



Characterization of enzymes that modify or degrade the *Pseudomonas* virulence factor, alginate
by Stephanie Ann Douthit

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy
in Department of Microbiology
Montana State University
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Abstract:

Biosynthesis of the polysaccharide alginate is important for *Pseudomonas aeruginosa* to establish chronic pulmonary infections in Cystic Fibrosis patients.

Alginate is a linear polymer of β 1-4 linked D-mannuronate (M) interspersed with its C-5 epimer, L-guluronate (G). Initially D-mannuronate residues are polymerized into the periplasm as polymannuronic acid. In the periplasm, some polymannuronate residues are converted to L-guluronate residues by the C-5 epimerase, AlgG. Alginate is further modified by the addition of O-acetyl groups to the D-mannuronate residues AlgI, AlgJ, and AlgF. The focus of this research was to further characterize the alginate modifying enzymes, AlgG and AlgJ. We found that AlgG contains a repeating sequence that is characterized as a CARbohydrate-binding and Sugar Hydrolases (CASH) domain.

Proteins containing this domain fold as right-handed β -helices (RH β H) and bind to long chain linear polysaccharides. AlgG was predicted to fold as a RH β H by the 3D-PSSM structural prediction program. RH β H models of AlgG predict that the identified 324-DPHD-327 motif lies in the long shallow groove that may accommodate alginate. Site-directed mutations of this motif disrupt enzymatic activity, but not structural integrity, suggesting that these mutations lie in the epimerase catalytic domain. Asparagines 362 and 367 are predicted to stack with other asparagine residues along the β -helix. Results obtained from site directed mutants of N362 or N367 suggest that these mutations disrupt asparagine stacking and protein stability. Original attempts to identify alginate binding motifs were made using phage display peptide libraries. This technique proved unsuccessful in identifying binding motifs in AlgG or AlgJ, as discussed in Chapter 3. However, we were able to characterize AlgG with structural modeling, and identified two potentially important motifs in AlgJ. Two putative guluronate specific lyases were also identified in *P. aeruginosa*, PA1167 and PA1784. Overexpression of PA1167 in mucoid strains FRD1 and FRD1153 results in a non-mucoid phenotype, suggesting this acts as an alginate lyase. The experiments also show the *P. aeruginosa* cannot use alginate as a carbon source. This research provides a greater understanding of carbohydrate/protein interactions between alginate modifying enzymes and alginate.

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Stephanie Ann Douthit

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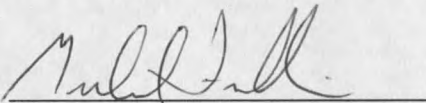
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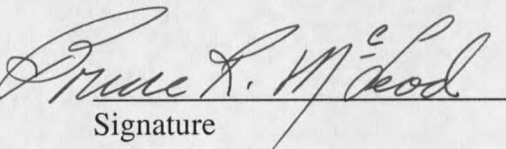
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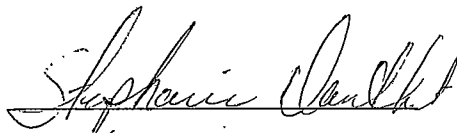
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CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that infects immunocompromised individuals, patients with burn wounds, and cystic fibrosis (CF) patients. Cystic fibrosis is the most common heritable disease among Caucasians affecting one in every 2500 births with a carrier frequency of one in 25 (37). Cystic fibrosis was originally described from post mortem children with pancreatic disease whose pancreas formed fibrils (37, 151). Historically, this disease was associated with the endocrine system. Most children died before the age of one year due to malnutrition. Advances in nutrition have allowed children to survive longer. However, this genetic disorder predisposes these patients to bacterial pulmonary infections. Today pulmonary failure due to chronic infection with *P. aeruginosa* is the leading cause of morbidity and mortality of this patient group, with 80-95% succumbing to bacterial infections (37, 151). Chronic infection usually occurs following conversion of *P. aeruginosa* to a mucoid phenotype, where the bacteria over-produce the extracellular polysaccharide alginate (Fig.1.1) (192). This organism is also thought to exist as a biofilm, a community of organisms encased in an extracellular matrix, in the CF lung (251). Even with antipseudomonal treatments, *P. aeruginosa* persists in chronic pulmonary infections of CF patients (10, 91, 112). Both the conversion to mucoid phenotype and biofilm mode of

growth contribute to the persistence of *P. aeruginosa* in this environment (91, 112, 151, 192, 194). This review focuses on how *P. aeruginosa* is able to colonize and persist in the CF lung, the unique host-parasite interactions that occur between the two, and the role alginate plays in chronic disease.



Figure 1.1. Mucoïd CF isolate FRD1 (alginate overproducing strain) and non-mucoïd burn wound isolate PAO1

Cystic Fibrosis, the Disease

Significant advances in treating this disease have been made in the past four decades. The mean lifespan of patients with CF in 1969 was 14 years, and now most patients can expect to survive into their 30's (151). This increase of lifespan can be attributed to 1) the discovery of the mutant gene, cystic fibrosis transmembrane conductance regulator (CFTR), and 2) a better understanding of host /parasite

ABSTRACT

Biosynthesis of the polysaccharide alginate is important for *Pseudomonas aeruginosa* to establish chronic pulmonary infections in Cystic Fibrosis patients. Alginate is a linear polymer of β 1-4 linked D-mannuronate (M) interspersed with its C-5 epimer, L-guluronate (G). Initially D-mannuronate residues are polymerized into the periplasm as polymannuronic acid. In the periplasm, some polymannuronate residues are converted to L-guluronate residues by the C-5 epimerase, AlgG. Alginate is further modified by the addition of O-acetyl groups to the D-mannuronate residues AlgI, AlgJ, and AlgF. The focus of this research was to further characterize the alginate modifying enzymes, AlgG and AlgJ. We found that AlgG contains a repeating sequence that is characterized as a CARbohydrate-binding and Sugar Hydrolases (CASH) domain. Proteins containing this domain fold as right-handed β -helices (RH β H) and bind to long chain linear polysaccharides. AlgG was predicted to fold as a RH β H by the 3D-PSSM structural prediction program. RH β H models of AlgG predict that the identified 324-DPHD-327 motif lies in the long shallow groove that may accommodate alginate. Site-directed mutations of this motif disrupt enzymatic activity, but not structural integrity, suggesting that these mutations lie in the epimerase catalytic domain. Asparagines 362 and 367 are predicted to stack with other asparagine residues along the β -helix. Results obtained from site directed mutants of N362 or N367 suggest that these mutations disrupt asparagine stacking and protein stability. Original attempts to identify alginate binding motifs were made using phage display peptide libraries. This technique proved unsuccessful in identifying binding motifs in AlgG or AlgJ, as discussed in Chapter 3. However, we were able to characterize AlgG with structural modeling, and identified two potentially important motifs in AlgJ. Two putative guluronate specific lyases were also identified in *P. aeruginosa*, PA1167 and PA1784. Overexpression of PA1167 in mucoid strains FRD1 and FRD1153 results in a non-mucoid phenotype, suggesting this acts as an alginate lyase. The experiments also show the *P. aeruginosa* cannot use alginate as a carbon source. This research provides a greater understanding of carbohydrate/protein interactions between alginate modifying enzymes and alginate.

disease. However, even with antibiotic treatments and suppression of the inflammatory immune response, *P. aeruginosa* cannot be eradicated and remains the leading cause of mortality in CF patients.

The gene responsible for the CF disorder was identified in 1989. It is located on chromosome 7 (227), and encodes the cystic fibrosis transmembrane conductance regulator (CFTR). The most common mutation in defective CFTR is the deletion of phenylalanine 508 ($\Delta F508$) (133, 303). This mutation occurs in 70% of patients and over 50% of patients are homozygous for this mutation. The frequency of $\Delta F508$ varies among different geographical locations. For example, this mutation occurs in 27% of patients in Turkey; while in Denmark 87% of the CF population has this mutation (115, 243). Over 1000 different mutations in the CFTR gene have now been identified (CFTR mutation database by Cystic fibrosis Genetic Analysis Consortium, <http://www.genet.sickkids.on.ca/cftr/>). There are large phenotypic differences between patients that are homozygous or heterozygous for $\Delta F508$ (146).

Originally, there was controversy surrounding the function of CFTR. Patients with CF characteristically have overly salty sweat, which is used as a diagnostic standard. Therefore, it was thought that the protein was a chloride channel, namely an outwardly rectifying chloride channel (ORCC). However, CFTR resembles a large family of regulatory transporters (223). It was later clarified that CFTR acts both as a membrane-bound regulator of ion channels, including the ORCC, and also acts as a chloride channel (2, 223, 226, 304). CFTR is expressed on epithelial cells primarily in the pancreas, salivary glands, sweat gland, intestine, and reproductive tract (37, 60, 127). Besides

regulating ORCC, CFTR interacts with epithelial sodium channel (ENaC), renally derived potassium channel (ROMK2), Aquaporin 3, a water channel in airway cells, Na^+ , K^+ , 2Cl^- co-transporter (NKCC-1) and electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBC-1) (151, 200). Deregulation of these ion channels facilitates the uptake and retention of water into the epithelial cells resulting in dehydration of the mucous layer of the CF lung. This characteristic dehydration of the mucous layer impairs mucociliary escalation, which has been attributed to the increased susceptibility of CF patients to bacterial pulmonary infections.

Not only does CFTR affect ionic balance, but it also plays a role in pH balance. CFTR regulates the NBC-1 co-transporter and is a cotransporter of HCO_3^- . Loss of this transport affects cytosolic pH (216). Inadequate acidification of cells leads to under sialylation of glycoproteins, namely gangliotetraosylceramide, on epithelial cell surfaces, and disrupts cyclic AMP dependant exo and endocytosis, both contributing to increased pulmonary infections (200, 214, 235).

Symptoms of CF are multi-factorial and include physiological processes, which are not well understood. As mentioned, the increased susceptibility of CF patients to *P. aeruginosa* pulmonary infections remains the major concern of the disease and will be discussed in detail later in this review. The CFTR defect causes many other physiological abnormalities, namely pancreatic insufficiency in 90% of patients, biliary disorders affecting the liver and gall bladder in 30% of patients, and infertility in 98% of males (218). Pancreatic insufficiency is attributed to a reduced volume of pancreatic secretions and low HCO_3^- concentrations, causing inadequate acidification. The pro-

enzymes are therefore retained in pancreatic ducts and are prematurely activated leading to tissue damage and the formation of fibrotic tissue (218), resulting in malnutrition due to the pancreatic disease. Before modern care, most patients died within one year due to malnutrition and complications of the gastrointestinal tract.

Correlations have been made between malnutrition and the incidence of lung infection in CF patients. Yu et al. (300) showed that well nourished CFTR^{-/-} mice and CFTR^{-/-} mice corrected for the CFTR mutation in the intestine showed no differences in clearance of *P. aeruginosa*, whereas malnourished mice had decreased bacterial clearance. These researchers also associated malnutrition with host defenses showing that levels of TNF α and NO₃⁻ were lower in malnourished mice and that TNF α and iNOS knockout mice had reduced bacterial clearance. Malnourished mice also had excess inflammation and did not produce the IL-10 anti-inflammatory cytokine. Studies have also shown that well nourished patients are able to prolong onset of *P. aeruginosa* infection, possibly due to maintenance of proper immune responses (246). Treatments to enhance nutrition such as pancreatic enzyme replacement have greatly improved the overall health of these patients (1, 151).

Pulmonary Infection

Historically, the leading cause of death was due to malnutrition. Now, chronic pulmonary infections are the leading cause of patient mortality. This section will discuss the microbiology of the CF lung and focuses on the role of *P. aeruginosa* infections in CF patients.

Bacteria infecting CF lungs include *Staphylococcus aureus*, *Hemophilus influenza*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa*. In the past 2 decades *Burkholderia cepacia* has also been an emerging pathogen infecting <10% of CF patients (rev in Govan and Deretic (91)). A recent study by Rogers et al. (224) characterized the bacterial communities in adults with CF. The dominant organism was *P. aeruginosa* followed by *Stenotrophomonas maltophilia*. Interestingly, many of the organisms identified were anaerobes, often associated with oral or gut communities. How these bacterial communities contribute to the dominance of *P. aeruginosa* and to lung deterioration has yet to be studied. Even though the subject size of that study was small, it indicates that other organisms may contribute to pulmonary disease.

Bacterial infection of young CF patients (0-5 years) is dominated by *Staphylococcus aureus*, *Hemophilus influenza*, and *Stenotrophomonas maltophilia* with 60-80% of patients having these infections. Infections with these organisms can be successfully treated with antibiotics. Between the ages of 5-9 years the ratio of infection favors that of *P. aeruginosa* and by adolescence or early adulthood this organism dominates pulmonary infections (91, 151). Anti-pseudomonal treatments are unable to eradicate this bacterium and it remains that mucoid *P. aeruginosa* is the primary organism that appreciably contributes to decline in lung function and death. The factors that promote the selection and dominance of mucoid *P. aeruginosa* strains are not well understood. With the complex immune responses and effects of CFTR mutations, it is an intriguing question that is difficult to address, but critical in understanding and preventing these infections.

Many hypotheses exist on why *P. aeruginosa* is specifically selected for and eventually converts to the mucoid phenotype. It is certain that it is a multifactorial phenomenon and will incorporate many of the models discussed below. In the next section, I will discuss deficiencies in the CF immune response, how *P. aeruginosa* interacts with the CF host, and how the bacteria initially colonize the pulmonary tissue. The following section, I will review the information that is known regarding the conversion to mucoid phenotype and the immune responses to this conversion. These section will then be followed by a discussion of the biosynthesis and enzymology on the major player of the mucoid phenotype, alginate.

Establishment of *Pseudomonas aeruginosa* Infection

Microbial "Priming"

In CF chronic pulmonary infections with acute exacerbations of viral and bacterial infections, lungs may be continuously damaged, promoting colonization of *P. aeruginosa*. However, this hypothesis is not well supported. Burns et al. (20) found that 97% of patients less than three years of age had positive cultures for *P. aeruginosa* or had antibody response to this bacterium. Why *P. aeruginosa* does not produce chronic infection in young patients is not understood. Upon infection with *S. aureus*, it is common practice to treat patients with antibiotic therapy. Studies have been conducted using prophylactic treatments with anti-staphylococcal drugs to prevent *S. aureus* infection in an attempt to stall *P. aeruginosa* colonization. Surprisingly, the opposite effect occurred. Patients receiving the prophylactic treatments were colonized with *P.*

aeruginosa faster than those patients receiving treatments only upon onset of *S. aureus* infection or with no treatments (10, 219). This argues that the “priming” hypothesis is not correct. How infection with other bacteria and viruses affect lung function is still a mystery and may have an undiscovered impact in the progression of the disease.

Deficiencies in the Host Immune Response

Several groups have shown airway inflammation without positive bacterial cultures, suggesting that CF airways inherently have high levels of inflammatory mediators such as neutrophil elastase and IL-8 and low levels of anti-inflammatory cytokines such as IL-10 (134, 175). However, evidence suggests that bacterial infections occur in very young CF patients. One report indicated that 17% of patients less than one year of age had been infected with *P. aeruginosa*. The detection of immune mediators in these patients was likely due to these infections (4, 20, 132). Birrer et al. (13) studied 27 children with CF and showed normal levels of anti-proteases, α -anti-trypsin and leukoprotease inhibitor. However, these patients had an excess of active neutrophil elastase indicating an imbalance between proteases and anti-proteases. It is interesting that chronic infections had not established in these younger patients. Noah et al. (178) showed IL-10 levels were normal in very young uninfected infants.

It is unknown how the CFTR defect is associated with immune deficiencies, but a prolonged inflammatory response does occur, especially in chronic infections. This response is a major contributor to pulmonary tissue damage. CF airways show increased levels of proinflammatory cytokines such as IL-8, IL-1, IL-6 and other mediators such as TNF α (192). All are important for neutrophil recruitment. The inflammatory response is

enhanced due to decreased concentrations of IL-10, an anti-inflammatory cytokine that inhibits the pro-inflammatory cytokines (14, 66, 174). IL-10 knockout mice have increased lung inflammation yet no greater bacterial burden than wild-type mice, supporting the theory that CF patients have a prolonged inflammatory response even when infections have been cleared (28). A more detailed study showed that in IL-10 knockout mice, the neutrophil and proinflammatory cytokine concentrations were greater than wild-type mice even six days after the infection was cleared (29), explaining why infants have inflammatory cytokines even without evidence of infection. Supplementing the mice with IL-10 greatly increased the survival rate and decreased the neutrophil and inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid (28).

The immune response in CF with *P. aeruginosa* infection is a Th2 response and involves IL-4, IL-5, IL-6, IL-10, IgG1 and IgE (173). A study by Song et al. (259) suggests that the immune response in acute mucoid *P. aeruginosa* infections resembles a Th1 type response. The Th1 type immune response may therefore be the normal response to *P. aeruginosa* infections and the Th2 response seen in CF patients may play a role in chronic *P. aeruginosa* infections. However, this response does not explain why *P. aeruginosa* is selected for in this airway environment. DiMango et al. (54) showed that *P. aeruginosa* gene products stimulate secretion of IL-8, which increases expression of NfκB, important in neutrophil recruitment. *P. aeruginosa* lipopolysaccharide (LPS) is an important stimulator of the inflammatory response since it activates NfκB. Constant recruitment of neutrophils and release of their proteases and elastases contributes to tissue

damage. Neutrophil elastase also compromises the immune response by cleaving C3b and CR1 receptors in complement cascade (53, 281).

The nitrogen balance in the CF airway is also abnormal, with the levels of NO lower and NH_4 higher than in non-CF lungs (149, 287). This imbalance is in part due to lowered expression of the inducible nitric oxide synthase (iNOS), lowering the NO concentration (131). NO also acts as an antimicrobial, and reduction of NO may have an effect on persistence of bacteria. The NO and NH_4 imbalance also contributes to the imbalance of other ion concentrations within the airways. For instance, high levels of NH_4 and low levels of NO inhibit Cl^- transport and contribute to the dehydration and viscosity of the mucous. In normal airway surface fluid the high levels of NO enhances Cl^- transport and inhibits Na^+ transport into cells, which helps maintain the fluidity of the mucous layer (212).

Studies suggest that bacteria are able to persist in the CF lung due to inadequacies in the host immune response. The altered ionic balances of the CF lung may affect primary defenses such as macrophage and neutrophil activity, and the activity of antimicrobial proteins and cationic peptides (8, 35, 88, 151, 250, 257). Higher Cl^- concentrations have been found in airway surface fluids from the trachea, main stem bronchi and in nasal mucous in CF patients compared to normal persons (125, 257). Smith et al. (257) found that the airway surface fluids of CF lungs had a reduced ability to kill bacteria and this was attributed to the high NaCl concentrations in this fluid. The elevated NaCl concentrations have been shown to affect phagocytic killing, inactivate the cationic peptides, human β -defensins, as well as the antimicrobial proteins, lysozyme,

lactoferrin, and the secretory leukocyte protease inhibitor (SLP1) (8, 45, 88, 252, 272, 278). Lysozyme and lactoferrin are the most abundant antimicrobial factors in airway surface fluid, and lysozyme is the most effective antimicrobial factor against *P.*

aeruginosa (19, 34, 278). Their inactivation may have a significant affect on bacterial infections. Singh et al. (250) also reported that lactoferrin inhibits *P. aeruginosa* biofilm formation, and could possibly play an important role in innate host response toward *P. aeruginosa* biofilm formation. However, if the lactoferrin activity is reduced in the CF lung, prevention of biofilm formation may also be reduced. Anionic peptides are not affected by high ionic concentrations, but are not as abundant in airway surface fluid (19). Their role in preventing bacterial infection in CF needs to be addressed.

Considering the complexity and diversity of the antimicrobial factors in the lungs it is difficult to assess the impact they have in CF infections and warrants more vigorous studies.

P. aeruginosa contains several proteases that interfere with innate host defenses. These include LasA (staphylolytic) protease, elastase, and alkaline protease, which are up-regulated in biofilms, and mucoid isolates (68, 242, 267). Elastase has a large repertoire of host molecules it can degrade, such as, elastin, collagen, cytokines, complement components like opsonin C3 and chemotactic protein C5. It also inactivates IgG (79). LasA has a unique effect on host tissue by inducing the shedding of syndecan-1, a cell surface heparin sulfate molecule that is anchored in the cell membrane (188, 189). These extracellular domains are shed during tissue injury and interfere with host defenses by binding to neutrophil elastase, cathepsin G, surfactants A and D, and cationic

peptides. Soluble heparin sulfate released from the shed domains, also inhibits cytokines that recruit phagocytes. In a study by Park et al. (188) addition of the syndecan-1 ectodomains along with challenge by *P. aeruginosa* into the lungs increased the incidence of infection resulting in increased mortality of mice compared to control treatments. Furthermore, *P. aeruginosa* elastase was recently shown to degrade surfactant protein A and D (SP-A and SP-D), whose levels are decreased in the BAL fluid of CF patients (156, 179, 211). Degradation of SP-A was also shown to reduce macrophage phagocytosis of *P. aeruginosa* (156). Interestingly, surfactant A levels in CF lungs with bacterial infections are inversely correlated with inflammation and surfactant D levels were inversely correlated to inflammation regardless of infection (179). These data indicate that lower levels of surfactants, induced by the presence of elastase or a consequence of the CF defect, play a role in progression of the disease by altering host responses.

Adherence

Adhesion of bacteria to epithelial cells usually initiates endocytosis, followed by desquamation of the cells from the epithelial layer and destruction of cells by apoptosis or by the activity of cytotoxic T cells. Understanding the alterations that occur in CF lungs in initial adhesion events of bacteria, specifically *P. aeruginosa*, may provide novel therapies to prevent infection. Three main theories exist describing adhesion of bacteria to pulmonary tissue of CF patients.

The most prominent defense mechanism in the lungs is the mucocilliary escalator, where bacteria and debris are trapped in the mucus layer that lines the lumen and are extracted from the lungs via the action of the ciliated epithelium. The viscous mucous layer in the CF lung is responsible for this defective clearance mechanism. The thicker mucous is a result of ionic imbalance that dehydrates the mucous and induces its production. Once infection occurs, mucous levels increase due to the inflammatory responses mediated by $\text{TNF}\alpha$ and $\text{Nf}\kappa\text{B}$ (53). High levels of DNA and actin contribute to pulmonary viscosity due to the persistent recruitment and lysis of neutrophils and macrophages. The most common and standard treatments with regard to thick mucous are chest percussions which loosens the thick, viscous mucous so that it can be expelled (37, 218). Even though this is still standard, more modern treatments are now used to correct the characteristic viscous pulmonary mucous. Amiloride aspirated into the lungs has been a successful treatment. Amiloride blocks Na^+ uptake by respiratory epithelium thereby reducing water loss in lumen, which helps regain proper mucocilliary clearance. Similar treatments include Dnase, which decreases viscosity of mucous by relieving the lungs of the overburden of DNA from neutrophils and PMNs infiltrating the lungs (37).

Critics of the mucous entrapment hypothesis state that it does not explain the selection and dominance of *P. aeruginosa* in CF airways. However, others suggest that *P. aeruginosa* may have special advantages with regard to mucous adhesion. Li et al. (147) demonstrated that *P. aeruginosa* LPS upregulated the Muc-2 gene, which participates in mucin production in epithelial cells. This up-regulation may also contribute to the increased mucous in the CF airway and decline in lung function.

