zn-dependent protease (MucP) play a central role in the alginate production pathway in *P. aeruginosa*. Identification of specific proteinase inhibitors of MucD, AlgW, AlgO, and MucP could be useful therapeutic agents to prevent alginate production. Commercial proteinase inhibitors from G-biosciences or Sigma that can act on either of MucD, AlgW, AlgO, and MucP. The available literature showed that between two inner membrane proteases AlgW and MucP, the MucP is the essential protease for the proteolytic cleavage of both MucA or its mutant allele MucA22. MucP is Zn metalloproteinase. Thus, it may be worthwhile to use screening of Zn metalloproteinase inhibitors to impede alginate production by *P. aeruginosa*. Fortunately, a database for wide range of metalloproteinase inhibitors are available (Muvva *et al.*, 2016). Culturing mucoid *P. aeruginosa* in the presence of the protease inhibitors and screening for the

loss of mucoidy could be a strategy to identify potential inhibitors of specific protease proteins. Finally, the mechanism of action of the protease inhibitors can be explored.

Identification of small molecule inhibitors of pyocyanin production

Culturing *P. aeruginosa* in a media supplemented with PLP increased pyocyanin production. Pyocyanin is one of the virulence factors of *P. aeruginosa*. By identifying the proteins affecting pyocyanin production due to PLP, screening for small molecule inhibitors of the proteins can be done that could ultimately make *P. aeruginosa* less virulent. It is also important to note that PLP acts as a co-factor for 23 *P. aeruginosa* proteins. Thus, by inhibiting the activity of PLP synthesis, we can affect the metabolic activity of the microbe such that its growth and/or its virulence can be inhibited.

Mechanism of cleavage MucA in the membrane

Generally, the membrane-spanning segment of a protein forms α helices. Hydrogen bonds preclude the proteolytic cleavage of peptide bonds present in the α helices (Paetzel *et al.*, 1998). How AlgW and MucP can get access to the peptide bond of MucA for the cleavage is still unresolved. It might be possible that due to the release of MucB, the MucA might undergo a conformational change or it could form a β strand-like conformation that becomes more susceptible to proteolysis as previously predicted (Hubbard *et al.*, 1994; Matthews, 1988).

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Final conclusions and implications

This dissertation identified an outer membrane protein referred to as Lipopolysaccharide transport protein D (LptD), as a component of alginate production pathway. The LptD is likely to interact with periplasmic protease protein AlgO and inner membrane protease protein AlgW for restoration of the alginate production by suppressor of alginate production (*sap*) mutant *sap27*. The non-mucoid *sap27* is derived from PDO300 that consecutively mucoid phenotype. The *sap27* has a mutation in the AlgO.

The last gene of *lptD-surA-pdxA* forms an operon. It is well established that *pdxA* is involved in pyridoxal 5'-phosphate synthesis (PLP), an active form of vitamin B$_6$. Culturing the *P. aeruginosa* in excess PLP and aromatic amino acids phenylalanine and tyrosine increases pyocyanin production.

This investigation led to the finding of an outer membrane protein (OMP) that is important in alginate gene regulation in *P. aeruginosa* and this discovery could increase the chance of a potential treatment option. OMPs are an easier target because the effect of an efflux pump that might reduce the efficacy of the drug is bypassed. Additionally, data in this dissertation show that PLP and aromatic amino acids; phenylalanine and tyrosine enhance the production of pyocyanin. Targeting the proteins involved in the biosynthesis of PLP and aromatic amino acid could be a potential way to limit the infection caused by *P. aeruginosa*.

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PUBLICATIONS AND PRESENTATIONS

Sundar Pandey, Camila Delgado, Laura Florez, Hansi Kumari, and Kalai Mathee (2018). Outer membrane protein (PA0595) plays a role in the regulation of alginate synthesis in *P. aeruginosa*. Journal of Medical Microbiology.DOI:10.1099/jmm.0.000752

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DOI: 10.1002/9781119004813.ch32

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