Recently, Arora et al. (5) demonstrated that the flagellar cap protein of *P. aeruginosa* binds to mucin, explaining an increased affinity of this bacterium for the CF lung environment.

An alternative explanation the persistence of *P. aeruginosa* in the CF airway relies in the physiological lifestyle of *P. aeruginosa* once it is entrapped in airway mucous. The anoxic airway hypothesis suggests that *P. aeruginosa* is trapped in the thick isotonic mucous where oxygen is depleted. The partial pressure of O₂ of mucopurulent masses is greatly reduced in the CF lung and it has been suggested that CF epithelial cells have an abnormal consumption of oxygen, thereby contributing to steep oxygen gradients. The increased oxygen consumption in CFTR defective cells was related to the increased turnover rate of deregulated ion channels such as K⁺ and Na⁺. These channels are ATP-consuming pumps, and therefore, require more oxygen (290). These same investigators suggest that since *P. aeruginosa* is not found on epithelial cells, but rather, imbedded in the mucous, this organism has weak, if any, interactions with airway cells. The embedded cells found were of the typical mucoid microcolony morphology and these investigators suggest that the anaerobic environment contributes to the conversion of the bacteria to a mucoid phenotype (290). Even though it may be feasible that an established chronic infection persists in the anoxic mucous, this theory lacks convincing support for the initial colonization of *P. aeruginosa* in the CF airways. The next two hypotheses for adhesion discuss specific interactions of *P. aeruginosa* and host cell surfaces.

P. aeruginosa is 10-15% more adherent to CF cells than to normal epithelial cells (301). This phenomenon has been attributed to the increase of aGM1 (asialo-

gangliotetraosylceramide) sites observed on the apical membranes of CF epithelial cells. The tetrasaccharide (Gal- β 1-3-GalNAc- β 1-4-Gal- β 1-4Glc) of aGM1 acts as a receptor for *P. aeruginosa* (116). The inadequate acidification of CF cells causes undersialylation on epithelial cell surfaces and increases the aGM1 concentration. This relationship has only been demonstrated using *in vitro* studies, and has not been shown to occur *in vivo*. In addition, the putative binding mechanism was thought to be mediated by the bacterial pilus. However, structural data indicate that the pilus binding site is buried within the protein and would not interact directly with the aGM1 (200, 235).

Pier and colleagues have promoted the idea that CFTR is a receptor specifically for *P. aeruginosa* and loss of CFTR on epithelial surfaces decreases bacterial clearance. Pier et al. (207) demonstrated that cultured human Δ F508 cells are defective in uptake of *P. aeruginosa* compared to wildtype cells. The core LPS was determined to be the ligand, when exogenous core LPS was able to displace binding of *P. aeruginosa* to cells expressing CFTR. Further studies by this group identify CFTR as the receptor for LPS and identified amino acids 108-117 of CFTR as the recognition site (206). *In vivo* data supporting the CFTR-LPS hypothesis show that CF mice with no expressed CFTR had reduced uptake and clearance upon bacterial challenge and increased bacterial loads compared to wildtype mice. Also tracheas of wildtype mice infected with *P. aeruginosa* showed bacterial uptake and desquamation, but this was not observed in CFTR deficient mice (234). On the other hand, Chroneos et al. (31) previously showed no difference in clearance between CFTR over expressed mice and wildtype mice when challenged with PAO1, a non-mucoid burn wound isolate of *P. aeruginosa*. Coleman et al. (36) showed

the opposite results when mice with over expressed CFTR had accelerated clearance. To add to this controversy, it has also been shown recently that a major adhesion site for *P. aeruginosa* LPS is CD-14 on epithelial cells rather than CFTR (53).

Further research investigating CFTR-LPS interactions was conducted with an LPS deficient mucoid strain of *P. aeruginosa*. It was found that LPS deficient strains are retained equally well in CF mouse models and wildtype mice (31, 161, 234). However, when wildtype mice are challenged with an LPS positive strain the infection was more readily cleared (298). Contradicting this finding, Kalin et al. (126) indicated that CFTR is expressed on CF epithelial cells at normal levels. If this is true, then it may not be the loss of interaction between CFTR and LPS in the CF lung that inhibits uptake and clearance, but rather, the inability for defective CFTR cells to ingest the bacteria and/or go through apoptosis.

Several researchers have attempted to describe the apoptotic events of CF cells. Rajan et al. (217) showed no differences in apoptotic ability between CF cells and wildtype cells. Gallagher and Gottlieb (78) show that CFTR is independent of apoptotic events confirming the results of Rajan et al. (217). However, Pier and colleagues show otherwise in a detailed study where CFTR defective cells are delayed in apoptosis and have lower concentrations of the CD95/CD95 ligand, important for apoptosis of CFTR -/- epithelial cells (24, 94).

Supporting data for bacterial/CFTR specific interactions are seen in studies of *Salmonella enterica* serovar *typhi*. Disease by this organism requires translocation to the submucosa. Interaction between *S. enterica* and CFTR allows the epithelial cells to

desquamate and to expose the submucosa. Decreasing CFTR expression heightens the resistance to disease. In CF homozygous mice, no translocation of *S. enterica* was observed. In heterozygous mice translocation was reduced 86% compared to wildtype (205). Interestingly, this may provide evidence for the high frequency of mutant CFTR alleles in the Caucasian population, since this allele may confer resistance to diseases such as typhoid fever, cholera, tuberculosis and influenza (151).

Controversy regarding the role that CFTR has in *P. aeruginosa* clearance is far from resolved. It is likely that many of the above mentioned factors and undiscovered interactions between the pathogen and the host participate in a complex cascade of responses and reactions that allow *P. aeruginosa* to colonize and persist in the CF lung.

Alginate has not been shown to contribute to adherence to epithelial cells, however, its role in establishment of chronic infection is extremely important and will be the focus of the next section (161, 214).

Significance of Alginate Production and Mucoïd Phenotype

Alginate and the Immune Response

The most striking and clinically important manifestation of chronic *P. aeruginosa* infections is the conversion of the bacteria to the alginate over-producing, mucoïd, phenotype. The mucoïd phenotype was first observed in pancreatic infections of CF patients by Doggett et al. (56). Lam et al. (141) first described the connection between chronic pulmonary infection in CF patients and the mucoïd phenotype. Alginate contributes to lung infection in several ways, namely in avoidance of host defenses (201).

Even in the presence of extremely high titers of IgG and IgA, effective killing of *P. aeruginosa* does not occur. High titers of these antibodies are actually an indicator of poor pulmonary function (201, 213). The presence of high IgA is indicative of the inflammatory response. Alginate may enhance this response by stimulating B-cells and by contributing to the hypergammaglobulinemia often seen in CF patients (41, 193). Alginate also contributes to the inflammatory response by inducing proinflammatory cytokines such as Il-1, Il-8 and TNF α and by enhancing neutrophil oxidative bursts (195, 260). Ironically alginate has been shown to suppress neutrophil and polymorphonuclear (PMN) chemotaxis (195).

Alginate contributes to avoidance of immune responses by inhibiting opsonic and non-opsonic mediated killing, inhibiting phagocytosis, and scavenging reactive oxygen compounds (145). Inhibition of immune killing is partly due to the physical nature of mucoid strains and their ability to form large microcolonies that are difficult to be engulfed by macrophages. Alginate also blocks opsonic antigenic sites, such as the core oligosaccharide of LPS. Alginate inhibits stimulation of the alternative pathway of complement, and suppresses lymphocyte function and opsonic antibody production (80, 103, 155, 195). In the presence of non-opsonic antibodies to alginate, opsonic antibodies are not normally formed in either CF patients or non-CF individuals (80, 203, 204). Patients that have opsonic antibodies have better lung function (165, 187, 208). Non-opsonic antibodies can mediate opsonic complement, but complement is not deposited on cell surfaces, possibly due to the cleavage of C3bi by neutrophil elastase (195, 204, 277). More evidence to support poor capabilities of non-opsonic antibodies is that they do not

have any killing affect on *P. aeruginosa* biofilms (203). On the other hand, opsonic antibodies promote phagocytic killing, and complement-mediated killing by deposition of complement components C3bi and C3b (187, 204). Therefore, promoting opsonic antibody production would be extremely advantageous for CF patients.

In acute infections, mucoid *P. aeruginosa* promotes a Th1 response with higher levels of IFN γ and IL-12 (259). However, in CF patients with chronic infections the immune response is dominated by a Th2 response as seen with higher levels of IL-4 and lower levels of IFN γ . Patients with Th1 mediators have better lung function and reduced incidence of chronic infection (173). Promotion of a Th1 response may be helpful in treating chronic infections in CF patients.

Theilacker et al. (273) have created a vaccine against alginate by conjugating alginate to keyhole limpet hemocyanin, KLH. CF mice vaccinated with this conjugate produced opsonic antibody to alginate even in the presence of non-opsonic antibody to alginate. Subsequent vaccinations produced a T-cell independent response indicative of a Th1 response. This vaccine seems promising in promoting a more efficient immune response. However, even with a Th1 response the mucoid strains are not cleared well in acute infection models, confirming the significance of alginate and mucoid phenotype in disease (17, 159, 259).

Interestingly, Theilacker et al. also found that the opsonic antibodies had epitopes to the acetyl groups located on the mannuronate O2 or O3 residues of alginate. The presence of this epitope was essential in initiating a killing response (202). Others have shown that opsonic antibodies to serogroup A polysaccharide of *Neisseria meningitides*,

and type 5 and type 8 capsular polysaccharide of *Staphylococcus aureus* contain epitopes to acetyl groups (12, 63). Acetate groups are essential in resisting non-opsonic antibody mediated phagocytic killing as shown by Pier et al. (202) where non-opsonic antibodies are unable to kill strains with wild type alginate, but are able to kill acetate deficient strains. On the other hand, the investigators also demonstrated that opsonic antibodies containing epitopes to acetyl groups are able to kill both the wildtype strains and acetate deficient strains. In contrast, the presence of the acetyl groups on alginate are also essential in inhibiting the activation of alternative pathway of complement and blocks the deposition of C3b and C4b onto the hydroxyl groups of the uronic acid residues, interfering with the host immune defenses. Acetate residues also contribute to the physical properties of the polymer by enhancing its viscosity (177, 202, 277) and promoting the formation of microcolonies, essential for the establishment of biofilms and chronic infection (107, 177).

Alginate and Biofilm Phenotype of *P. aeruginosa* in Cystic Fibrosis

Besides the unique mucoid phenotype of *P. aeruginosa* in CF infections this organism appears to exist as a biofilm, as well. Biofilms are described as being a cohesive community of organisms surrounded by an extracellular matrix, and attached to a surface. (reviewed in (57, 112, 181, 191, 265, 283, 284). Biofilm microcolonies that are encased in alginate (141) have been observed in CF lungs. Alginate is responsible for the three dimensional architecture seen in biofilms produced by CF isolates. This structure is not observed in non-mucoid biofilms of PAO1 (107, 177).

The presence of quorum sensing (QS) molecules, global regulatory molecules essential for biofilm formation, in CF sputum supports *P. aeruginosa* existing as a biofilm in the CF lungs (44, 251). Two quorum-sensing signals are produced by *P. aeruginosa*, N-3-oxododecanoyl-homoserine lactone (3OC12-HSL) and N-butanoyl-homoserine lactone (C4-HSL). The LasI and RhII enzymes produce these molecules, respectively. These molecules, when bound to the regulatory proteins LasR and RhIR, initiate transcription of genes essential for biofilm formation (75, 190, 285). QS systems also regulate a number of virulence factors including elastase, LasA protease, alkaline phosphatase, and hydrogen cyanide (68). Recently microarray data have shown that these factors, with the exception of LasA, are also upregulated in biofilms produced by mucoid strains. Transcription of *lasA* and *lasB* has been observed in CF sputum and is positively coordinated with *algD* transcription, a gene essential for alginate biosynthesis (267). Other reports show an inverse relationship between alginate production and activity of LasA and elastase (162, 184). Mathee et al. (162) showed that upon conversion to mucoid phenotype, LasA and elastase activity diminished. However, the strains used in that study were not CF isolates, which may explain the differences observed. These data signify the complex nature of, and difficulty in understanding the *P. aeruginosa* mucoid phenotype in relation to its life in the CF lung.

Additional evidence for biofilm formation in the CF lung is the presence of the *P. aeruginosa* quinalone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, in CF sputum (96). PQS is an important component in biofilm regulation (96). In addition, *P. aeruginosa* CF isolates often lack LPS core oligosaccharide (142), and flagella (150). Most

significantly, these bacteria are highly resistant to antibiotics, typical of a *P. aeruginosa* biofilms. A study using an isogenic mucoid PDO300 (*mucA22* allele) and non-mucoid, PAO1 (wildtype) strains growing as biofilms showed that the mucoid biofilms were 1000 times more resistant to tobramycin than were the non-mucoid strains. When grown planktonically, no difference between the strains was observed, and both strains were sensitive to 1µg/ml of tobramycin (107). These data support the hypothesis that *P. aeruginosa* grows as a biofilm in the CF lung, and that alginate over-production by these strains in combination with biofilm growth work synergistically to establish chronic infection. These studies also signify the complexity of chronic infection with *P. aeruginosa*.

Conversion to the Mucoid Phenotype: Regulation and Reasons

Much research has been done to identify how *P. aeruginosa* converts to a mucoid phenotype. The locus on the *P. aeruginosa* genome that is responsible for alginate control was identified by Fyfe et al. (76). Mutations in this locus were suspected to initiate alginate synthesis. Martin et al. (159) linked alginate conversion to the *mucA22* allele. They showed that several mutations occur within *mucA*, resulting in frameshifts of the regulatory protein MucA. The most frequent mutations were a deletion of one cytosine in a string of five cytosines, or in an eight base pair repeat. It was later discovered that mutations in *mucB*, *mucC* and *mucD* also could induce mucoid conversion (15, 85, 157, 238). Mutations of these *muc* genes result in the deregulation of the alternative sigma factor AlgT (also called AlgU, σE , or $\sigma 22$). AlgT positively

regulates alginate expression by direct binding to the *algD* promoter, the first gene of the twelve gene operon that encodes the alginate biosynthetic proteins (27, 69, 293). MucA and MucB act as anti-sigma factors that negatively regulate AlgT activity (85, 163, 240). MucA was shown to directly bind to AlgT and is localized to the bacterial inner membrane (240). MucB is a periplasmic protein that possibly acts as a sensory protein (163, 240). *algT*, *mucABCD* are encoded on one operon that is positively autoregulated by AlgT (52, 108). The *muc* locus is not the only region that has an effect on AlgT regulation. Mutations in AlgW, a protein that resembles a serine protease, also results in a mucoid phenotype (15). AlgW is not encoded on the *muc* operon.

Evidence suggests that AlgT activity is strongly tied to environmental stresses. The harsh environment of the CF lung provides a selective pressure to maintain the expression of *algT*. AlgT is not only involved in expression of alginate genes, but is also required for expression other stress-related factors. AlgT has 66% identity to σ^E of *E. coli*, a sigma factor that responds to heat stress (51). *algT* is upregulated during heat shock and positively regulates *rpoH*, a sigma factor required for heat shock response (237, 239). AlgT and σ^E of *E. coli* can be interchanged, and they recognize the same promoter sequence (108). AlgT is also similar to Spo0H, an alternative sigma factor that controls sporulation in *Bacillus subtilis* (158). AlgT and the alternative sigma factor, RpoH are expressed simultaneously during heat shock. The presence of RpoH in mucoid CF isolates and in a mucoid derivative of PAO1, suggests that RpoH expression may be a result of AlgT deregulation and not a result of heat stress (68, 237). Conversion to mucoidy was observed when PAO1 was exposed to both heat shock and osmotic shock,

but not when each stress was applied separately (241). Upregulation of *osmC* and *osmE* by AlgT supports AlgT as an important factor in modulating the effects of osmotic shock (68).

AlgT expression may be upregulated by oxidative stress. Mutants with deletions of *algT* have increased sensitivity to paraquat, a superoxide-generating redox cycling compound. Conversion to the mucoid phenotype was observed in PAO1 biofilms that were grown with constant exposure to H_2O_2 . These conditions resulted in *mucA* mutations (160, 162). High levels of superoxide dismutase, which neutralizes superoxide oxygen radicals, were seen in mucoid strains (98, 102). The high level of oxygen radicals and H_2O_2 released by PMN in the lungs may therefore contribute to the conversion of *P. aeruginosa* to mucoidy.

The outer membrane porin, OprF, is another gene upregulated by AlgT (67, 68). This porin is expressed under anaerobic conditions and is expressed in CF isolates (98, 297). Some argue that OprF is present due to mucoid strains living anaerobically in mucopurulent masses and is evidence that anaerobic growth selects for mucoid isolates (101, 290). However, the presence of OprF may only be a result of AlgT deregulation. If *P. aeruginosa* is living anaerobically, then selection for AlgT deregulation may enable expression of genes needed for anaerobic growth. However, as stated earlier, mucoid conversion occurs under oxidative stress as well. Therefore, it appears that several factors of the CF environment would contribute to AlgT deregulation and conversion to the mucoid phenotype.

Other genes upregulated by AlgT are *lptA* and *lptB*, lipoproteins that stimulate the release of Il-8 from PMNs; *osmC* and *osmE*, important in high osmolarity; and regulator genes involved in alginate synthesis (67, 68). AlgT negatively regulates *fliA* expression and abrogates flagella synthesis (68, 81, 286), suggesting that environmental stress, biofilm formation, and AlgT activity are interconnected. Some AlgT regulated proteins are seen in the expression profiles of growing biofilms, such as *osmC*, *osmE* and *pfp1*, a putative protease, suggesting that AlgT plays a role in biofilm development (68, 242).

The mucoid phenotype is rarely found in *P. aeruginosa* isolates from non-CF sources. Alginate production is not necessarily upregulated upon exposure to environmental stresses. Schurr et al. (241) showed that when PAO1 was grown with heat stress, AlgT was expressed, but did not result in a mucoid phenotype. When the cells were grown with both heat and osmotic stresses, alginate production was induced. This indicates that regulation of alginate production is complex. RpoS is a global stress response regulator that has been shown to be important in the biofilm phenotype (144), and in alginate production. Alginate production is reduced by 70% in an *rpoS* mutant (268). AlgT is at the top of the alginate regulatory hierarchy. AlgT in combination with the RNA polymerase binds to the *algD* promoter and induces transcription of the alginate biosynthetic operon. In addition, AlgT controls the expression of other alginate regulatory factors, including *algR* and *algB*. AlgR and AlgB are response regulators of two-component regulatory systems, where AlgZ and KinB are their respective cognate kinases (49, 84, 87, 153, 292, 299). Phosphorylation by the cognate kinases was shown not to be required for AlgR or AlgB activity in regulation of alginate production (152).

AlgR has been shown to be necessary for alginate production in mucoid strains and directly binds to the *algD* promoter (47, 49, 128). AlgR also regulates *algC*, a phosphomannomutase and a phosphoglucosemutase, needed for both alginate synthesis and LPS synthesis (40, 305, 306). AlgB expression does not rely entirely on AlgT activation, but on other stress signals that are regulated by integration host factor (IHF) (291). Other alginate regulatory proteins include AlgP and AlgQ whose roles are not yet clear (49, 50, 129). Additional regulation is observed with an IHF binding site located upstream of the *algD* promoter (170). Recently, it has been shown that RpoN (σ_{54}) participates in sigma factor antagonism and binds to the *algD* promoter, preventing activation by AlgT (16).

Even though alginate regulation is complex, mucoid phenotypes emerge in CF infections primarily due to the *mucA* mutations. As stated previously, H₂O₂ exposure could induce these mutations. But why is *P. aeruginosa* prone to such mutations? Oliver et al. (185) suggest that CF isolates have become hypermutable. Their study looked at the mutation rate in CF isolates compared to non-CF strains. They showed that 36% of CF patients were colonized with a hypermutable strain, while non-CF acutely infected patients were not. Of the strains more closely studied, two had deletions in the *mutS* gene and four had deletions in *mutY*. Another explanation for the mutations observed in *P. aeruginosa* is the presence of large chromosomal inversions (LCI) (225). Kresse et al. (140) identified strains containing insertion sequence IS6100 in *wbpM*, *pilB* or *mutS*, which explains the LPS deficiency, pilin deficiency and the hypermutability seen in these strains, respectively. This insertion element was originally identified in *Mycobacterium*

fortuitum, and has been shown to cause genetic rearrangements in a heterologous host (97). LCIs are often seen in pathogenic bacteria such as *Bordetella pertussis*, *Salmonella typhi*, and *Neisseria meningitidis* (114). Even though the genomes of CF isolates are similar to that of PAO1, whose genome is completely sequenced, variations exist in the form of large genetic re-arrangements, including insertions and deletions (261, 288), Wolfgang et al. (288) showed that clusters of strain specific genes are sites for insertions of novel genetic material. One example is the presence of the *exoU* virulence island. *P. aeruginosa* isolated from late-stage CF infections contain additional genetic material in comparison to the PAO1 reference strain, accounting for 10% of their entire genome. The G+C content in many of these additional regions is much lower than the average content for PAO1. This indicates that these regions were obtained through horizontal gene transfer, which may contribute to the genetic variation among different CF isolates (261).

Another selective pressure is the presence of antibiotics, used to treat the bacterial infections. Bacterial biofilms are 100-1000 times more resistant to antibiotics than their planktonic counterparts (112, 265). Therefore, antibiotics may be one factor in the conversion to mucoid phenotype. When non-mucoid PAO1 was grown in a chemostat with 3 μ g of ciprofloxacin or other fluoroquinolones, conversion to mucoidy was observed. When the antibiotic was removed the strains reverted to a non-mucoid phenotype (209). However, alginate production alone was not enough to resist antibiotic challenge indicating that other cellular modifications occur in CF isolates that allow them to become antibiotic resistant (162, 209). The hypothesis that mucoid phenotype is selected for under antibiotic pressure is supported by the study of Hentzer et al. (107),

that showed higher antibiotic resistance in biofilms of mucoid strains than in biofilms of nonmucoid strains, suggesting that over-production of alginate and biofilm growth are required for high levels of antibiotic resistance. Recently, Drenkard and Ausubel (58) also showed a high frequency of antibiotic resistant rough-small colony variants isolated from CF patients treated with antibiotics. Patients not treated did not have these variants. These variants were highly resistant to antibiotics, and formed biofilms quickly in presence of antibiotics. This group also identified a two component regulatory system PvrR that is directly involved in the antibiotic resistance of these variants. Their results indicate that antibiotic use may be partially responsible for the persistence of *P. aeruginosa* in the CF lung.

Alginate Biosynthesis

Alginate is a non-branching, non-repeating copolymer composed of D-mannuronic (M) acid linked to its C-5 epimer L-guluronic (G) acid by a β 1-4 glycosidic bond. Alginate was originally described as a polymer produced by marine algae and seaweed. Alginate is also produced by *Azotobacter spp.* (38, 90, 92, 148). Alginates form gel like substances, where the arrangement of M and G residues along the polymer gives it certain gelling properties (266). These gelling properties have been exploited in industrial processes, especially in the food and cosmetic industries (254). Alginate produced by seaweed, algae and *Azotobacter* contain regions within the polymer composed of only guluronate residues. These regions are referred to as G-blocks. G-blocks form an 1C_4 conformation (Figure 1.2) which positions the residues in what is

called an egg box structure. These structures bind calcium ions, and contribute to the stiffness of the alginate (93). The advantages of these varied gelling properties is seen in seaweed plant structure where the high G-block content exists in the stiff stipes and low G content exists in the more flexible blades that bend with high ocean currents (6, 105, 137). Alginates from pseudomonads do not form G-blocks, rather, they have alternating M/G blocks or M/M blocks (236, 247). M blocks form a 4C_1 conformation that is less stiff than G blocks and M/G blocks are the most flexible form (Fig.1.2). *Azotobacter vinelandii* is able to form a variety of different alginate types, including all three basic combinations, G/G, M/M and M/G. The different ratios of M/G residues are used in producing different layers in the dormant cyst of *Azotobacter spp* (186). *P. aeruginosa* alginate, however, forms a flexible or fluid like gel that is detachable from the surface of the cells. This might provide *P. aeruginosa* with a survival advantage since the alginate will form a protective barrier, yet allows essential nutrients and water to pass to the bacteria. A unique feature of the bacterial alginates is that they are acetylated at O2 or O3 on mannuronate residues (253). Acetylation occurs on about 40% of the mannuronate residues of alginate produced in *P. aeruginosa* (236, 253). The presence of acetyl groups contributes to greater viscosity of the polymer and the three dimensional biofilm structure seen in *P. aeruginosa* FRD1 biofilms (177).

Alginate synthesis in *P. aeruginosa* and *A. vinelandii* is dependent on the Entner-Doudoroff metabolic pathway (9, 11). Sugar components, such as mannitol, glucose, and gluconate, are broken into trioses, glyceraldehyde-3-phosphate and pyruvate. Glyceraldehyde-3-phosphate can go directly into alginate synthesis, but pyruvate is

converted into oxaloacetate and into phosphoenolpyruvate. An aldolase converts these two trioses into the six carbon sugar, fructose-6-phosphate. Fructose-6-phosphate is considered the initial component in alginate production.

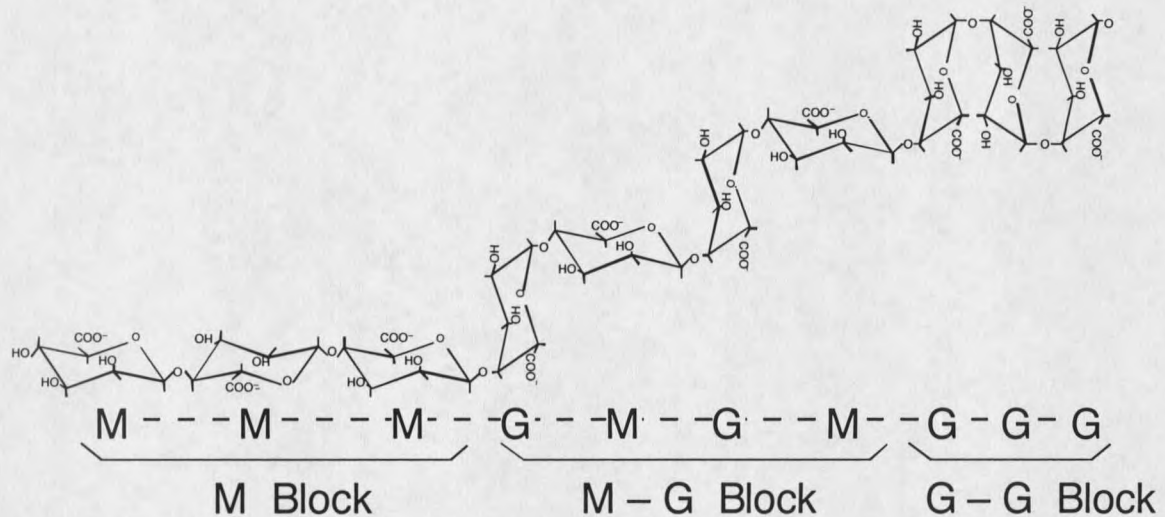


Figure 1.2. Alginate structure. M represents mannuronate residues. G represents guluronate residues.

As mentioned earlier, alginate is synthesized from fructose-6-phosphate by thirteen proteins, twelve of which are encoded on a single operon, the *algD* operon (Fig.1.3) (27). AlgC is encoded elsewhere on the chromosome (305). AlgA, AlgD and AlgC convert fructose-6-phosphate to GDP-mannuronate. AlgA has two roles in alginate synthesis, one, as a phosphomannose isomerase converting fructose-6-phosphate into mannose-6-phosphate, and two, as a GDP-D-pyrophosphorylase, converting mannose-1-

phosphate into GDP-D-mannose (42, 82, 249). AlgC also has dual activities. In alginate synthesis, AlgC acts as a phosphomannose mutase, converting mannose-6-phosphate into mannose-1-phosphate. In LPS biosynthesis, it acts as a phosphoglucose mutase (40, 86). AlgD, whose crystal structure was recently solved, is a GDP-mannose dehydrogenase that converts the GDP-mannose into GDP-mannuronic acid, the monomer precursor to the alginate polymer (48, 258). These monomers are polymerized into the periplasm by an unknown protein or complex, possibly involving Alg8, which resembles β -glycosyl transferases (27, 43, 154, 231). AlgK may also play a role, since deletion mutants of *algK* result in non-mucoid phenotype (118).

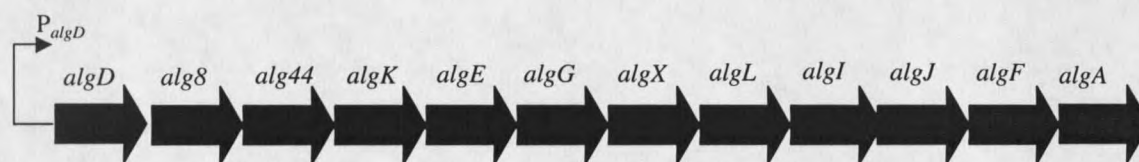


Figure 1.3. Alginate biosynthetic operon

The polymannuronate polymer in the periplasm is modified in two ways, one, by the conversion of some D-mannuronate residues to L-guluronate residues to create a M/G ratio of approximately 3:1 (236), and two, by the addition of acetyl groups on some of the D-mannuronate residues at positions O2 or O3 of the uronic acids. This polymer modification process is unusual for polysaccharide biosynthesis. In most carbohydrate polymers, the unique monomer components are produced separately and then assembled in repeating units. In alginate biosynthesis, the epimerization of D-mannuronate to L-guluronate is carried out by the periplasmic C-5 epimerase, AlgG (70). AlgG may also

play a role in alginate polymerization. A recent study by Jain et al. (118) has shown that like *algK*, deletion of *algG* results in a non-mucoid phenotype. Their studies show that in these mutants, uronic acids are secreted from the cells. The secreted uronic acid residues contain unsaturated ends, indicative of alginate lyase activity. These authors suggest that AlgK and AlgG are important in polymerization by protecting the polymer from AlgL, the alginate lyase encoded on the alginate biosynthetic operon. They also proposed that alginate synthesis occurs within a scaffold or protein complex. AlgL is a periplasmic protein whose function is required for alginate synthesis, but its exact role is unclear (personal communication with D. E. Ohman, 18, 233). The addition of acetyl groups onto mannuronate residues also occurs in the periplasm by the acetylation complex consisting of AlgI, AlgJ and AlgF (71, 72). AlgI is a membrane spanning protein, AlgJ is a periplasmic protein whose leader sequence is not cleaved and remains bound to the inner membrane, and AlgF is a periplasmic protein (73). How these proteins work to acetylate the polymer is not yet clear. Finally, it is thought that AlgE, a protein resembling an outer membrane secretin, secretes the final alginate polymer (32, 220). The functions of AlgX and Alg44 that are also encoded on the twelve-gene operon are not yet known. Figure 1.4 illustrates a model for alginate biosynthesis.

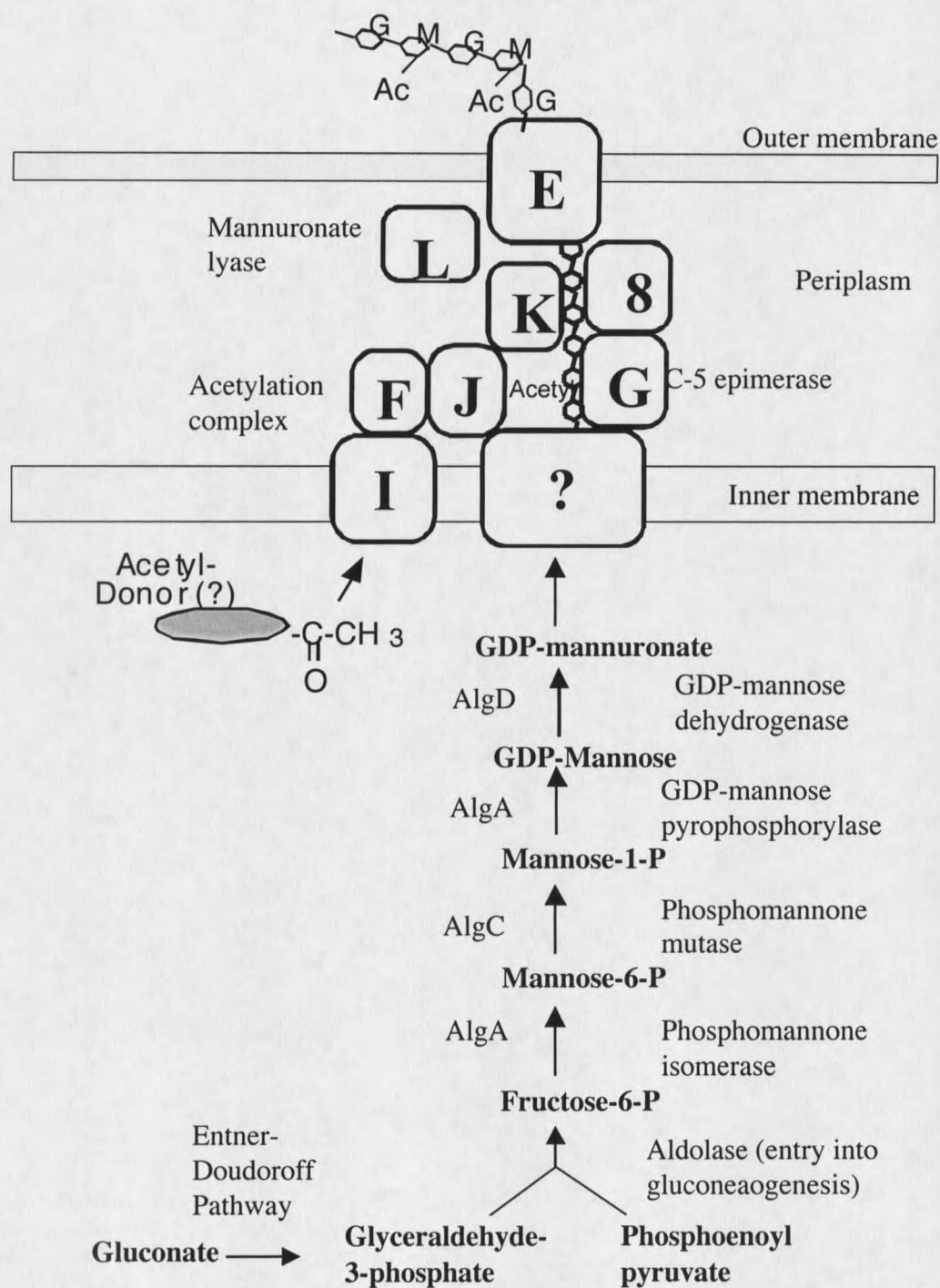


Figure 1.4. Alginate biosynthesis from gluconate. Modified from Banerjee (9) and Beale (11).

The research described here, focuses on enzymes involved in alginate modification, in particular enzymes involved in alginate acetylation, AlgI, AlgJ, and AlgF as well as the C-5 epimerase AlgG.

The mature form of AlgG is a 55kD periplasmic protein, encoded by 1.6kb gene. AlgG homologues exist in several bacterial species that produce alginate, such as *P. fluorescens*, *P. syringae*, and *A. vinelandii* with 67%, 65% and 66% identity, respectively (172, 196, 221). Recently a C-5 epimerase resembling AlgG has been characterized in the marine brown alga *Laminaria digitata* (180). *A. vinelandii* has eight additional extracellular epimerases (AlgE1-7 and AlgY), encoded by a cluster of genes on the *A. vinelandii* chromosome (61, 269). Haug and Larson (104) first identified C-5 epimerization in *A. vinelandii* in 1969. These epimerases contain two distinct modules that can be repeated several times in one gene, and are termed A and R modules (61). R-modules are important in binding calcium and A modules are involved in catalysis. The A modules have ~40-55% identity to AlgG of *P. aeruginosa*. Even though the A-modules of the AlgE proteins are highly similar to one another their epimerase activity varies. AlgE6 produces long strings of G-blocks, AlgE2 produces short G-blocks, and AlgE4 produces M/G blocks (61, 62, 99, 269).

C-5 epimerases specific for other carbohydrates exist. The glycosaminoglycans (GAG) heparin, heparan sulfate and dermatan sulfate are modified by C-5 epimerases (279). These polymers produced by higher eukaryotes consist of glucuronic acid linked via 1-4 glycosidic bonds to α -D-glucosamine or β -D-galactosamine for heparin and dermatan, respectively. As in alginate synthesis where mannuronate is converted to

guluronate and O-acetylated post polymer formation, the glucuronic acids are epimerized to α -L-iduronic acid and then sulfated after these polysaccharides are polymerized (279). While little sequence similarity is seen between alginate epimerases and GAG epimerases, Valla et al. (279) showed similarities between them in hydrophobicity plots.

O-acetylation of *P. aeruginosa* alginate occurs via the AlgI, AlgJ and AlgF O-acetylation complex (71, 72). AlgF is a 23kD protein that is exported to the periplasm. In the periplasm the leader sequence is cleaved and the final protein is ~20kD. AlgI is an integral membrane protein with seven membrane spanning helices. AlgJ is a 43 kD protein that is exported into the periplasm, but remains linked to the inner membrane via its uncleaved leader peptide (73), and therefore, is classified as a type II membrane protein (215). AlgJ has little sequence similarity to proteins other than AlgJ and AlgX from other pseudomonads. Although these proteins are all important for acetylation little is known about the mechanism of O-acetylation in bacterial alginates.

As stated earlier, the function of several of the alginate biosynthetic proteins is known, however little data exist on the interactions between alginate and its modifying enzymes. Likewise, there is little information about the biochemical activities of these enzymes. The intention of this research was to investigate the carbohydrate binding interactions and amino acid residues involved in these interactions. In addition, these studies were designed to identify important catalytic regions within the alginate modifying proteins. These studies primarily focused on characterizing AlgG, secondarily on AlgJ, and finally on the two putative lyases identified in *P. aeruginosa*. As a result, this research provides a greater understanding of carbohydrate/protein interactions of

AlgG and AlgJ, as well as provides a greater understanding of alginate biosynthesis in the opportunistic pathogen *P. aeruginosa*, and possibly contributes to the development of novel treatments against these infections in CF patients.

CHAPTER TWO

THE PREDICTED STRUCTURE OF ALGG, THE ALGINATE C-5 EPIMERASE OF
P. AERUGINOSA, AND ITS FUNCTIONALLY IMPORTANT DOMAINSIntroduction

Alginate is a viscous polysaccharide produced by brown seaweeds and by certain bacteria including *Pseudomonas* and *Azotobacter* species. Alginate is a high molecular weight linear copolymer composed of β -D-mannuronic acid (M) and its C-5 epimer α -L-guluronic acid (G) linked by β 1-4 glycosidic bonds. In bacterial but not in algal alginates, the M residues are modified by the addition of O-acetyl groups at the O2 and/or O3 positions (253). The block structures of alginate vary depending on the organism producing the polymer. Algal and *A. vinelandii* alginates contain continuous stretches of G residues (G-blocks), while alginates from pseudomonads contain primarily M residues randomly interspersed with G residues (105, 236, 253). The G-blocks chelate calcium and give the polymer its gelling properties (93, 105, 137, 253, 254), important in *A. vinelandii* for building the capsule of the cysts (229). In *P. aeruginosa*, however, G blocks are not found, most likely because the alginate epimerase of pseudomonads, AlgG, is not capable of introducing a G residue adjacent to another G-residue (236, 253). This results in a more flexible gel than the *A. vinelandii* alginates (77). In *A. vinelandii*, G-blocks are likely introduced into the alginate polymer by a series of extracellular mannuronan epimerases, AlgE1-AlgE7, that are encoded by a tandem repeat of genes on

the *A. vinelandii* genome(61, 269). Genes similar to those for the *A. vinelandii* extracellular epimerases are not found on the *P. aeruginosa* genome.

In the opportunistic pathogen *P. aeruginosa*, alginate is an important virulence factor, particularly in patients with the genetic disorder cystic fibrosis (CF), where conversion of strains to the alginate overproducing (mucoid) phenotype often results in chronic *P. aeruginosa* infections (194). Alginate acts as a virulence factor in these chronic infections by contributing to the matrix material of the *P. aeruginosa* biofilms, and by protecting the bacteria from opsonic phagocytosis. Alginate also neutralizes oxygen radicals produced by inflammatory immune cells (145), and stimulates the production of inflammatory cytokines indicative of a Th2 type immune response (192, 195). Alginate structure contributes to its activity as a virulence factor. For example, the M/G block structure is important for the chemical and physical properties of the polymer, including its viscosity and its interaction with divalent cations (253, 254). In addition, the presence of O-acetyl groups on the O2 and O3 positions of the D-mannuronate residues (253) has been shown to be essential for the formation of microcolonies, for the avoidance of antibody-independent opsonic mediated killing, and for conferring resistance to alginate specific antibodies (177, 202).

The bacterial alginate biosynthetic pathway can be thought of in four parts, 1) synthesis of the alginate precursor, GDP-mannuronic acid, 2) polymerization of GDP-mannuronic acid into β 1-4 linked polymannuronate, 3) modification of the polymannuronate into its final alginate structure, and 4) polymer secretion. AlgA, a phosphomannose isomerase-GDP-D-mannose phosphorylase, AlgC, a

phosphomannomutase, and AlgD, a GDP-mannose dehydrogenase, carry out precursor synthesis. Precursor synthesis has been well characterized, and the crystal structures of AlgD and AlgC as well as the active sites within these enzymes have been characterized (258). Less is known about the polymerization step, which may be carried out by Alg8 and Alg44, putative inner membrane proteins (154). The periplasmic proteins, AlgX and AlgK, whose functions have not yet been determined, may also play a role in polymerization (27, 118). Enzymes that primarily localize to the periplasm carry out modifications of polymannuronic acid to its structurally mature and functionally active form. These enzymes include the AlgI, AlgJ, AlgF, alginate O-acetylation complex, AlgG, the periplasmic mannuronan epimerase, and AlgL, an alginate lyase (26, 70-73, 233). Jain et al. (117) proposed that the alginate biosynthetic complex might form a multicomponent scaffold, involved in polymer modification and export. Evidence supporting this hypothesis includes the deletion mutation studies of *algG* and *algK* (117, 118). These deletion mutants secrete depolymerized alginate, suggesting that removal of either of these components from the putative alginate biosynthetic scaffold results in access to and degradation of the alginate by the periplasmic alginate lyase, AlgL. Further support for the scaffold model is the work of Gimmestad et al. (83) who suggest that one epimerase is required for each alginate molecule synthesized, rather than multiple epimerases acting at random sites on the polymer. Again, this suggests that the alginate is shuttled across the periplasm through an alginate biosynthetic complex, and that mannuronan epimerization occurs during this shuttling process. To further characterize AlgG and its interaction with the alginate precursor, polymannuronate, and to gain a

better understanding of the alginate biosynthetic scaffold, we performed structural predictions of AlgG. AlgG contains a repeated sequence in its C-terminus identified as a carbohydrate and sugar hydrolases (CASH) domain (33). We show that these repeats are predicted to fold into a right-handed β -helical structure (RH β H), similar to other proteins containing the (CASH) domain (33). The putative RH β H structure of AlgG contains a shallow groove that may accommodate the alginate polymer as it is shuttled through the periplasm. Site directed mutagenesis studies suggest that this groove contains an active site for mannuronan epimerization.

Methods

Bacterial Strains, Plasmids, Mutagenic Oligonucleotides and Media

Bacterial strains, plasmids, and mutagenic oligonucleotides are listed in Table 2.1. *P. aeruginosa* and *E. coli* were cultured on L broth (LB) (10g tryptone, 5g yeast extract, 5g NaCl per liter). Triparental matings were used to transfer plasmids from *E. coli* to *P. aeruginosa* using the helper plasmid pRK2013 and pseudomonas isolation agar (PIA) (Difco) as the selective medium. Antibiotics when used were at the following concentrations ($\mu\text{g/ml}$): carbenicillin 300, ampicillin 100, tetracycline 20, and kanamycin 60.

Table 2.1 Strains and Plasmids

Strain or Plasmid	Genotype, phenotype or mutagenic oligonucleotide	Reference
<i>E. coli</i>		
HB101	proA2, leuB6, thi-1, lacY1, hsdR, hsdM, recA13, supE44, rspL20	
<i>P. aeruginosa</i>		
FRD1	Cystic fibrosis isolate Alg ⁺	(183)
FRD462	algG4 S272N Alg ⁺	(26)
FRD1200	Δ algG:: <i>Gm</i>	(117)
Plasmids		
pRK2013	ColE1-Tra(RK2) ⁺ Km ^R	(65)
pALTER-Ex1	phagemid Tc ^R	Promega
pMF54	P _{trc} ColE1 replicon with oriV _{SF} oriT(RK2) lac ^q Ap ^R	(70)
pMF55	pMF54 with 2.2kb algG NcoI-NcoI fragment	(70)
pSAD51	pALTER Ex-1 with 2.2kb algG insertion with D324 point mutation to A	This study
pSAD1	pALTER-Ex1 with 2.2kb algG NcoI-NcoI fragment	This study
pJS209	pMF54 with 1.7kb algG4 fragment from FRD462 cloned into XbaI -Xho sites	(117)
pMF106	pMF54 with algL	(117)
pRC5	contains aly guluronate lyase of <i>Klebsiella aerogenes</i> Ap ^R	(25)
pSAD20	pMF54 with algG E94A CCGCCGCGCGGTGGCCGCAAGATCGTGC	This study
pSAD83	pMF54 with algG R99A AAGCCAAGATCGTGGCCAAGCCGGGTGGCC	This study
pSAD94	pMF54 with algG deletion of amino acids 89-102 GCCCGACCTCTCCGCGGTGGCCGCGCCAGCG	This study
pSAD21	pMF54 with algJ K58A CGAAGCCCCTACGACGCGGAATTCCCGATCAAG	This study
pSAD22	pMF54 with algJ E53A CTGGCGCACGCCTTCGCCGCCACTACGACAAGG	This study
pSAD32	pMF54 with algJ H55A GCACGCCTTCGAAGCCGCTACGACAACCAATTC	This study

Abbreviations: Alg⁺, alginate overproduction; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R tetracycline resistance; Tra, transfer by conjugation.

DNA manipulations

General cloning procedures were carried out using the protocols described in Ausubel et al. (7). For site-directed mutagenesis of AlgG the Altered Sites protocol was used (Promega). The NcoI fragment containing the *P. aeruginosa* algG open reading frame, described previously (70) was ligated into the pALTER Ex1 vector (Promega)

producing plasmid pSAD1. Single stranded DNA was produced by subjecting pSAD1 to R408 phage packaging. Single stranded DNA was isolated from phage and subjected to mutagenesis as described in Promega pALTER protocol. Briefly, *E. coli* JM109 (pSAD1) clones were grown in LB with tetracycline and with R408 phage for 6 hours. Phage particles were precipitated from the supernatant on ice with 20% polyethylene glycol and 3.5M ammonium sulfate, pH 5.3. Contaminating proteins were removed, first, with the addition of equal volume of phenol chloroform, vortexed and centrifuged for 5 minutes at 13,000 rpm. The aqueous layer was transferred to a new tube. Second, two more proteins extractions were conducted with chloroform/isoamyl alcohol (24:1), in equal volume to the sample, to remove the phenol and proteins. Single stranded DNA in the aqueous layer was then precipitated with ethanol and centrifuged. The DNA pellet was dried and resuspended in deionized H₂O. Mutagenic oligonucleotides (Table 2.2) were annealed to the purified pSAD1 single stranded DNA to introduce point mutations into *algG*. Ampicillin repair oligonucleotide and a tetracycline knockout oligonucleotide were also annealed to the pSAD1 single stranded DNA and used as a selection method. The ampicillin oligonucleotide repairs a mutation in the ampicillin gene to recover ampicillin resistance and the tetracycline oligonucleotide introduces a mutation in the tetracycline gene to knock out resistance. A phagemid is synthesized *in vitro* following the Altered Sites protocol and amplified in *E. coli* in the presence of ampicillin. These are then plated onto agar containing LB and ampicillin for selection of possible site directed mutants. Phagemid DNA from individual colonies was purified and the region containing the point mutation was sequenced. Once mutant DNA was identified, the

NcoI fragment containing *algG* was cloned into the *P. aeruginosa* P_{trc} expression vector pMF54. Plasmids were introduced into *P. aeruginosa* FRD462 *algG4* and *P. aeruginosa* FRD1200 $\Delta algG::Gm$ by triparental matings. The resulting transformants were tested for the recovery of a mucoid phenotype in FRD1200, which would indicate that the mutant AlgG is able to restore the polymerization defect in the FRD1200 *algG* deficient strain. The transformants were also tested for the recovery of guluronate residues to the alginate polymer in the epimerization deficient strain FRD462, as well as in FRD1200.

Overlap extension PCR was used to construct plasmids with deletions of amino acids 37 to 137, or amino acids 37 to 161 from the N-terminal α -helical region of AlgG, while retaining the native AlgG signal peptide. The resulting plasmids were labeled pSAD149 and pSAD151. For these deletion experiments, two partial *algG* fragments were constructed, encoding the leader fragment and the β -helical region. The leader fragment was amplified from pMF55 with the forward primer 5' CGACTGCACGGTGCACCAATGCTTC -3' and reverse primer 5'- GAAGATCGCCTGGGGCGCCGCCACGCCTGGCCGTGCAG 3'. The reverse primer allowed PCR of the cleavage site of the leader sequence and an additional 15 nucleotides that acted as an overhanging primer for the 5' end of the β -helical region. The β -helical fragment was primed with a forward primer 5'- CAGGCGTGGGCGGGCGCCCCAGGCGATCTTCATCGAAGGC-3' containing the nucleotides that code for 137-PQAIF-141 and the 15 overlapping nucleotides to prime the 3' end of the cleavage site. The reverse primer 5'- GGGCCATCTAGAGGCCGGCGGCC-3' was engineered to introduce an *XbaI*

restriction site 58 nucleotides downstream from the stop codon. The two PCR products were confirmed by agarose gel electrophoresis and gel purified. A second PCR amplification was conducted by combining the first two PCR products, allowing annealing of the overlapping regions. One large fragment was amplified by using the forward primer of the leader fragment and the reverse primer of the β -helical fragment. The final 1.5kb fragment was confirmed by agarose electrophoresis. This fragment was gel purified and cloned into the pMF54 plasmid using the *NcoI* and *XbaI* restriction sites creating pSAD149. Figure 2.1 shows a schematic representation of this procedure. This procedure was also used to construct a larger N-terminal deletion, using primers listed in Table 2.2. The resulting plasmid was labeled pSAD151.

Table 2.2 Mutagenic oligonucleotides and PCR primers for overlap extension PCR

AlgG Mutation	pMF54 clone	Mutagenic oligonucleotide 5'-3'
R316A	pSAD84	AAGGGCAACACCTACGCCGACAACATCGTCTAC
D317A	pSAD86	GGCAACACCTACCGCGCCAACATCGTCTACGGC
Y321F	pSAD68	CGCGACAACATCGTCGGCGGCATCGACCCG
D324A	pSAD53	ATCGTCTACGGCATCCGCCCCGCACGACCCGT
P325A	pSAD56	GTCTACGGCAATCGACGCGCACGACCGTTCGCAC
H326A	pSAD47	TACGGCATCGACCCGGCCGACCGTTCGCAC
D327A	pSAD70	TACGGCATCGACCCGCACGCCCGTTCGCACCCGCT
D336A	pSAD88	CGCCTGATCATCGCCGCCAACACCGTCCACGGG
N361A	pSAD85	GACAGCTTCATCTTCGCCAACCGCAGCTACGAG
N362A	pSAD92	AGCTTCATCTTCAACGCCCGCAGCTACGAGAAC
S364A	pSAD110	ATCTTCAACAACCGCGCCTACGAGAACAAGCTT
Y365F	pSAD107	TTCAACAACCGCAGCTTCGAGAACAAGCTTTCC
E366A	pSAD137	AACCGCAGCTACGCGAACAAGCTTTCC
N367A	pSAD90	AACCGCAGCTACGAGGCCAAGCTTTCCGGGATC
V383A	pSAD102	AGCGAGGGCAACCTGGCCGCCTACAACGAGGTC
Y385F	pSAD105	GGCAACCTGGTGGCCTTCAACGAGGTCTATCGC
D324A.H326A.	pSAD157	TACGGCATCGCCCCGGCCGACCGTTCGCAC
D324A. D326A	pSAD158	TACGGCATCGCCCCGCACGCCCGTTCGCAC
deletion mutant	pSAD94	GCCCGACCTCTCCGGCGGTGGCCGCGCCAGCG
89-102		
<u>N-terminal</u>		
<u>deletions</u>		
37-137	pSAD149	Leader fragment forward primer CGACTGCACGGTGCACCAATGCTTC Leader fragment reverse primer GAAGATCGCCTGGGGCGCCGCCACGCCTGGCCGTGCAG Beta helix fragment forward primer CAGGCGTGGGCGGCGCCCCAGGCGATCTTCATCGAAGGC Beta helix fragment reverse primer TGGGCCATCTAGAGGCCGGCGGCC
37-161	pSAD151	Leader fragment forward primer CGACTGCACGGTGCACCAATGCTTC Leader fragment reverse primer CTCGACCTGTTCCAGCGCCGCCACGCCTGGCCGTGCAG Beta helix fragment forward primer CAGGCGTGGGCGGCGCTGGAACAGGTCGAGCCGGGGGTG Beta helix fragment reverse primer TGGGCCATCTAGAGGCCGGCGGCC

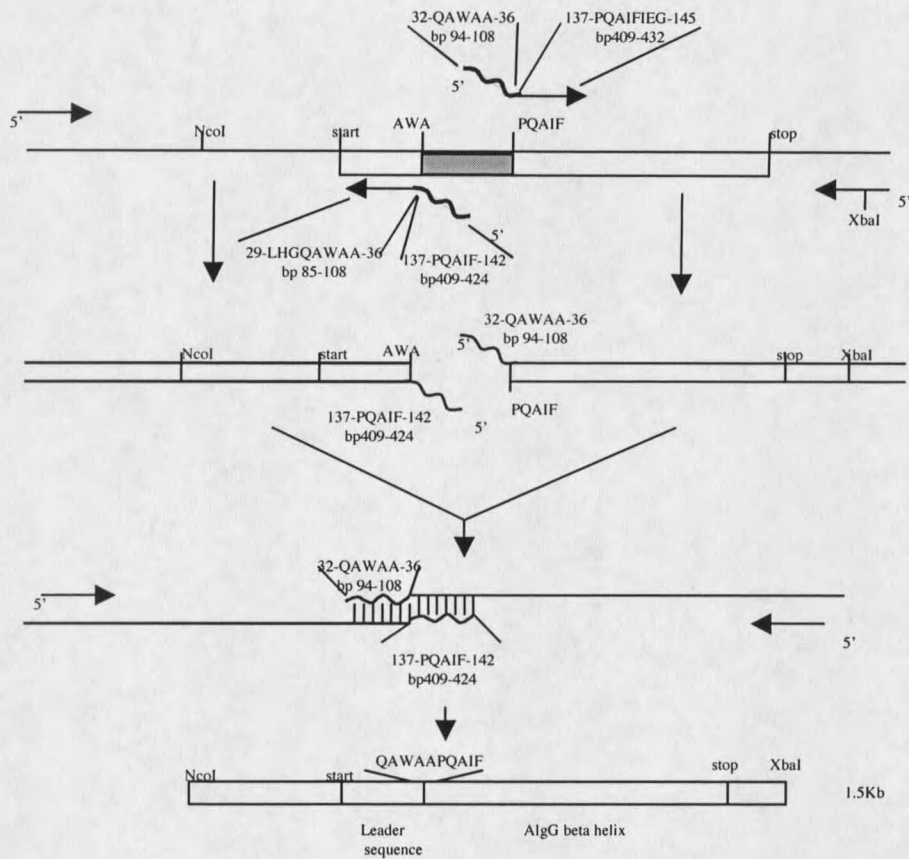


Figure 2.1. Construction of truncated AlgG missing N-terminal α helical region. Two fragments of *algG* were constructed. The first fragment contains the leader sequence of *algG* and the second contain the beta helical portion. The dashed box represents bp109-408 that will be deleted from the final *algG* product. The reverse primer for fragment one contains an overhang sequence from bp 409-432 that encodes for 137-PQAI F-142. The forward primer for the beta helical fragment contains an overhang sequence from bp 94-108 encoding for the cleavage site 32-QAWAA-37. The individually amplified fragments

Alginate Concentrations and Epimerization Assays

Alginate concentrations were assayed using a modification of the Knutson and Jeanes (138) protocols. *P. aeruginosa* FRD462 or FRD1200 with plasmids containing

algG point mutations were incubated for 24 hours in 10ml of LB with 300 μ g/ml carbenicillin. AlgG expression was induced with 1mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cultures were diluted with an equal volume saline, mixed and centrifuged at 8,000 x gravity (g) for 15 min. Culture supernatants were precipitated with 1/3 volume of 2% (w/v) cetyl pyridinium chloride (CPC). Pellets were resuspended in 10ml 1M NaCl and shaken overnight at 37°C. The polymers were precipitated again with 10 ml cold isopropanol, and the pellet resuspended in 4ml of saline. Twenty μ l of alginate was mixed with 1.0 ml of borate-sulfuric acid reagent (10mM H₃BO₃ in concentrated H₂SO₄) and 30 μ l of carbazole reagent (0.1% in ethanol). The mixture was heated to 50°C for 30 mins and concentration determined spectrophotometrically at 535 nm, using *Macrocystis pyrifera* alginate as a standard.

Alginate epimerization was determined using the alginate lyase assay described previously (70). Alginate lyase (Aly) of *Klebsiella aerogenes* cleaves L-guluronate at MG or GG blocks. A colorimetric assay described by Chitnis and Ohman (26) determines the relative number of G residues by determining the abundance of cleaved, unsaturated residues created by Aly. For this assay, 65 μ g of total uronic acids measured from purified alginate was adjusted to 150 μ l in deionized water. The alginates were deacetylated using 50 μ l of 1M NaOH and heating to 65°C for 15 minutes. Alginates were neutralized to pH 7.2 with 50 μ l of 1M HCl and 100 μ l of lyase buffer (50mM Tris-HCl pH 8.0, 10mM MgCl₂.6H₂O, 5mM CaCl₂). Ten μ l of purified guluronate specific lyase (see below) was added and the mixture was incubated for 1 h at 25° C. Periodic acid solution (250 μ l of 0.2M) was added to the lyase reaction and incubated for 40 min at

room temperature. Sodium arsenite (2% in 0.5N HCl) was then added at 100 μ l per reaction and allowed to incubate at room temperature for one min. One ml of a 0.6% thiobarbituric acid solution (pH 2.0) was added and the reaction incubated at 65°C for 30 min. The samples were cooled for 1 hour and centrifuged at 12,000 rpm for 5 minutes to remove the precipitate that formed. Abundance was measured spectrophotometrically at 535 nm. Results were normalized to OD/ng uronic acid and compared to wild-type alginate as a percentage of wildtype activity. Students t-tests were performed between the results obtained for the AlgG mutants and wildtype alginate with $p < 0.05$.

Preparation of Alginate Lyases

Crude extraction of guluronate specific alginate lyase, Aly, of *Klebsiella aerogenes* has been previously described (117). The mannuronate specific alginate lyase from *P. aeruginosa* was extracted from *E. coli* containing the *algL* expression plasmid pMF106. The cells were sonicated, followed by centrifugation to remove cellular debris. The supernatant with lyase activity was stored at 4°C.

Protein Structural Predictions

The amino acid sequence of AlgG was modeled using 3D-PSSM at www.bmm.icnet.uk/~3dpssm (130). This is a threading program where known structures from Protein Data Bank (PDB) are compared to the unknown protein and scored on sequence similarity, secondary structural predictions using Psi-Pred (<http://globin.bio.warwick.ac.uk/psipred/>), and solvent accessibility. The best fitting proteins are then used as models to thread the unknown protein and create a coordinate

file for the unknown protein. The unknown protein can then be viewed in 3D on RASMOL, a computer program allowing the 3 dimensional analysis of chemical structures. Support for the predicted right-handed beta helical structure of AlgG was generated using FFAS (<http://ffas.ljcrf.edu/ffas-cgi/cgi/login.pl>) (228) and BETAWRAP (<http://betawrap.lcs.mit.edu/>) (39).

AlgG Sequence Alignments

Sequences of β -helical repeats were aligned using the secondary structural predictions of AlgG and secondary structures of the known RH β H proteins. Residues that stack in RH β H fold are aligned in the repeat alignments presented. Alignments with the A modules of AlgE1-AlgE7 C-5 epimerases of *Azotobacter vinelandii*, the coat protein GP1 of *Ectocarpus siliculosus* virus, and of AlgG homologs from *P. fluorescens* and *A. vinelandii*, were performed using CLUSTAL X multiple sequence alignment (109, 119).

Results

Identification and Characterization of AlgG Sequence Repeats

Jain et al. (117) described AlgG as having a α -helical rich region at its N-terminus and a β -rich region at its C-terminus. Sequence analysis of the AlgG C-terminus shows a conserved repeating sequence of approximately 24 amino acids with the general consensus sequence – I.X.Z.(+/-).X.(S/A/V).X.(+/-).X.(3Z).X.N.(4X).N. (3X).G, where Z represents an aliphatic residue, (+/-) represents a charged residue, and X represents any amino acid (Fig. 2.2A). The letter designation for amino acids are given in the Appendix.

	<u>PB1</u>	<u>T1</u>	<u>PB2</u>	<u>T2</u>	<u>PB3</u>	<u>T3</u>																		
	1 3 5	9	12	15	17	20	24																	
A. P.a.1	<u>FLISW</u> -----	<u>GG</u>	<u>AEVYLS</u>	N---	<u>STFTS</u>	<u>FGYNAS</u> (2X)	--YG																	
P.a.2	<u>ISISQYS</u> -----	<u>PG</u>	<u>MDK</u>	---Q---	<u>MKRPR</u>	<u>PKGWVI</u> (8X)	--YG																	
P.a.3	<u>FYCY</u> -EA-----	<u>DD</u>	<u>LVVK</u>	--GN---	<u>TYRD</u>	---NIV-----	YG																	
P.a.4	<u>IDPHDRS</u> -----	<u>HR</u>	<u>LIIA</u>	--DN---	<u>TVHG</u>	---TRKK-----	HG																	
P.a.5	<u>IIVSREV</u> -----	<u>ND</u>	<u>SFIF</u>	--NN---	<u>RSYE</u>	---NKL-----	SG																	
P.a.6	<u>IVLDRNSEG</u> -----	<u>N</u>	<u>LVAY</u>	---N---	<u>EVYR</u>	---NHS-----	DG																	
P.a.7	<u>ITLYE</u> -SG-----	<u>DN</u>	<u>LLWG</u>	---N---	<u>QVLA</u>	---NRR-----	HG																	
P.a.8	<u>IRVRN</u> -SV-----	<u>N</u>	<u>IRLY</u>	---EN---	<u>LAAG</u>	---NQL-----	IG																	
P.a.9	<u>VYGH</u> IKDLTNTDRN-	<u>I</u> ALD	---	PF																				
B. 1AIR.1	<u>VEIK</u> -EF-----	<u>TK</u>	<u>GITII</u>	--GA---	<u>NGSS</u>	---AN-----	FG																	
1AIR.2	<u>IWIK</u> -K-----	<u>SS</u>	<u>DVVVQ</u>	--NM---	<u>RIG</u>	---YLP (7X)---	M																	
1AIR.3	<u>IRVD</u> -D-----	<u>SP</u>	<u>NVWVD</u>	--HN---	<u>ELFA</u>	---ANH (13X)-	SA																	
1AIR.4	<u>VDIK</u> -GA-----	<u>SN</u>	<u>TVTVS</u>	--YN---	<u>YIH</u>	---GVKK-----	VG																	
1AIR.5	<u>LD</u> ---GS (5X)-	<u>GR</u>	<u>NITYH</u>	--HN---	<u>YYN</u>	---DVMAR-----	LP																	
1AIR.6	<u>LQ</u> -R-----	<u>GG</u>	<u>LVHAY</u>	--NN---	<u>LYT</u>	---NITG-----	SG																	
1AIR.7	<u>LNVR</u> -QN-----	<u>G</u>	<u>QALIE</u>	--NN---	<u>WFE</u>	---KAIN-----	P																	
1AIR.8	<u>VTS</u> -RYDGKNF-G-	<u>TWVLK</u>	---	GN---	<u>NIT</u>	---K																		
C. 1DBG_1	<u>QLIVSN</u> - (6X)-	<u>P</u>	<u>ITIKA</u>	--LN	<u>PGKVFF</u>	<u>TGD</u>	-----A																	
1DBG_2	<u>KVELR</u> -GE-----	<u>H</u>	<u>LILE</u>	---GI---	<u>WFK</u>	---DGN (11X)	GL																	
1DBG_3	<u>VA</u> -IY-GS-----	<u>YN</u>	<u>RIT</u>	---AC---	<u>VFD</u>	---CFD (4X)-	AY																	
1DBG_4	<u>IT</u> -TS-L (8X)-	<u>HC</u>	<u>RID</u>	---HC---	<u>SFT</u>	---DKITFD--	QV																	
1DBG_5	<u>IN</u> -LN-N (15X)	<u>YH</u>	<u>RVD</u>	---HC---	<u>FFS</u>	---NPQ (6X)-	GG																	
1DBG_6	<u>IR</u> -IG-Y (6X)-	<u>RC</u>	<u>LVD</u>	---SN---	<u>LFMR</u>	---QDSEA---	EI																	
1DBG_7	<u>IT</u> -SK-SQ-----	<u>EN</u>	<u>VYY</u>	---GN---	<u>TYLN</u>	---C-----	QG																	
1DBG_8	<u>TMNFR</u> HGD-----	<u>HQ</u>	<u>VAI</u>	---NN---	<u>FYIG</u>	---NDQ (4X)-	GG																	
1DBG_9	<u>MF</u> -VW-GS-----	<u>RH</u>	<u>VIA</u>	---CN---	<u>YFEL</u>	---SET (6X)-	AA																	
1DBG_10	<u>LY</u> -LNPG (10X)	<u>D</u>	<u>MLIA</u>	---NN---	<u>AFIN</u>	---VNG-----	YA																	
D.	1	2	3	4	5	6	7	9	12	15	17	20	24											
AlgG P.a.	Z	X	Z	X	+	+	S	X	+	Z	Z	Z	X	+	N	X	X	X	X	N	X	X	X	G

Figure 2.2 Repeat alignments between putative repeats of AlgG and repeats of known right-handed β -helices. A. *Pseudomonas aeruginosa* putative repeats, B. 1AIR, pectate lyase C *Erwinia chrysanthemi*, C. 1DBG, Chondroitinase B *Flavobacterium heparinum*, Beta regions PB1, PB2, PB3 are underlined. Turn regions are T1, T2 and T3. Extended loop regions are truncated and represented in parentheses with the number of residues in the loop. Repeats of each protein are numbered from first to last coil. The residues from 1AIR and 1DBG are stacked as they would be in their crystal structure. D. Consensus sequence for coil repeats of AlgG. Numbers at top represent residue position in repeat.

The sequence of AlgG contains at least nine of these 24mer repeats, with six repeats easily identified, and three (two upstream of the six and one downstream) that are more difficult to align, due to extra amino acids that exist between the conserved residues of the repeat. This repeating sequence motif is characteristic of the CARbohydrate binding and Sugar Hydrolases (CASH) domain, often contained in carbohydrate binding proteins, such as polygalacturonases, pectate and pectin lyases, surface layer proteins of Archaea and alginate epimerases (33) and Ciccarelli, 2002 supplemental information). Several proteins containing the CASH domain have been crystallized and form a parallel right-handed beta helix (RH β H) and are classified in the pectin-like lyase superfamily (176). The repeats of AlgG also align to the repeats of the crystallized RH β H proteins. Figures 2.2 B and C show these repeat alignments between AlgG, pectate lyase C (1AIR) of *Erwinia chrysanthemi*, and chondroitinase B (1DBG) of *Flavobacterium heparinum*.

Structural Modeling of AlgG Predict a Right Handed β -Helix Fold

To determine if AlgG could have a similar structure to other CASH proteins, we conducted predictive structural modeling. The web based structural prediction program 3D-PSSM predicted the C-terminal region of AlgG to fold as a right-handed β helix, with the most probable match threading to pectate lyase C of *Erwinia chrysanthemi* with an E value of 4.0×10^{-5} . The E-value (Expect value) is the measure of the number of hits one can expect by chance when searching a database; as the number approaches zero the less likely the hit is by chance and increases the confidence in the result. The next six proteins that matched AlgG were also RH β H proteins with E-values ranging from 1.9×10^{-3} to 1.8

$\times 10^{-2}$ (Table 2.3a). The FFAS web-based predictive program also matched AlgG to RH β H proteins with the most significant matches to polygalacturonase from *Erwinia carotovora* and pectate lyase A of *Aspergillus niger*. The scores for these predictions were -9.88 and -9.58 respectively (Table 2.3B). Results with scores less than -9.5 have a less than 3% chance of being a false positive. Results from BETAWRAP, another web based program that identifies proteins that form β -helices, also gave AlgG a good score for folding as a RH β H. AlgG had a raw score of -19.6 with a P-value of $2.0e^{-4}$. The P-value indicates the likelihood that a randomly chosen non-beta helix protein would give a similar raw score, meaning it would be unlikely that AlgG would match with a non- β -helical structure.

Table 2.3A: 3D-PSSM, Top structural hits for AlgG

PDB	Protein	Organism	E-value	% identity	Structure
1air	pectate lyase C	<i>Erwinia chrysanthemi</i>	$3.98e^{-5}$	14	RH β H
1dbg	chondroitinase B	<i>Flavobacterium heparinum</i>	0.00195	12	RH β H
1kcc	endopolygalacturonase	<i>Stereum purpureum</i>	0.00263	13	RH β H
1hg8	endopolygalacturonase	<i>Fusarium moniliforme</i>	0.00282	15	RH β H
1bhe	polygalacturonase	<i>Erwinia carotovora</i>	0.00418	12	RH β H
1idk	pectin lyase A	<i>Aspergillus niger</i>	0.00479	10	RH β H
1ib4	polygalacturonase	<i>Aspergillus aculeatus</i>	0.0186	14	RH β H
1l0q	surface layer protein	<i>Methanosarcina mazei</i>	0.019	13	β propeller
1qcx	pectin lyase B	<i>Aspergillus niger</i>	0.045	12	RH β H
1qjv	pectin methylesterase	<i>Erwinia chrysanthemi</i>	0.056	14	RH β H
1cfz	endopolygalacturonase II	<i>Aspergillus niger</i>	0.070	12	RH β H
1pcl	pectate lyase E	<i>Erwinia chrysanthemi</i>	1.79	13	RH β H

Significant hits have E value less than 1

Table 2.3B: FFAS , Top structural hits for AlgG

PDB	Protein	Organism	Score	% identity	Structure
1bhe	polygalacturonase	<i>Erwinia carotovora</i>	-9.88	12	RH β H
1idk	pectin lyase A	<i>Aspergillus niger</i>	-9.58	10	RH β H

Scores less than -9.5 have a less than 3% chance of being a false negative

The most distinctive characteristic of the RH β H fold is that it forms a spring like structure with a shallow groove on one face that accommodates the long chain of linear polysaccharides. One coil or rung of the spring is made of one sequence repeat and the length of the helix depends on the number of coils or repeats present in the protein. In RH β H proteins, the coils can be repeated 8-13 times (as is the case with the 9 identified repeats of AlgG). The essential components in one RH β H coil and repeat are the three β -sheets (PB1, PB2, and PB3) that are separated by turn regions (T1, T2, and T3), where PB1, PB2, and PB3 of one repeat will stack with its respective PB1, PB2 and PB3 of the next repeat (120, 295). The shallow groove is composed of PB1 regions flanked by T1 and T3 regions (Fig. 2.3). Predictive secondary structural modeling shows that the AlgG repeats likely contain three β -sheets separated by three turning regions, which align with structures of the known RH β H proteins (Fig. 2.2). The alignments also show that AlgG likely contains primarily aliphatic residues in the β -sheets, which is also typical of other RH β H proteins. Positions 1, 3, and 10-12 of each repeat are usually aliphatic residues. In RH β H proteins, the aliphatic residues of the β -sheets stack in the interior of the helix creating a hydrophobic core (120). The second and fourth positions in each predicted coil of AlgG contain charged and/or aromatic residues typical in RH β H PB1 strands. These charged or aromatic residues on the PB1 grooved face often interact with the carbohydrate substrate (3, 143, 232, 248). Asparagine residues dominate the turn regions of AlgG at positions 9, 15, and 20. Another feature of the RH β H fold is the extended loops observed between PB2 and PB3 or between PB1 and PB2 in some repeats. These

extended loops are highly divergent among all RH β H (106). Extended loops likely occur in repeats 1, 2 and 9 of AlgG. Even though the amino acid sequence identity between AlgG and many of the identified RH β H proteins is less than 14%, the similar features shown between the repeat sequences of AlgG and these proteins lend significant support for AlgG folding as a RH β H.

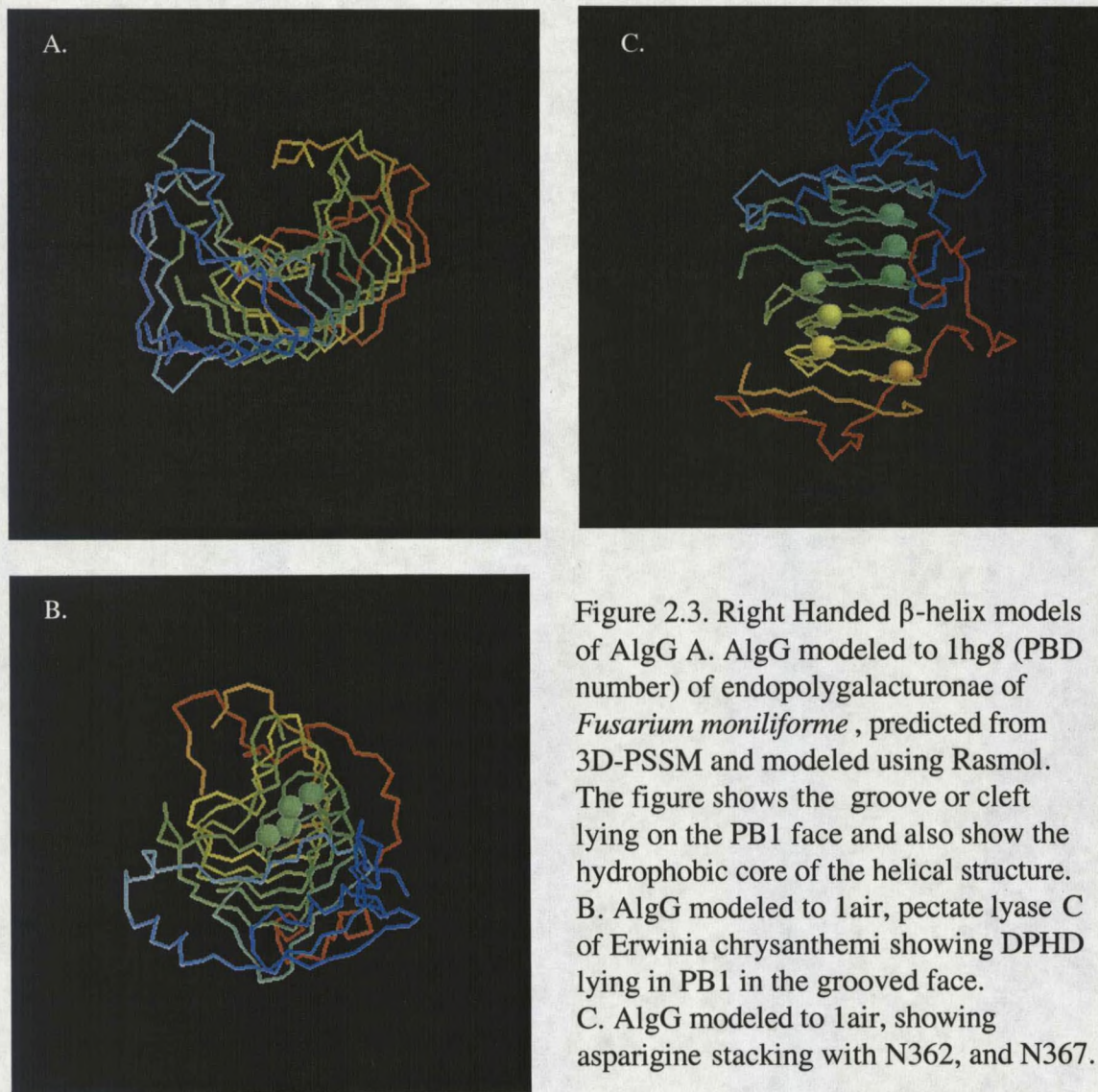


Figure 2.3. Right Handed β -helix models of AlgG A. AlgG modeled to 1hg8 (PBD number) of endopolygalacturonase of *Fusarium moniliforme*, predicted from 3D-PSSM and modeled using Rasmol. The figure shows the groove or cleft lying on the PB1 face and also show the hydrophobic core of the helical structure. B. AlgG modeled to 1air, pectate lyase C of *Erwinia chrysanthemi* showing DPHD lying in PB1 in the grooved face. C. AlgG modeled to 1air, showing asparagine stacking with N362, and N367.

Site Directed Mutagenesis Studies of Three Conserved Motifs in C-5 Epimerases

Three motifs within AlgG have been previously shown to be conserved in alginate epimerases. These motifs are 324-DPHD-327, 361-NNRSY-365 and 381-NLVAY-385 (83, 270). These motifs align to the extracellular epimerases (AlgE 1-7) of *A. vinelandii* and to the coat protein of the GP1 virus that infects the marine brown algae *Ectocarpus siliculosus* (Fig 2.4A and B). Svanem et al. (270) showed that the amino acid corresponding to D324 in AlgE7 is a catalytically important residue. According to the repeat alignments and structural models of AlgG the DPHD motif is located on PB1 in the center of the substrate binding groove, (Fig 2.3B). Based on repeat alignments and models N361 and N362 of NNRSY motif occur in T2 of coil 5, N367 occurs in T3 of the same coil, and RSYE make up PB3 between these two turns. N381 of the NLVAY motif occurs in T1 of coil 6 and LVAY follow in PB2. These motifs would be located on the opposite side of the predicted alginate binding shallow groove, and suggest that these residues are involved in structural integrity of the protein, rather than in direct interactions with the alginate polymer. The 3D-PSSM AlgG model threaded to pectate lyase C stacks N362 with N313, N337, N386, N409, and N432, stacks N367 with N391, N414 and N437, and stacks N381 with N404 (Fig. 2.3C). A similar stacking pattern is seen in many of the 3D-PSSM RH β H models produced for AlgG, suggesting that the asparagines in the NNRSY and NLVAY motifs are important in asparagine stacking, and likely not involved in mannuronan catalysis.

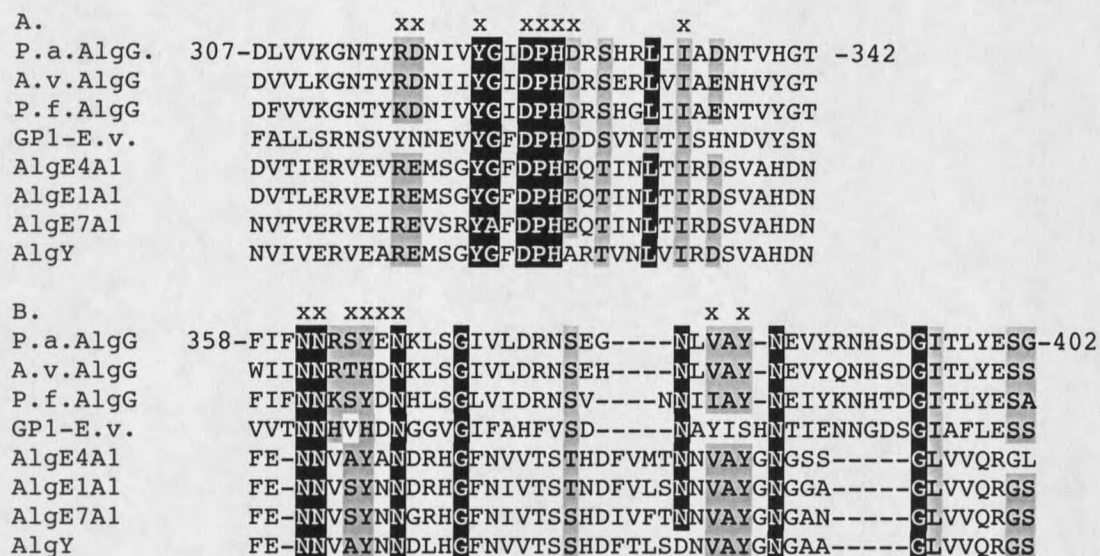


Figure 2.4. Amino acid linear sequence alignments of AlgG homologues. *P. aeruginosa*, *A. vinelandii*, *P. fluorescens*, and Gp1-ESV (coat protein of *Ectocarpus siliculosus* virus), and the A modules of AlgE 1,2 and 7 of the extracellular C-5 epimerases of *A. vinelandii*. Black are identical amino acids and grey are similar amino acids. X indicate those amino acids subjected to site directed mutagenesis. A. represents the DPHD motif. B. represents the NNRSY and NLVAY motifs.

To investigate the importance of these motifs and surrounding amino acids site directed mutagenesis was conducted. Amino acids targeted for site directed mutagenesis are marked with an x in Figure 2.4. Plasmids containing *algG* point mutations were introduced into *P. aeruginosa* FRD1200, an *algG* deletion mutant, which secretes depolymerized alginate, and is defective in the polymerization of the alginate polymer. They were also introduced into FRD462, which contains an *algG* point mutation and only produces polymannuronate (26, 117). Assays were performed to determine if expression of the AlgG mutants resulted in recovery of the alginate polymer phenotype in FRD1200 and recovery of the epimerization defect in FRD462 and FRD1200.

The mutants obtained by site directed mutagenesis studies were classified into four groups based on the resulting phenotypes of the complemented strains. Class I mutants include D324A, P325A, H326A and D327A. AlgG containing these mutations complement the alginate polymerization defect in FRD1200. However, the alginate produced in these transformants was only polymannuronate alginate and contained no or little guluronate residues. These proteins were also not able to complement the epimerase defect in FRD462 (Fig 2.5A). These data demonstrate that these mutations are functional in maintaining a polymerized alginate polymer, and therefore have likely retained their structural integrity. However, proteins with these mutations are defective in epimerase activity, implying that these amino acids may be important catalytically for epimerization.

Class II mutants contain D317A, Y321F, N362A, N367A and V383A. AlgG proteins with these mutations did not complement the alginate polymerization defect of FRD1200. In addition, they did not complement the epimerase defect in FRD462 (Fig 2.5B). The results suggest that either these mutant proteins have lost affinity for the alginate polymer, are impaired in protein/protein interactions with other alginate biosynthetic proteins or are not folded correctly.

Class III mutants contain R316A and N361A and are not able to recover the epimerase activity in FRD462, but recover polymer formation and epimerase activity in FRD1200 to wild-type levels (Fig 2.5C). These results suggest that a dominant negative phenotype may exist in FRD462 *algG4*, since the epimerization defect was recovered in FRD1200 but not in FRD462. In this case, the chromosomally encoded AlgG from

FRD462 *algG4* may out-compete the plasmid-encoded AlgG for binding sites on the polymer or for sites within the proposed alginate scaffold.

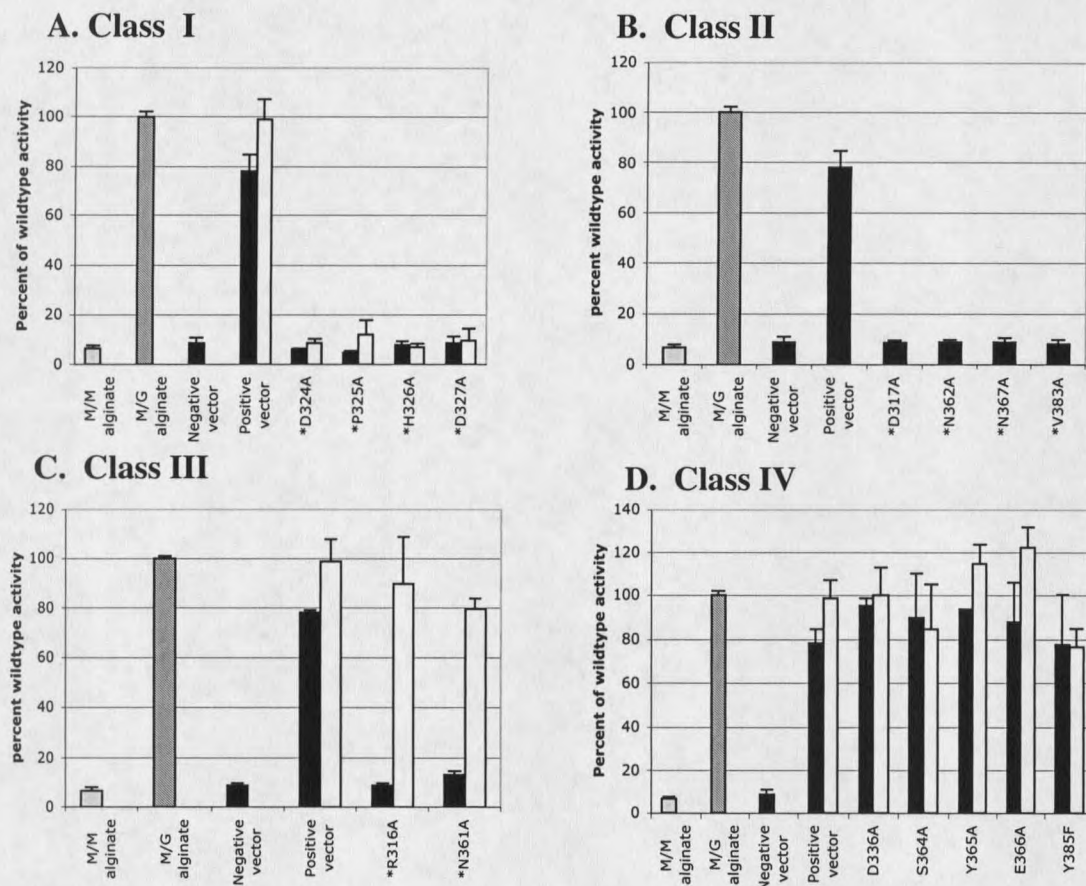


Figure 2.5 Complementation of site directed mutants in FRD462 and FRD1200. Measurement of guluronic acid from alginate purified from FRD462, FRD1200, or FRD1 with expression of AlgG on the chromosome or *in trans* from plasmids with site directed mutations. Purified alginate was subjected to degradation by the G- specific lyase from *Klebsiella*. The unsaturated bonds produced were measured with the thiobarburetic acid assay spectrometrically at 535nm. The results are presented as a percentage of reaction activity seen in wildtype alginate produced in FRD1. M/M is polymannuronate purified from FRD462 and M/G is wildtype alginate purified from FRD1. Negative vector is pMF54 and positive vector expressing native *algG* is pMF55. Black columns represent plasmids expressed in FRD462 and White columns represent plasmids expressed in FRD1200. Asterisks indicate significant difference from wildtype activity and were determined with a students t-test ($p < 0.05$).

Class IV mutants include D336A, S364A, Y365A, E366A and Y385F which fully complement both FRD462 and FRD1200 to wildtype levels (Fig 2.5D). These mutants appear to override the dominant negative effect of the FRD462 *algG4*, and suggest that these amino acids have no functional role in alginate epimerization *in vivo*.

The AlgG protein with mutations in the DPHD motif has a similar phenotype to that of *algG4*, the FRD462 mutant *algG* allele, in that they are able to maintain polymer formation, yet cannot epimerize it. Therefore, these mutations may also impart a dominant negative phenotype. We tested this hypothesis by expressing these mutant proteins in the wildtype background of FRD1. Surprisingly, D324A reduced alginate epimerization 4.8 fold. The other mutant proteins did not affect epimerization significantly (Fig 2.6). Tests without induction of the D324A plasmid with IPTG yielded a 3.3 fold reduction in epimerization, suggesting that even with low levels of expression this mutant is dominant over wildtype AlgG. We also determined if AlgG4 from FRD462 was dominant negative in the wildtype background, by transforming the *algG4* allele into FRD1 and measuring the guluronate residues on the purified alginate. Interestingly, expression of the *algG4* allele (S272N) did not affect epimerization in the wildtype strain (Fig 2.7). The results suggest that the S272N mutation does not have an enhanced capacity for the alginate polymer or in binding to the proposed alginate biosynthetic scaffold. Rather, the dominant negative effect observed with N361A and R316A mutants is likely due to a compromised enzymatic function of these proteins.

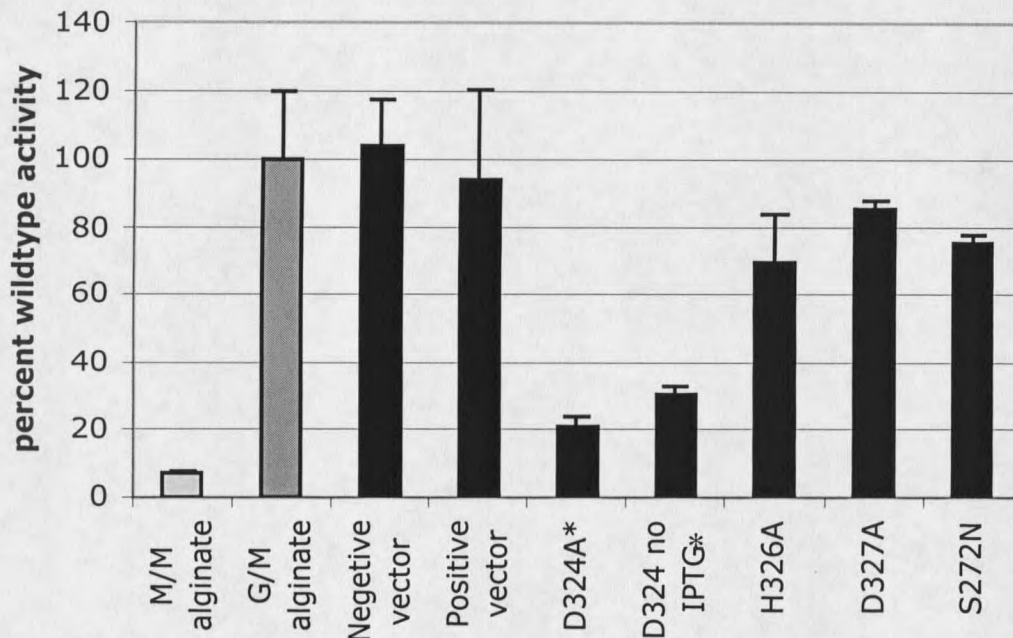


Figure 2.6. Expression of site directed mutants in FRD1 wildtype background. Measurement of guluronic acid from alginate purified from FRD1 with expression of AlgG from the chromosome or *in trans* from plasmids with site directed mutations. 65 μ g of deacetylated polymer from each sample was treated with 10 μ l of G-specific lyase from *Klebsiella*. The unsaturated bonds formed at the reducing ends were measured with the thiobarburetic acid assay spectrometrically at 535nm. The results are presented as a percentage of reaction activity seen in wildtype alginate produced in FRD1. M/M is polymannuronate purified from FRD462 and M/G is wildtype alginate purified from FRD1. Negative vector is pMF54 and positive vector expressing native *algG* is pMF55. Black columns represent plasmids expressed in FRD1 Asterisks indicate significant difference from wildtype activity and were determined with a students t-test ($p < 0.05$).

The N-terminal α -Helical Domain of AlgG

The structures obtained from 3D-PSSM do not model approximately the first 120 amino acids of AlgG, post cleavage, to the RH β H proteins. This amino-terminal region is dominated by α -helices from amino acid 37-156 (117). Domain architecture for RH β H

proteins has been suggested to exist in the glycosidic hydrolase families 55 and 87 (222), and in the surface layer protein B of *Methanosarcina mazei* (124). Two crystallized RH β H proteins, the tailspike protein of phage P22 (245) and dextranase from *Penicillium minioluteum*, have been shown to contain multiple domains in addition to the CASH domain (143). The large α -helical region predicted in the amino terminus of AlgG suggests that it is a separate domain from the putative RH β H fold. An AlgG deletion mutant missing amino acids 89-102, a putative α helix, fully complemented the *algG* deletion strain, FRD1200 (Fig 2.6), suggesting that this N-terminal region is not important for AlgG epimerase activity. To determine the importance of the entire N-terminal putative helical region in protein function, mutants were constructed deleting the regions from amino acid 37-137 (pSAD149) and from 37-161 (pSAD151). These mutants were not able to complement FRD1200 in polymer production, indicating that some portion of the N-terminal region is required for AlgG activity (data not shown). Therefore, this region may be important in maintaining structural integrity of the RH β H or may be important in the protein/protein interactions of the proposed alginate biosynthetic scaffold.

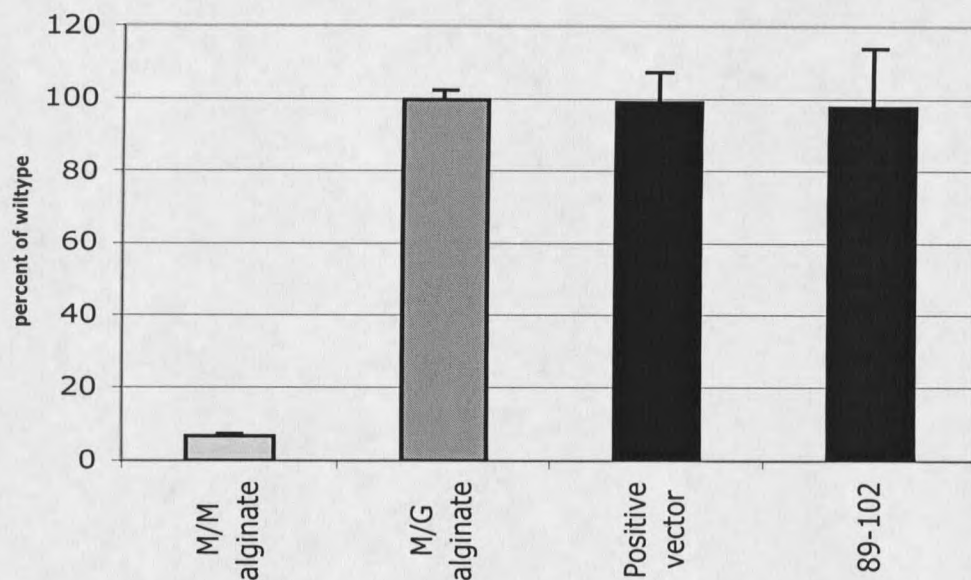


Figure 2.7 Complementation of the deletion mutant from amino acid 89-102 in FRD1200. Measurement of guluronic acid from alginate purified from FRD1200, white columns, with expression of *algG* *in trans* from plasmids pMF55 (positive vector) or pSAD88 (deletion mutant). 65 μ g of deacetylated polymer from each sample was treated with 10 μ l of G- specific lyase from *Klebsiella*. The unsaturated bonds formed at the reducing ends were measured with the thiobarburetic acid assay spectrometrically at 535nm. The results are presented as a percentage of reaction activity seen in wildtype alginate produced in FRD1. M/M is polymannuronate purified from FRD462 and M/G is wildtype alginate purified from FRD1.

Discussion

From 3D-PSSM modeling, AlgG is predicted to form a RH β H. The RH β H is a common fold for carbohydrate binding proteins. To date, the crystal structures of 19 RH β H proteins in the pectin-like lyase superfamily have been solved (176). This superfamily consists of pectin and pectate lyases of *Erwinia chrysanthemi* (294), *Aspergillus niger* (282) and *Bacillus subtilis* (198), and the galacturonases (polygalacturonase and rhamnogalacturonase) of *Aspergillus aculeatus* (30, 197),

Erwinia carotovora (199), *Sterum purpureum* (248) *Aspergillus niger* (280) and *Fusarium moniliforme* (64). Other carbohydrate binding proteins in this family include the dextranase of *Penicillium minioluteum* (143), chondroitinase B of *Flavobacterium heparinum* (113), ι -carrageenase of *Alteromonas sp.* (166), methylsterase of *Erwinia chrysanthemi* (121), the tail spike protein of phage P22 (264) and the P69 pertactin of *Bordetella pertussis* (59). This structure is found in proteins of all three domains of life, although as of yet, it has not been found in higher eukaryotes. All of these proteins, with the exception of pertactin, cleave polysaccharides that compose the extracellular matrix of plants and animals. The high charge densities of the non-methylated component of plant pectins, polyglacturonates, and ι -carrageen, a gel like polysaccharide produced by marine red alga, is similar to the highly charged alginic acid (120) of *P. aeruginosa* and other organisms. The pectin lyases, P22 tailspike and pertactin do not act on charged polymers, yet they have the RH β H protein structure. This protein fold appears to be a general carbohydrate binding structure that accommodates high molecular weight linear polysaccharides, and occurs in several glycosidic hydrolases, and now in the AlgG epimerase.

Evidence presented in this thesis supports the hypothesis that AlgG folds as a RH β H. First, the scores obtained from 3D-PSSM modeling, BETAWRAP analysis and FFAS analysis indicate that AlgG has a high probability of folding as a RH β H. Second, the similarities between the repeats found in AlgG and known RH β H proteins support this model; the repeats between AlgG and known RH β H have similar secondary structure alignments of the three β -sheets and the three turn regions. Similar amino acids in these

repeats also align, including the aliphatic residues in the β -sheets, the asparagines in the turn regions, and the charged residues in PB1 that are involved in binding interactions with polysaccharides (3, 143, 232, 248). Of particular significance is the asparagine stacking observed in repeat alignments and in the structural models. Asparagine stacking is commonly seen and is of structural importance in RH β H proteins (120, 222). The association that RH β H proteins have with linear polysaccharides gives credibility to this fold. A recent study using hydrophobic cluster analysis (HCA) shows that AlgG from *P. aeruginosa*, AlgE5 from *A. vinelandii*, GP-1 coat protein, and MannC5-E1, an alginate C-5 epimerase from the marine brown alga *L. digitata*, have similar hydrophobic distributions that predict a succession of β -strands with a small amphipathic α -helix at the amino terminus of the β -strands. They also identified that this resembled the secondary structure of RH β H proteins (180).

Another structural possibility for AlgG is a β -propeller. The surface layer protein (Slp) β -propeller domain of the Archaea *Methanosarcina mazei* was identified as a possible fold for AlgG by 3D-PSSM. A β -propeller contains numerous β -sheets folded into a six or seven bladed propeller. For the Slp protein, the blade sequences are repeated and contain the YVTN motif (homologous to the YWTD motif of the LDL receptor in metazoans) (124). However, the repeated sequences of AlgG do not match that of the repeats of the Slp, and are more similar to RH β H repeats. The high content of β -sheets in both structures make it difficult to distinguish, with a high degree of certainty, the proper fold. Considering the evidence described above, the repeated regions of AlgG support a RH β H structure over a β propeller. In addition, of the two known β propeller proteins one

is not a carbohydrate binding protein and the function of the other is not yet known. Therefore, the association RH β H proteins have with carbohydrates provides further support for the RH β H fold. The β propeller fold, however, cannot be totally discounted as a structural possibility for AlgG.

Site directed mutagenesis of the DPHD motif indicates that it is important for catalytic function, and supports the results of Svanem et al. (270) that suggest amino acid D324 (D152 in AlgE7) is important for catalytic activity. In all the RH β H models for AlgG this motif lies in the center of the grooved face and most often on PB1 (Fig. 2.3B). This position corresponds to the location of the catalytic centers for all the known RH β H, where the reactive residues are either on PB1 or adjacent to it (3, 136, 143, 222, 230, 232, 248, 262). The modeling of these amino acids in or near the catalytic centers of other RH β H proteins gives support that this motif resides in the catalytic center of AlgG.

Two mechanisms exist in this structural family for polysaccharide cleavage. Polygalacturonases cleave via acid/base hydrolysis, usually involving three aspartate residues (197, 199, 222, 248, 280). One aspartate acts as a general acid catalyst and donates a proton directly to the glycosidic oxygen. The other two aspartates act as bases activating a water molecule for nucleophilic attack on the anomeric carbon. The pectate and pectin lyases, as well as many other polysaccharide lyases, use β -elimination for degrading carbohydrate polymers (113, 164, 232, 296). In these reactions a proton is abstracted from C5 by a base and is donated to the glycosidic oxygen at the cleavage site by an acid. This results in the formation of an unsaturated C4-C5 bond (95). The base of the reaction in pectin lyases is an arginine residue or in the case of pectate lyases a Ca⁺

ion, and the acid is often an aspartate residue (113, 122, 198, 232). It has been proposed that the lyase reaction by β -elimination is mechanistically similar to alginate epimerization, since both reactions form a similar intermediate (Fig 2.8)(77). This suggests that catalytic residues in AlgG, such as aspartates D324 and/or D327 or arginines, would resemble those in the lyases. A study by Gimmestad et al.(83) showed that the residues in *P. fluorescens* corresponding to D375 and R422 of *P. aeruginosa* are important in epimerase activity. These residues are also possible catalytic residues in that they reside on or adjacent to PB1 near the center of the helix, similar to the DPHD motif.

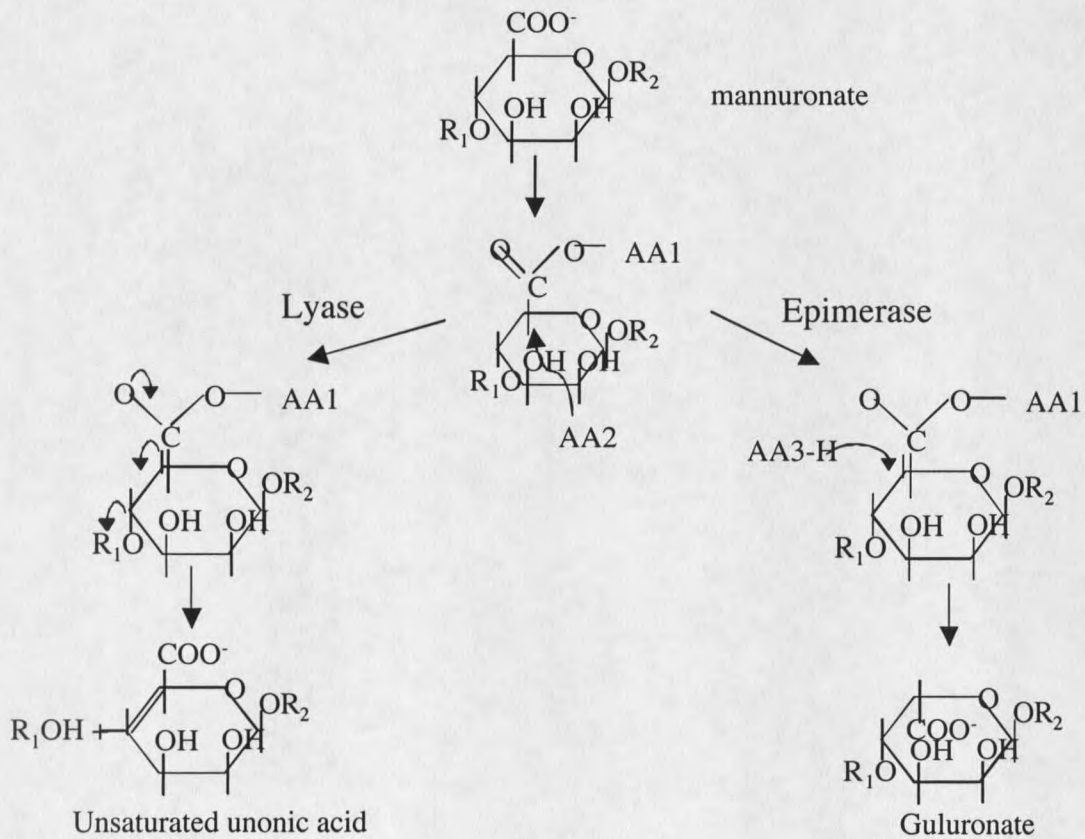


Figure 2.8. Proposed mechanism of alginate epimerization. Epimerization resembles β -elimination of alginate lyases. AA1, AA2, and AA3 represent separate amino acids involved in the mechanisms.

An alternative explanation to the role of the DPHD motif being involved in catalysis is that these amino acids as well as D375 and R422 may be important in polymer binding. Several RH β H proteins have been co-crystallized with their respective substrate. The results of those studies demonstrate that amino acids, often charged or aromatic, are located along the groove of RH β H proteins and are in contact with the substrate ((3, 55, 143, 232, 248)). It was also demonstrated in cellobiohydrolase I of *Trichoderma reesei* that at least seven sugar residues are in contact with the protein along its binding face, suggesting that many amino acids in the shallow groove are required for polysaccharide binding (55). In RH β H proteins, identifying binding motifs from linear sequence alignments is unlikely (described in Chapter 3). Alignments based on repeated coils would identify a higher number of putative binding residues, since the binding residues would localize to the PB1 region. Otherwise, they would appear scattered throughout the linear sequence of the protein.

Amino acids that usually interact with carbohydrates are aspartates, arginines, histidines, lysines, tyrosines, and serines. Two potentially important binding residues in AlgG are Y301 and Y303, which, in the model, lie on PB1 of coil 3 next to the putative active site (DPHD motif). Tyrosine residues have been shown to bind to the OH group on the C2 carbon and contribute to stabilization of the carbohydrate transition state (30, 302). In the A1-III lyase of *Sphingomonas* sp. Y246 is important in abstracting the C5 proton by β elimination, thereby arguing that Y301 and/or Y303 could be important catalytically for AlgG. To test this hypothesis, future studies are needed to determine the

interactions of the DPHD residues and the other residues along the PB1 face with the alginate precursor, polymannuronate.

The NNRSY motif and the NLVAY motif have been identified previously (83) and appear to be conserved among all of the C-5 epimerases, including the extracellular epimerases of *A. vinelandii*. These extracellular epimerases have a similar putative RH β H repeating sequence to that predicted for AlgG (Fig 2.9). When taking this into consideration, the NNVAY motif of AlgE does not align with the NLVAY motif of AlgG, but rather aligns to NEVY (Fig 2.9). BETAWRAP also gave the first A module of AlgE1 a high probability of folding as a RH β H, supporting the alginate C-5 epimerases as a non-hydrolytic class of RH β H proteins. The NNRSY motif is also similar to the NNHSY motif found in many alginate lyases such as AlgL and A1-III of *Sphingomonas* sp. (289). The crystal structure of the alginate lyase of *Sphingomonas* demonstrates protein-carbohydrate interactions with N191 and H192 of this motif at the catalytic center (296). The results suggest that the NNRSY motif of AlgG may play a similar catalytic role. However, the structure of alginate lyase from *Sphingomonas* sp. forms an α_6/α_5 barrel consisting of many α -helices. Structural predictions show no predicted α -helices in AlgG beyond amino acid 154. Evidence presented here supports the role of N362 and N367, within this motif, in asparagine stacking. S364A and Y365F showed no affect on AlgG activity *in vivo*, lending little support for this motif being a catalytic center. These data along with the structural predictions and alignments do not support AlgG folding in a α_6/α_5 conformation. Therefore, these data do not support the hypothesis that the NNRSY motif is important in polymannuronate binding or in catalytic activity. These

observations also reveal the limitations that linear sequence alignments have in predicting the function of amino acid motifs.

	<u>PB1</u>		T1	<u>PB2</u>	T2	<u>PB3</u>	T3
	1 3 5		9	12	15	17	20 24
P.a.4	<u>DPH</u> DRS-----		HR- <u>LI</u> IA-DN- <u>TV</u> HG-TRKKHG				
P.a.5	<u>IIV</u> SREV-----		ND- <u>SF</u> IF- NN - <u>RS</u> YE-NKL-SG				
P.a.6	<u>I</u> VLDNRSEG-----		N- LV AY--N- <u>EV</u> YR-NHS-DG				
P.a.7	<u>IT</u> LYE-SG-----		DN- <u>LL</u> WG--N- <u>QV</u> LA-NRR-HG				
P.a.8	<u>IR</u> VRN-SV-----		N- <u>IR</u> LY-EN- <u>LA</u> AG-NQL-IG				
P.a.9	<u>VYGH</u> IKDLTNTD--RN- <u>IAL</u> D						
AlgE.1	F <u>DPHE</u> QTI-----		N-LTIR-DS-VAHD-NSL-DG				
AlgE.2	FVADYQV-----		G-GVFE- NN - <u>VS</u> YN-NDR-HG				
AlgE.3	FNIVT-ST-----		ND-FVLS- NN - <u>VAY</u> G-NGG-AG				
AlgE.4	LVVQRGSYDLPHPY-D-IIDG--G-AYYD-NAL-EG						
AlgE.5	VQLKMA-----		HD-VTLQ-NA-EIYG-NGL-YG				
AlgE.6	VRVYGA-----		QD-VQIL-DN-QIHD-NSQ-NG				
AlgE consensus	ZXZ+XXX-----		+-ZZZX-+N-XXY+-NXX-+G				
P.a. consensus	IXZX++S-----		+-ZZZX-+N-XXXX-NXX-XG				

Figure 2.9. Repeat alignment with AlgG of *P. aeruginosa* and AlgE1 of *A. vinelandii*. Showing the similarities between the two proteins in regards to a repetitive motif. Putative Beta sheets of AlgG are underlined. PB= beta sheets and T= turns of the predicted β -helical structure. Consensus sequences are listed for AlgE repeats and AlgG repeats. Z in consensus is aliphatic; + is charged. Bold are motifs NNRSY and NLVAY that aligned to the NNVSY and NNVAI motifs of AlgE, respectively, in figure 2.4. The shaded is the DPHD motif that aligns with the DPHE motif of AlgE. Repeats alignments began at the DPHD motif.

Three different types of stacking side chains occur in the RH β H, 1) hydrogen bond stacking, primarily by asparagine residues, 2) hydrophobic stacking by aliphatic residues and 3) planar stacking by aromatic residues (122). Many RH β H structures form asparagine stacks, which helps in maintaining the secondary structural elements (122).

AlgG is unique in that this would occur at all three turn regions, but most prevalent at T2

and T3. Site directed mutagenesis showed that N362A and N367A disrupted protein function in polymerization and epimerase activity. The repeat alignment predicts that these amino acids occur at T2 and T3 respectively, and many of the threaded models produced by 3D-PSSM support the repeat alignments and stack the asparagine residues. Mutations in these residues (Class II mutants) may have disrupted the structural stability of the protein and caused protein misfolding. Aromatic stacking may have been disrupted with the Y321F mutation, which according to repeat alignments would stack with Y254, and Y298 (Fig. 2.2). Another aromatic stack may occur between Y360, Y385 and Y430. One mutation (V383A) suggests disruption of aliphatic stacking. According to the models this residue would lie on PB2, where aliphatic stacking occurs in other RH β H proteins (122).

The predicted α -helices in the N-terminal region of AlgG indicate that this protein contains multiple domains. Several glycosidic hydrolases have multiple domains. Dextranase of *P. minioluteum* consists of a C-terminal RH β H and an N-terminal β -sandwich (143). Based on the crystal structure the β -sandwich interacts with the back face of the RH β H domain contributing to the protein's structural stability. The tailspike of P22 also contains multiple domains where the N-terminal domain contains a β -sandwich, but interacts and binds to the phage head rather than interacting directly with the RH β H domain (245, 263). This protein also exists as a homotrimer (245, 264). Other RH β H proteins also display multiple domain structures. The surface layer protein B of *Methanosarcina mazei* contains domains predicted to fold as a RH β H and as a polycystic kidney disease (PKD) domain β -sandwich. The PKD domains are involved in

surface-to-surface interactions between two *M. mazei* cells (124). Considering these examples, the N-terminal region of AlgG may provide structural stability and proper folding of the protein, like that of dextranase, or provide the protein/protein interactions necessary for the proposed alginate biosynthetic scaffold. Results here indicate that the N-terminal portion of the protein is required for proper alginate polymerization, but may not be necessary for epimerization. Future studies addressing the role of AlgG in the alginate biosynthetic scaffold are necessary to determine the functional roles of individual domains within AlgG.

An interesting observation in these studies is that AlgG with the D324A mutation is dominant negative over wildtype AlgG. To be dominant over the wildtype protein, it is likely necessary for the mutant protein to displace the wildtype protein, either from the alginate polymer or from the alginate biosynthetic scaffold. Therefore, this dominance can be explained in a two ways. First it must be noted that the mutant gene is plasmid encoded and overexpressed. Therefore, the mutant protein should have higher abundance in the cell periplasm than the chromosomally encoded wildtype protein. If this is the case, then the mutant protein could be occupying a limited number of sites on the alginate biosynthetic scaffold, thereby displacing the wildtype protein from the complex.

Alternatively, the increased concentration of mutant protein could be out-competing the wildtype protein for limited sites on the alginate polymer, and thereby interfering with the ability of the wildtype protein to epimerize the alginate. The studies conducted by Gimmetstad et al. (83) indicate that one polymer chain is epimerized by one AlgG, supporting the scaffold hypothesis, and thereby, supporting the hypothesis that the mutant

AlgG is occupying all of the sites in the scaffold. When this mutant is not induced we see the return of some epimerase activity, indicating that the wildtype AlgG is able to occupy some of the AlgG sites. However, this does not explain the lack of a dominant negative phenotype in the other overexpressed mutants where occupying the AlgG sites in the scaffold should also result in a dominant negative phenotype. Alternatively, the mutant AlgG may bind to the alginate polymer more strongly than the wildtype protein and not release the alginate, since epimerization activity is impaired. This may not allow access of the alginate polymer to the wildtype AlgG. This hypothesis could explain the lack of a dominant negative phenotype of the other catalytic domain mutants, since they may be defective in binding to the polymer. Further studies addressing the binding efficiency of these mutants and/or studies confirming a scaffold would provide insight into the nature of these mutations and a greater understanding of the role AlgG has in alginate biosynthesis.

CHAPTER THREE

THE USE OF PHAGE DISPLAY PEPTIDE LIBRARIES TO IDENTIFY ALGINATE BINDING MOTIFS IN ALGINATE MODIFYING ENZYMES

Introduction

Little is known about the interactions between alginate and its modifying enzymes. Several studies have been conducted on the C-5 epimerases of *A. vinelandii*, but the sites of specific protein/alginate interactions have not been mapped. The few studies that exist on the *P. aeruginosa* alginate modifying enzymes focus on the actions these proteins have on alginate or the alginate precursor, polymannuronate (26, 70-73, 117, 118, 233). The research described in this thesis focuses on characterizing the specific sites important for catalysis and alginate/protein interactions. Since the alginate modifying enzymes including AlgG and AlgJ have little sequence identity to other proteins, except for their homologs in other *Pseudomonas* and *Azotobacter spp*, it is difficult to identify putative alginate-binding domains based on amino acid sequence identity. Therefore, I used a phage display library approach in an attempt to identify putative alginate binding motifs in these enzymes.

Phage display peptide screening uses a library of random peptides fused to a phage capsid protein. Peptide motifs that are potentially important for interaction with a target molecule are then selected by applying the phage display library to the immobilized molecule of interest. Selection for peptides that bind the immobilized

molecule is conducted in several steps; 1) the molecule is immobilized to a surface or bead matrix, 2) the phage display library is incubated with the immobilized molecule, 3) unbound phage are washed from the matrix, 4) phage that have bound the molecule of interest are eluted in a high NaCl or high or low pH solution, 5) the eluted phage is amplified in *E. coli*, 6) the amplified phage are reapplied to the matrix and the process is repeated. After several rounds of enrichment and amplification, the DNA of the phage capsid gene from individual phage plaques is sequenced. The enriched peptide sequences fused to the phage capsid can then be identified. The motifs identified are searched for in proteins of interest, in this case, the alginate modifying enzymes.

George Smith first proved that phage display technology was useful. He showed that a foreign DNA sequence could be inserted into the pIII gene of a filamentous phage and the subsequent peptide would be retained in the pIII protein without disrupting phage infectivity (255). Random phage display libraries have since been used to identify amino acids with high specificity for target molecules. This system was most commonly used to identify sites of protein-protein interactions (256). Jim Burritt and Al Jesaitis of Montana State University, Department of Microbiology identified peptide sequences that mimic discontinuous epitopes of the integral membrane protein cytochrome *b*. Monoclonal antibodies (mAbs) specific for the cytochrome *b* were used as a target molecule (22, 123). Similarly, peptides mimicking carbohydrate structure have been identified with mAbs of the group B streptococcal type III capsular polysaccharide (210), using this approach.

To identify potential amino acids motifs important in alginate binding, this study used the J404 phage display library with polymannuronate or polymannuronate/guluronate (M/G) alginate, as the target molecules. Although not used here, another approach for this technology would be to use mAbs to alginate, which have recently been developed by Pier and coworkers (273). The M13KBst library created by Jim Burritt (23) was used in this study. This library had a random nine amino acid peptide fused to the N terminus of the phage capsid protein, pIII. This library contains approximately 5×10^8 random peptide sequences (21, 23).

The phage display study began as a broad search to identify motifs in any of the alginate modifying enzymes and was eventually narrowed to AlgG and AlgJ. Motifs found in these proteins were similar to phage peptides identified in this study and suggested they have a role in alginate interactions. AlgG is an alginate C-5 epimerase with a molecular weight of 55kD for the mature protein. It is located in the periplasm of *Pseudomonas* spp. and *Azotobacter* spp. (70, 83, 221)). AlgJ is another periplasmic protein. It is 43kD, and is bound to the inner membrane via an uncleaved leader sequence. AlgJ is important in the O-acetylation of alginate on the mannuronate residues at positions O2 or O3. This protein is thought to work in concert with AlgI and AlgF, also required for O-acetylation (71-73).

Material and Methods

Strains, Plasmids and Bacterial Media

Strains, plasmids and mutagenic oligonucleotides are listed in Table 3.1. *E. coli* and *P. aeruginosa* were routinely cultured in L Broth (10g tryptone, 5g yeast extract, and 5g NaCl per liter). *P. aeruginosa* was selected after matings with *E. coli* by using *Pseudomonas* Isolation Agar (PIA) (Difco). Antibiotics were used at the following concentrations, in $\mu\text{g/ml}$: ampicillin (Ap) 100, carbenicillin (Cb) 300, and tetracycline 20.

The phage library used was J404 nonapeptide library supplied by Jim Burritt Montana State University, Department of Microbiology.

Preparation of Epoxy Sepharose Alginate Beads

Epoxy-activated sepharose 6B beads (Pharmacia) were chosen as a matrix to conjugate polymannuronate alginate or M/G alginate by creating a stable ether linkage with the alginate hydroxyl groups. Beads were prepared as per manufacturer's instructions. Beads were swelled and washed with 500 ml of deionized H_2O . Purified deacetylated polymannuronate (2.5 ml) was added to 0.5 ml of swollen beads and the pH adjusted to 12.4 with 1N NaOH. The reaction mixture was incubated at 37°C for 16 hours. The beads were washed with 50 ml of 0.04N NaOH. The unconjugated groups were blocked in 10 ml of 1M ethanolamine at pH 8.0 and incubated overnight at 40°C. The beads were washed with 20 ml of 0.1M acetate buffer (0.05 M acetic acid, 0.05 M sodium acetate, 0.5M NaCl, pH 4.0), followed by a 20mls of 0.1M Tris-HCl (pH 8.5) with 0.5M NaCl. Alternating washes were repeated three times. After a final wash with

phage buffer (50mM Tris-HCl, 1.5M NaCl 0.5% Tween 20 v/v, 1mg/ml BSA, pH 7.5)

the beads were resuspended in 10 ml phage buffer. Control beads followed the same procedure but without the addition of polymannuronate or alginate.

Table 3.1 Strains, Plasmids and Mutagenic Oligonucleotides

Strain or Plasmid	Genotype, phenotype or mutagenic oligonucleotide	Reference
<i>E. coli</i>		
HB101	<i>proA2, leuB6, thi-1, lacY1, hsdR, hsdM, recA13, supE44, rspL20</i>	
K91		
<i>P. aeruginosa</i>		
FRD1	Cystic fibrosis isolate Alg ⁺	(183)
FRD462	<i>algG4 S272N</i> Alg ⁺	(26)
FRD1200	Δ <i>algG::Gm</i>	(117)
FRD1176	<i>AlgJ6</i> Alg ⁺	(73)
Plasmids		
pRK2013	ColE1-Tra(RK2) ⁺ Km ^R	(65)
pALTER-Ex1	phagemid Tc ^R	Promega
pMF54	P _{trc} ColE1 replicon with <i>oriV_{SR} oriT(RK2) lac^{iq}</i> Ap ^R	(70)
pMF55	pMF54 with 2.2kb <i>algG</i> NcoI-NcoI fragment	(70)
pMF150	pMF54 with 1.1kb <i>algJ</i> NcoI-HindIII fragment	(73)
pSAD1	pALTER-Ex1 with 2.2kb <i>algG</i> NcoI-NcoI fragment	This study
pSAD3	pALTER-Ex1 with 1.1kb <i>algJ</i> NcoI-HindIII fragment	This study
pMF106	pMF54 with <i>algL</i>	(117)
pRC5	contains aly guluronate lyase of <i>Klebsiella aerogenes</i> Ap ^R	(25)
pSAD20	pMF54 with <i>algG</i> E94A CCGCCGCGCGGTGGCCGCAAGATCGTGC	This study
pSAD83	pMF54 with <i>algG</i> R99A AAGCCAAGATCGTGGCCAAGCCGGGTGGCC	This study
pSAD94	pMF54 with <i>algG</i> deletion of amino acids 89-102 GCCCGACCTCTCCGCGGTGGCCGCGCCAGCG	This study
pSAD21	pMF54 with <i>algJ</i> K58A CGAAGCCCACTACGACGCGGAATTCCCGATCAAG	This study
pSAD22	pMF54 with <i>algJ</i> E53A CTGGCGCACGCCTTCGCCGCCCCACTACGACAAGG	This study
pSAD32	pMF54 with <i>algJ</i> H55A GCACGCCTTCGAAGCCGCGCTACGACAACCAATTC	This study

Abbreviations: Alg⁺, alginate overproduction; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R tetracycline resistance; Tra, transfer by conjugation.

Preparation of Non-conjugated Polymannuronate Beads

Alginates were purified as described in Chapter 2. Purified polymannuronate was aerosolized using an airbrush into a large pan containing 2% cold CaCl_2 . Droplets of alginate gelled following exposure to the CaCl_2 . The beads were collected and stored at 4°C in the 2% CaCl_2 . Phage display was conducted as before with an altered phage buffer (50mM Tris-HCl pH7.5, 10mM CaCl_2 , 0.5% Tween 20 v/v, 1mg/ml BSA). NaCl (1M) was used to dissolve the beads. The eluent was added directly added to *E. coli* K91 for phage amplification.

Phage Display

This procedure has been described previously (23), and was performed similarly here, but with some modifications. Alginate-conjugated beads (300 μ l) were added to a polystyrene column and washed with 5ml of phage buffer. Phage buffer (1ml) was added to the column and the column capped at both ends to allow incubation with slow rocking for 16 h at 4°C. The beads were then washed with 100 ml of phage buffer. Phage elution buffer (1M NaCl) (2ml) was added to the column, and the eluent collected. A second elution was conducted using M-specific alginate lyase to disassociate the polymer from the matrix. Lyase buffer (50mM Tris-HCl, 10mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5mM CaCl_2 , pH8.0) (1ml) containing 100 μ l of partially purified M-specific alginate lyase (described below) was added to the beads. The reaction was incubated at 25°C for 1 hour and the eluent collected. The collected fractions were serial diluted to obtain a titer of the eluted phage. The remaining eluent was added to 20ml of LB, which was inoculated with 200 μ l of *E. coli* K91. The phage were allowed to infect the cells for 5 hours at 37°C with shaking.

The phage were purified from cell supernatants by centrifugation for 10 minutes at 8,000 x gravity. Phage in the supernatants (16 ml) were precipitated by the addition of 2.7 ml of 20% PEG/2.5M NaCl. The mixture was incubated on ice for one hour. The precipitated phage were collected by centrifugation (8,000 x gravity for 15 min) and resuspended in 500µl of 50mM Tris-HCl pH 7.5. The phage were either added to another column for enrichment or used for sequencing.

Phage Sequencing

After three rounds of enrichment, individual phage plaques were isolated. *E. coli* K91 (5ml) were inoculated with the individual plaques and incubate for 5 hours at 37°C. The phage were precipitated as described above with the exception that 1.5 ml of culture volume was used and the phage were resuspended in 100µl of TE buffer (10mM Tris-HCl, 1mM EDTA, ethylene-diamine-triacetic acid). Single stranded DNA from the purified phage was isolated by adding 200µl of phenol saturated chloroform (5:1) to the phage and vortexing. The aqueous phase was then collected and added to a new microfuge tube. Two chloroform extractions were then conducted using 100µl of chloroform, isoamyl alcohol (24:1). The ssDNA was precipitated with 10 µl 3M sodium acetate and 250 µl 95% cold ethanol followed by incubation at -80°C for 30 minutes. The ssDNA was collected by centrifugation (13,000 rpm for 10 min). The DNA pellet was washed once with 70% ice cold ethanol, centrifuged for 5 min and air dried. The pellet was resuspended in 25 µl of deionized H₂O.

The Applied Biosystems Sequencing reaction mix contained 4µl of ABI Big Dye, 2 µl of 5X sequencing buffer, 200 ng of ssDNA, 1.5µl of J534 primer

(5'GTTTTCTCGTCTTTCCAGACG-3') (23), and deionized water for a final volume of 20 μ l. Following the PCR reaction, DNA was precipitated with isopropanol, resuspended in formamide and analyzed using the ABI thermal sequencer. The amino acid sequence was determined by first identifying the DNA sequence of the random 27 base pair insertion and translating it using EDITSEQ of the DNASTAR[®] software. Sequences obtained were compared to one another and compared to sequences in AlgG, AlgJ, AlgI, AlgF or AlgL. Sequence alignments were conducted using the CLUSTALX program (109, 119).

Site Directed Mutagenesis

The *Nco*I DNA fragment containing *algG* from plasmid pMF55 was subcloned into the phagemid vector pALTER-Ex1. The resulting plasmid was labeled pSAD1. The *Nco*I-*Hind* III *algJ* fragment was also cloned into pALTER-Ex1, forming plasmid pSAD3. These plasmids were then used for site-directed mutagenesis. The protocol used was described by Promega and in Chapter 2. Mutations were confirmed by DNA sequencing, and the mutant *algJ* or *algG* genes were cloned into the *P_{trc}* expression vector pMF54, using the *Nco*I restriction site for *algG*, and the *Nco*I-*Hind* III sites for *algJ*. The plasmids were mobilized from *E. coli* to the *P. aeruginosa* mutant strains FRD462 (*algG4*), FRD1200 (*algG* deletion), or FRD1176 (*algJ6* Δ) using triparental matings with the conjugative helper plasmid pRK2013. Plasmids and oligonucleotide sequences are shown in Table 3.1.

Site-directed mutant plasmids were over-expressed in mutant strains of *P. aeruginosa* grown in LB and induced with 1mM IPTG. The IPTG induces the *P_{trc}*

promoter and results in high levels of expression of the cloned genes in *P. aeruginosa*. The alginates from the resulting mutant strains were purified and quantified, as described in Chapter 2. Students t-tests were performed between the results obtained for the AlgG mutants and alginate produced by positive vector containing wildtype *algG* with $p < 0.05$.

Alginate O-acetylation Assay

The assay used for determination of O-acetylation was modified from the assay described previously (71). Briefly, 500 μ l of purified alginate was incubated with 500 μ l alkaline hydroxylamine (0.35 M NH_2OH , 0.75M NaOH) for 10 minutes at 25°C. Perchloric acid (500 μ l of 1M solution) was added followed by 500 μ l of 70mM ferric perchlorate in 0.5M perchloric acid. O-acetyl group concentration was determined spectrophotometrically at 500 nm. The percentage of acetylation per mannuronate residues was determined by using ethyl acetate as a standard. Results were converted to percentage of acetylation compared to the positive control.

Preparation of Alginate Lyases

Crude extraction of guluronate specific alginate lyase, Aly, of *Klebsiella aerogenes* is described in Chapter 2. The mannuronate specific alginate lyase from *P. aeruginosa* was extracted from *E. coli* containing the *algL* expression plasmid pMF106. The cells were sonicated, followed by centrifugation to remove cellular debris. The supernatant with lyase activity was stored at 4°C.

Results

Phage Display with Polymannuronate Alginate

In an attempt to identify motifs important for binding alginate by alginate modifying enzymes we used the J404 Phage Display Peptide Library (PDPL). This library was applied to columns with polymannuronate or M/G alginate conjugated to epoxy sepharose beads. The unbound phage were washed from the column and the bound phage were eluted with a high NaCl buffer or with M-specific alginate lyase (AlgL), for the polymannuronate prepared beads only. After three rounds of phage enrichments individual phage plaques were isolated and amplified and sequenced to identify the nine amino acid peptide sequence encoded by the phage that had affinity for the alginate polymers. Initially, seventy phage sequences were obtained. All phage sequences of the nine amino acid variable regions are shown in the Appendix. A search for these sequences in the alginate modifying enzymes AlgG, AlgJ, AlgF and AlgL identified a highly similar sequence in AlgG, AVEAKIVRKP (termed the VEAKE motif). Seven phage sequences matched this motif in AlgG (Fig 3.1). The amino acid sequence alignment of AlgG proteins from *P. fluorescens*, *P. aeruginosa* and *A. vinelandii* show that this motif is conserved in AlgG from these species. Interestingly, one of the seven phage sequences, AAFEKHRKE, that aligns with the VEAKE motif also aligns with a similar motif in AlgJ (Fig 3.2). Alignments of these regions from AlgG and AlgJ showed that the VEAKE motif of AlgG and the AFEAH motif of AlgJ had some similarity (Fig 3.2). Alignments created by CLUSTAL X of these two proteins do not align the VEAKE

two charged amino acids glutamate 94 (E94) and arginine 99 (R99) by converting these residues to non-polar alanines. Charged amino acids are expected to interact with the highly charged alginate polymer. pSAD20, and pSAD83 containing the AlgG mutations of E94A and R99A, respectively, were mated into FRD462 *algG4*, an epimerase deficient strain only producing polymannuronate alginate, and FRD1200, a non-mucoid *algG* deletion mutant that secretes depolymerized alginate (26, 117). The AlgG mutant containing a mutation in E94 was able to restore the epimerization defect of FRD462, as well as recover a mucoid phenotype in FRD1200. The alginate produced from the FRD1200 strains transformed with this mutant was epimerized. This suggests that mutations in E94 do not affect AlgG function (Fig 3.3). Mutations in R99 restored epimerization activity in FRD462, but only up to 76% of wildtype. This was not significantly different, using a student's t-test ($p < 0.05$), from the positive plasmid control containing a wildtype copy of *algG*. AlgG containing the R99 mutation restored a mucoid phenotype to FRD1200, which produced an epimerized alginate, suggesting that R99 did not affect AlgG function (Fig 3.3).

To determine if the putative VEAK motif has any effect on AlgG function the entire motif from amino acid 89-102 was deleted. AlgG with this mutation did not complement FRD462, but fully complemented the FRD1200 strain, both restoring the FRD1200 polymerization defect and producing an epimerized alginate (Fig 3.3). The effect seen in FRD462 transformed with this mutant may be attributed to a dominant negative phenotype similar to that observed in other FRD462 complementation studies

(Chapter 2). The full recovery observed in FRD1200 indicates that the deletion of the VEA motif does not play an integral role in AlgG activity *in vivo* (Fig 3.3).

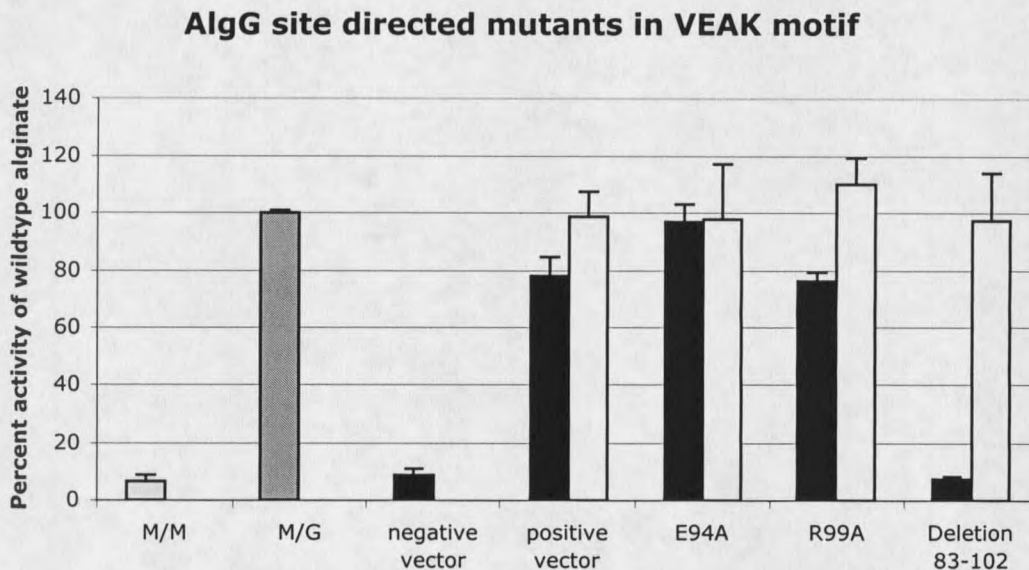
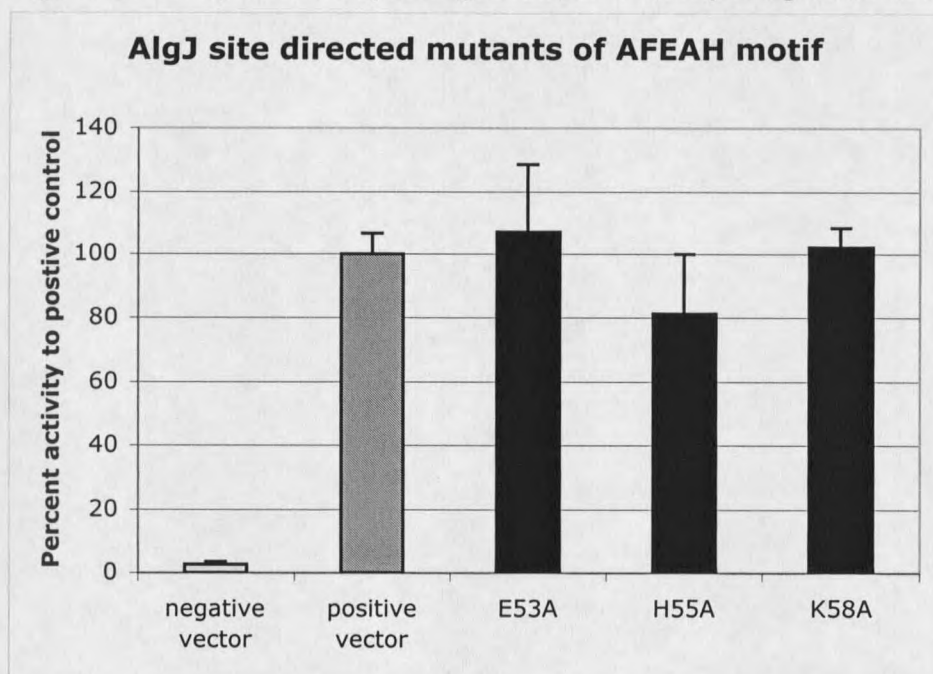


Figure 3.3. Epimerase activity of AlgG site-directed mutants. Activity is percentage of activity from M/G alginate purified from FRD1, striped bar. Grey bar is M/M alginate purified from FRD462. Black bars are alginate purified from FRD462 containing plasmids with AlgG point mutations, and the vector controls (pMF54 and pMF55). White bars are alginate purified from FRD1200 containing the AlgG mutant proteins and the control plasmids. No alginate was purified from FRD1200 with pMF54, as it does not produce alginate. The assay was conducted using the thiobarburetic colorimetric assay with guluronate specific lyase from *K. aerogenes* as described in the Materials and Methods. OD was measured as 535nm and normalized to ng uronic acid.

As in AlgG site directed mutagenesis, charged amino acids in the AlgJ AFEAH motif, E53, H55 and K58, were converted to non-polar alanines. Complementation in the acetylation mutant, FRD1176, showed that the mutants containing E53A and K58A were fully functional. Alginate produced from the FRD1176 transformant containing a mutation in H55A had a slight decrease in acetylation from wildtype alginate, but was not

significantly different from the positive vector control containing wildtype *algJ* (students t-test, $p < 0.05$) (Fig 3.4). These data suggest that the motifs identified from phage display sequences are not important for enzymatic activity, and are likely not alginate binding domains.

Figure 3.4. Acetylation assay of AlgJ mutants. Activity is a percent of acetylation



activity from alginate purified from *P. aeruginosa* 1176 (pMF150 *algJ*+), striped bar. Grey bar is alginate purified from *P. aeruginosa* 1176 (pMF54 *algJ*-). Black bars are alginate purified from FRD1176 contain plasmids with mutant AlgJ proteins. OD was measured as 500nm and normalized to ng uronic acid.

Additional Phage Display Studies

Since the motifs identified here by phage display peptide libraries were likely not important for function of AlgJ or AlgG, additional phage display was conducted to identify other motifs within these enzymes. In these studies, both polymannuronate and M/G alginate were investigated. In the M/G alginate phage display several similar

sequences to the VEAk motif were identified in the NaCl elution (Fig. 3.5). A consensus sequence of VXAKPXKKPPK was determined. However, sequences obtained from control beads, containing no alginate, were very similar to that of the experimental beads, with a consensus sequence of VXAKIKKPK (Fig 3.5). Interestingly, the lyase elution from the M/G alginate beads had very few sequences that resemble the consensus sequence of the NaCl elution of these same beads. A consensus sequence was not identified among the lyase eluted phage (data listed in the Appendix).

The high similarity between phage sequences obtained from alginate beads and control beads may be due to, 1) binding of phage to the unconjugated portions of the sepharose beads, or 2) binding of the VXAKPXKKPPK sequence to a broad range of carbohydrates including both sepharose and alginate. To eliminate the problem of cross reactivity to epoxy sepharose, beads composed entirely of alginate were produced. Alginate can be crosslinked by ionic interactions in the presence of Ca^+ (254). Alginate containing G residues creates hard, almost solid gels, while M/M alginates form pliable gels. Small resin like beads are needed to create a large surface area for phage binding. To produce alginate beads, purified polymannuronate alginate was aerosolized into cold 2% CaCl_2 . The droplets formed small non-uniform beads. These beads were tested to see if they retained their integrity with the buffers used for phage display. The 150mM NaCl in the phage buffer was found to dissolve the beads and therefore was replaced with 10mM CaCl_2 . The phage sequences obtained from these studies using alginate beads showed no recognizable consensus sequence, or similarity to the VXAKPXKKPPK consensus sequence. Sequences found in these studies are listed in the Appendix.

AlgG	AAAVEAKIVRKPGGR	<u>controls</u>	VHAKKDKWR
M/M	VEAKGHKKK		IHAK--KPKKP
1	VEAKQ--KPRR		VLGKIKKPK
2	VEAK--KRTAA		AVGKMKKPK
3	VEAK--KVRDS		SLAKVKKPK
4	VQAK--KQKNS		GINKPKKPK
5	VLAK--KGKLT		VMAK KLPKK
6	VYAK--KTRTA		VIAKIKKPK
7a	VPIKPTKKG	consensus	VXAKIKKPK
9	VKPKI--KPVK	alginate consensus	VXAKPXXXKPKP
10	SLAKV-KKP-K	control consensus	VXAKI-KKP-K
11	ALAK--KGP-KP	AlgG	AAAVEAKIVRKPGGR
12	ADAKT-RKP-K		
14	GWKA-KKPPK		
15a	IP-KE-KKV-KG		
16	KA--KPPKGGH		
17	AKGHKAPRK		
18a	AKIPRHK-KP		
18b	AKIPRHKKP		
20	GKLQ-KRPK		
15b	GKV-KKE-KPI		
22a	MKSPKKPRH		
23	EKPSRKPSK		
24a	PKPLKKVPK		
25	HRP-KKPDKM		
22b	HRP-KKPSKM		
26	IHA-KKPKKP		
31b	LVPRT--KPPK		
27	GWAKP--KAPK		
8	VWGAKTHKK		
28	KAPKRAPKE		
24b	KPVKKLPPK		
29	KPVKKLHKR		
21	KPRKPPSKG		
30	KPSKMMHVK		
31a	KPPKTRPVL		
7b	GKKTPKIPV		
13	VKKPPKVKG		
32	RKPQRKALC		
consensus	VXAKPXXXKPK		

Figure 3.5. Consensus sequence of M/G phage display. Phage display sequence alignment with phage obtained from M/G alginate and control epoxy sepharose beads. The identified AlgG VEA motif is included as well as the original phage sequence 1 from M/M alginate (figure 3.1). Consensus sequences are identified for both the M/G phage and control phage. Phage peptides with a letter designation can align in two different ways.

Discussion

Our attempts to identify alginate binding motifs using phage display peptides libraries was unsuccessful. Several conclusions and considerations can be made from these experiments. A couple of theories can explain the similar sequences identified between the alginate-conjugated beads and control beads. Either there were exposed sites on the alginate beads that the phage recognized, or the peptide motifs that we enriched recognize multiple types of polysaccharides, including both alginate and epoxy sepharose. Sequences obtained from phage display on aerosolized alginate beads show little or no similarity to the VXAKPXKKPPK consensus sequence obtained in the epoxy sepharose experiments. This does not support the hypothesis that this motif binds to both alginate and epoxy sepharose, but rather that the phage recognize the epoxy sepharose, possibly due to inefficient conjugation of M/G alginate to the beads.

What is so striking about this work is the presence of the consensus motif, VEAk, in AlgG. It is difficult to conclude this as a coincidence. This motif may actually have weak interactions with the polymer, where mutations in this region do not appreciably interfere with protein activity. Evidence does exist for this since the AlgG-VEAK deletion mutant did not complement FRD462, but did complement FRD1200. As seen in other AlgG mutants described in Chapter 2, the mutant AlgG encoded on the FRD462 (*algG4*) is dominant negative over some of the plasmid-encoded AlgG mutants. With regard to the VEAk deletion mutant, it appears that FRD462 *algG4* is dominant over the plasmid-based AlgG. This may result from the deletion mutant having a slightly diminished affinity for the alginate polymer compared to the chromosomally-encoded

enzyme. In order to address these possibilities AlgG-alginate interaction studies would be required. To conduct these assays properly it is necessary to purify AlgG, followed by isothermal calorimetry or affinity electrophoresis. Attempts to purify AlgG were made here but were not successful.

Phage display is ideal for the study of protein/protein interactions. These experiments prove the difficulty of this technique for determining carbohydrates/protein interactions. Carbohydrate binding domains that bind to high molecular weight polysaccharides often consist of 40-150 amino acids. The amino acids that interact with the polysaccharides often are located throughout the protein and form a binding domain following folding of the protein into its three-dimensional structure, rather than forming a linear sequence of amino acids (89). In the case of cellulose and xylane binding domains, the domain forms a shallow cleft or flat face composed of several β sheets often in hairpin turns. The conserved binding residues align on the β sheets in order to accommodate a long polysaccharide, where contact with more than one or two sugar residues is important (74, 89, 139, 275). A study by Divne et al. (55) demonstrated this with a cellulose chain bound in the cleft of cellobiohydrolase I. The polysaccharide lies across the beta sheets of the large beta sandwich where several interactions occur between the protein and at least seven glucosyl residues. A study with the *A. vinelandii* AlgE4 also shows that interactions with seven uronic acid residues would be necessary for catalytic activity (99). Considering this and the predicted right-handed β -helix structure of AlgG, one cannot expect that a short linear binding motif, like those obtained

from phage display, would exist in this protein. However, at the time of these studies the predicted structure of AlgG was not known.

Alternative methods to identify functional motifs in AlgG were employed and are addressed in Chapter 2. Recent studies conducted primarily by Dr. Michael Franklin and myself identified two functional motifs in AlgJ and are briefly discussed here. My participation in this study was in the construction of the site-directed mutations.

Research on AlgJ identified two putative functional motifs in AlgJ. Amino acid sequence searches identified AlgI homologs in a wide variety of bacteria not known to produce alginate. Some of these homologous proteins included the DltB proteins of Gram positive bacteria, which are required for O-alanylation of lipotichoic acids (LTA) (135). Both alanyl and acetyl groups are linked to their cognate polymers through ester linkages, and it is thought that AlgI and its homologs are used as carriers for acetyl or alanyl groups through the cytoplasmic membrane. The gene order among the AlgI homologs indicated that these proteins are genetically linked to type II membrane proteins, including AlgJ of *P. aeruginosa*. Amino acid sequence alignments of these putative type II membrane proteins and AlgJ, resulted in three distinct groups of proteins; the AlgJ like proteins, the NMA1479 proteins, and the DltD proteins. The AlgJ-like group consists of AlgJ of other pseudomonads, as well as AlgJ proteins from *D. vulgaris*, *Clostridia spp.* and *B. anthracis*. This group has conserved motifs, PXX and RTDXHW. The AlgJ-like group has no or very little sequence identity to the NMA1479 group or to the DltD group. However, the conserved motifs identified in each group do have some

similarities. Each contain a histidine residue followed by a large non-polar amino acid and a preceding aspartate residue (Fig 3.6).

Complementation studies in the alginate acetylation deficient strain FRD1176 with mutants in this RTDXHW motif showed that H195A and D193A abolished alginate acetylation activity. The W196F mutation reduced acetylation fourfold, and the T192A and T192G mutations reduced activity twofold and fivefold, respectively. The R191A mutation had no effect on alginate acetylation activity. Mutations P135A and K137A of the PXX motif also abolished or significantly reduced alginate acetylation. These recent findings are significant in that they identify a functional site in the type II membrane proteins and AlgJ. It is still not known if these motifs are involved catalyzing the transfer of acetyl groups to the polymer, but these mutations allow the testing of this hypothesis.

CHAPTER FOUR

IDENTIFICATION OF TWO GULURONATE SPECIFIC ALGINATE LYASES OF
*PSEUDOMONAS AERUGINOSA*Introduction

Alginate lyases are alginate modifying enzymes that cleave the 1-4 glycosidic bonds via β -elimination between uronic acid residues, forming an unsaturated C=C bond at the non-reducing end of the sugar subunit. In *P. aeruginosa*, the periplasmic mannuronate-specific alginate lyase, AlgL, is encoded on the alginate biosynthetic operon, and is important in alginate biosynthesis of alginate overproducing cystic fibrosis isolates (18, 27, 182, 233). AlgL is found in many *Pseudomonas* species and *Azotobacter* species, and *algL* mutants of *P. syringae* pv. *syringae* have a 50% decrease in alginate production (289). A wide variety of alginate lyases have been identified in alginate-degrading marine and soil bacterium. There are two main families of alginate lyases that are grouped by their activity on the alginate substrate. Both families are endoglycosylases. However, they differ since one family cleaves between two adjacent guluronate residues, and one family of lyases cleaves alginate at sites following a mannuronate residue (289). AlgL from *P. aeruginosa* is a mannuronate-specific alginate lyase.

Some Gram negative bacteria including *Alteromonas* spp., *Sphingomonas* spp., as well as several Gram positive bacteria, including *Sargassum fluitans* contain multiple

genes for alginate lyases and are able to break down alginate and use it as a sole carbon source. *Alteromonas* strain H-1 contains four intracellular lyases that vary in their cleavage specificity, cleaving either poly M, poly M/G or poly G alginates (289). The intracellular lyases of *Sphingomonas* sp. A1 have been studied extensively (169). *Sphingomonas* transports alginate into the cytoplasm through a large mouth-like pit and an ABC transporter system consisting of the periplasmic proteins AlgQ1 and AlgQ2, the cytoplasmic membrane bound ABC transporters AlgM1 and AlgM2, and the ATP binding protein, AlgS. In the cytoplasm, the alginate is degraded by the mannuronate specific lyase A1-III, the guluronate specific lyase A1-II and the oligoalginate lyase, Oal, which breaks down the alginate into dimers and trimers (100, 110, 167-169, 171).

Numerous polysaccharide specific lyases have been identified and are classified based on primary structural similarities (Carbohydrate-Active Enzymes server at URL: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). All polysaccharide lyases degrade uronic acids through a β -elimination reaction. Fourteen families of polysaccharide lyases exist. Pectate and/or pectin lyases dominate families 1, 2, 3, 9, and 10. The alginate lyases A1-II and A1-III belong to the polysaccharide lyase families PL-7 and PL-5, respectively. Crystal structures of proteins within these families reveal that pectate/pectin lyases fold into parallel right-handed β -helices. Other families also have β helical structures including chondroitinase B of family 6, and rhamnogalacturonan lyase of family 4. However, the right-handed β -helix is not the only structure of the polysaccharide lyases. A α/α barrel with antiparallel β sheets was assigned to chondroitinase AC in family 8, and a α/α barrel resembling that of chondroitinase AC

was assigned to the alginate lyase A1-III of *Sphingomonas* sp. A1 in family PL-5.

Alginate lyases compose 2 families, PL-5 and PL-7. PL-5 consists of the mannuronate specific lyases, such as AlgL of *P. aeruginosa* and its homologs in other pseudomonads and *Azotobacter* spp. The mannuronate-specific lyase A1-III of *Sphingomonas* sp. has some amino acid conservation with AlgL of *P. aeruginosa* (Fig 4.1). The PL-7 family of alginate lyases includes the guluronate-specific lyases of *Klebsiella aerogenes*, *Corynebacterium* sp., and *Vibrio halioticoli*. The *Sphingomonas* sp. lyase A1-II is also a member of this family. Here, I show that in addition to AlgL, two additional alginate lyases are encoded on the *P. aeruginosa* genome (genes PA1167, and PA1784). Based on amino acid sequence identity, these two putative alginate lyases fall into the PL-7 family of guluronate-specific alginate lyases. To determine if the product of these genes play a role in degradation of alginate, I performed mutagenesis and gene overexpression studies. The results of these studies are presented in this chapter.

```

Halomonas marina      IRPPQGYFAPVDKFKTGDKS-----DGCDAMPAPYTGPLQFRSKYEGSD
P. syringue AlgL     LVPPKGYDAPIEKMKTDGHN-----FSCEAIPKPYTDKLVFRSKYEGSD
P. aeruginosa AlgL   LVPPPGYYAAVGERKGSAG-----SCPAVPPPYTGSLVFTSKYEGSD
A. vinelandii AlgL  LVPPKGYAPVDIRKGEAP-----ACPVVPEPFTGELVFRSKYEGSD
A. chroomococcus    LVPPKGYAALEIRKGEAQ-----ACQAVPEPYTGELVFRSKYEGSD
Sphingomonas Al-III VKDPTASYVDVKARRTFLQSGQLDDRLLKAALPKEYDCTTEATPNPQQGEMVIPRRYLSGN
: * . . : : . * * . : : * * . .

Halomonas marina      KARATLNVQSEKAFRDTTKDITTLERGTAKRVMQFMRDGRPEQLECTLNWLTAWAKADAL
P. syringue AlgL     KARATLNAVSEEAFRDATKIDITTLERGVSKVVMQYMRDGRPEQLDCALNMMTWAKADAL
P. aeruginosa AlgL   SARATLNVKAEKTFRSQIKDITDMERGATKLVQYMRSGRDGDLACALNWMSSAWARAGAL
A. vinelandii AlgL  AARSTLNEEA EKAFRTKTAPIITQIERGVSRMVMRYMEKGRAGDLECTLAWLDAWAEDGAL
A. chroomococcus    SARSTLNKKA EKAFRAKTKPIETIERGVSRMVMRYMEKGRLLRAGMRPGLLDAWAEDDAL
Sphingomonas Al-III HG-----PVPNDYEPVVTLYRDFEKISATLGNLYVATGKPVYATCLLNMLDKWAKADAL
. : : : : * : : : : * : : : : * : : : : * : : : : * : : : : * : : : :

Halomonas marina      MSKDFNHTGKSMRKWALGSMASYYIRLKFSDSHPLAQHQEAEQLIEAWFSKMDAQVVSVDW
P. syringue AlgL     ESREFNHTGKSMRKWALGSMSSAYLRKLFSESHPLANRQDAKIIETWFSKLDQVVSVDW
P. aeruginosa AlgL   QSDDFNHTGKSMRKWALGSLSGAYMRLKFSRSSRPLAAHAEQSREIEDWFARLGTQVVRDW
A. vinelandii AlgL  LTTEYNHTGKSMRKWALGSLAGAYLRKLFSSQPLAAYPEQARRIESWFAKVGQVQIKDW
A. chroomococcus    LSTEYNHTGKSMRKWALGSLAGAYLRKLFSTSQPLAAYPEQAKRIEAWFAKVGQVQIKDW
Sphingomonas Al-III LNYDPKSQSWYQVEWSAATAAFALSTMMAEFN--VDTAORE--RVVKWLNLRVARHQS-F
. : : . * : : : : . : : . : : * : : : : * : : : : * : : : :

Halomonas marina      DNLPLEKINNHSYWAAWSVMATAVATNRRDLFDWAVKEYKVGVNQVDADGFLPNELKRRQ
P. syringue AlgL     SNLPLEKINNHSYWAAWSVMATAVATNRQDLFDWAVKEYKVAANQVDKDGFLPNEMKRRQ
P. aeruginosa AlgL   SGLPLKKNHSYWAAWSVMSTAVVTNRRDLFDWAVSEFKVAANQVDEQGFLPNELKRRQ
A. vinelandii AlgL  SDLPLKKNHSYWAAWAVMAAGVATNRRPLFDWAVEQFHIAAGQVDSNGFLPNELKRRQ
A. chroomococcus    SDLPLKQINNHSYWAAWSVMAAGVATNRRPLFDWAVEQFHIAAKQVDRPGLANELKRRQ
Sphingomonas Al-III PGGDTSCNNHSYWRGEATIIIGVISKDDLEFRWGLGRYVQAMGLINEDGSFVHEMTRHE
. . ***** . . * : : * * * : : . : : * : : * : : * : :

Halomonas marina      RALAYHNYALPPLAMIASFAQINGVDLRQ--ENNGALKRLGDRVLGAVKDPDEFEEKNG-
P. syringue AlgL     RALSYHNYALPPLAMIASFAQINGVDLRP--ENNGALKRLGDRVLGAVKDPSIFAEHNG-
P. aeruginosa AlgL   RALAYHNYALPPLAMIAAFAQINGVDLRQ--ENHGALQRLAERVVMKGVDDDETFEETG-
A. vinelandii AlgL  RALAYHNYSLPPLMMVAFAFALANGVDLRG--DNDGALGRLAGNVLAGVEKPEPFAERAGD
A. chroomococcus    RALAYHNYSLPPLMMIAAFAQINGVDLRG--DNDGALGRLAGNVLAGVEDPEPFAERAG-
Sphingomonas Al-III QSLHYQNYAMLPLTMIETASHQGDIDLYAYKENG RDIHSARKFVFAAVKNPDLIKKYAS-
: : * : : * : : * * * : : * : : * : : * : : * : : * : : * : :

Halomonas marina      KKQDMTDLKED-MKFAWLEPFCTLYTCAPDVIEKKRDMQPFKTFRLGGDLTKVYDPSHEK
P. syringue AlgL     EKQDMTDLKED-PKFAWLEPYCSLYTCSPDVLEEKHEKQPFKTFRLGGDLTKVYDPSHEK
P. aeruginosa AlgL   EDQDMTDLKVD-NKYAWLEPYCALYRCEPKMLEAKKDREPFNSFRLGGEVTRVFS--REG
A. vinelandii AlgL  EDQDMEDLETD-AKFSWLEPYCALYSCSPALRERKAEMGPFKNFRLGGDVTRIFDP-AEK
A. chroomococcus    EDQDMEDLETD-AKFSWLEPYCALYACSPALRERKAEMGPFKNFRLGGDVTRIFDP-QEK
Sphingomonas Al-III EPQDTRAFKPGRGLNWIEYQARARFGFADELG---FMTVPIDPRTGGSGTLLAYKPGQA
: * * : : . . * : : : : : * : : * * * : :

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Figure 4.1. Amino acid sequence alignment of PL-5 polymannuronate lyases. Boxed motifs are regions of high similarity among all the lyases represented. Sequences were aligned by CLUSTAL X.

Materials and Methods

Strains, Plasmids and Bacterial Media

Bacterial strains and plasmids are listed in Table 4.1. Bacteria were routinely cultured in L Broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) at 37°C. *P. aeruginosa* was isolated on Pseudomonas Isolation Agar (PIA) (Difco) with appropriate antibiotics. Antibiotics were used at the following concentrations, in µg/ml: ampicillin (Ap) 100, carbenicillin (Cb) 300, gentamicin (Gm) 100, and tetracycline 20.

Two different minimal media were used for the alginate growth experiments, medium 1 and medium 2. Medium 1 contained 0.02mM MgSO₄, 0.15 mM NaH₂PO₄·H₂O, 0.34 mM K₂HPO₄·H₂O, 145 mM NaCl, and trace metals (384 mg/ml FeCl₃·6H₂O, 432 mg/ml MgCl₂·4H₂O, 2.0 mg/ml CoCl₂·6H₂O, 66 mg/ml ZnSO₄·7H₂O, 342 mg/ml H₃BO₃, .037 mg/ml CuSO₄·5H₂O). Carbon sources were either 0.09 mM sodium glutamate plus 0.5 mM glycerol, 0.1% alginic acid from *Macrocystis pyrifera* (Sigma), or 0.1% hydrolyzed alginic acid. Alginate was hydrolyzed by incubating 1% alginate at 85°C for 90 minutes with 50 mM HCl and neutralized with equimolar NaOH. Medium 2 was similar to Medium 1, but contained 1mg/ml of (NH₄)₂SO₄ as a nitrogen source.

Table 4.1 Strains and plasmids

Strain or Plasmid	Genotype, phenotype or mutagenic oligonucleotide	Reference
<u>Strains</u>		
<u><i>E. coli</i></u>		
HB101	<i>proA2, leuB6, thi-1, lacY1, hsdR, hsdM, recA13, supE44, rspL20</i>	
K91		
SM10	<i>thi-1, thr, leu, tonA, lacY, supE recA::RP4-2-Tc::Mu Km^R</i>	(46)
<u><i>P. aeruginosa</i></u>		
FRD1	Cystic fibrosis isolate Alg ⁺	(183)
PAO1	Burn wound isolate Alg ⁻	
FRD462	<i>algG4 S272N</i> Alg ⁺	(26)
FRD1153	FRD1 derivative with <i>algJ</i> mutation Alg ⁺	(73)
Pao1167	Pao1 with $\Delta 1167::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
Pao1784	Pao1 with $\Delta 1784::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
Pao1300	Pao1 with $\Delta 1167::\Delta 1784::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
FRD484	FRD462 with $\Delta 1784::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
FRD485	FRD1 with $\Delta 1784::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
FRD486	FRD1153 with $\Delta 1784::\text{FRT-Gm}^{\text{R}}\text{-gfp}$ Alg ⁺	This study
<u>Plasmids</u>		
pRK2013	ColE1-Tra(RK2) ⁺ Km ^R	(65)
pMF54	P _{trc} ColE1 replicon with <i>oriV_{SF} oriT(RK2) lac^{lq} Ap^R</i>	(70)
pCR 2.1-TOPO		Invitrogen
pEX100T	<i>oriT sacB</i> Ap ^R blunt end <i>SamI</i> site	(244)
pSAD116	PA1167 <i>NcoI</i> - <i>Hind</i> -III fragment in pMF54	This study
pSAD118	PA1784 <i>NcoI</i> - <i>Hind</i> -III fragment in pMF54	This study
pSAD130	PA1167 with Gm cassette in pEX100T	This study
pSAD132	PA1784 with Gm cassette in pEX100T	This study
pPS858	FRT, <i>aacC1, gfp</i> , with mcs, Ap ^R , Gm ^R	(111)
pPS911	Flp recombinase for FRT target Ap ^R	Schweizer

Abbreviations: Alg⁺, alginate overproduction; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R tetracycline resistance; Gm^R gentamicin resistant Tra, transfer by conjugation: FRT, Flp recombinase target

DNA Manipulations

Primers used in DNA manipulations are listed in Table 4.2. PA1167 was PCR amplified from purified PAO1 genomic DNA by using forward primer GTCTCCATGGCTGACCTGAGTACCTGGAAC and reverse primer GCGAAGCTTGCGGCGCAGACGCCTCGACCG, creating a 690bp PCR product with *NcoI*-*Hind*III restrictions sites at the 5' and 3' ends, respectively. PA1784 was PCR

amplified with forward primer CTCACCATGGTCGATCTCAGCACCTGGAAC and reverse primer CGGAAGCTTGCACAGGGCTGGGGCGGCGCG also incorporating *NcoI* and *HindIII* restriction sites at the 5' and 3' ends of the 740bp fragment. The PCR products were gel purified and ligated into TOPO TA[®] vector (Invitrogen). Blue/white selection was used to select for insertions in the TOPO TA[®] vector. White colonies indicate loss of β -galactosidase activity and PCR product insertion into the vector. The fragments were digested from TOPO TA with *NcoI* and *HindIII*, gel purified, and ligated into the P_{trc} expression vector, pMF54. These plasmids resulted in isopropyl- β -D-thiogalactopyranoside (IPTG) inducible expression of PA1167 and PA1784. The resulting plasmids were labeled, pSAD116 for PA1167 expression and pSAD118 for PA1784 expression. The plasmids were introduced into *P. aeruginosa* strains, PAO1, FRD1, FRD462 and FRD1153 by triparental mating. Genes were induced by the addition of 1mM IPTG final concentration.

Gene knockout mutations were performed on PA1167 and PA1784, using a strategy illustrated in Figure 4.2. The PA1167 knockout mutant was constructed by PCR by first amplifying DNA fragments 931 base pairs (bp) upstream and 128 bp downstream of the ATG start codon of PA1167. The forward primer for this PCR product contained a the *PvuII* restriction site and the reverse primer a *HindIII* restriction site. The resulting PCR product was ~1.1 kb. A second fragment (fragment 2) was amplified with primers 28 bp upstream from the PA1167 stop codon and 823 bp downstream of stop codon. The forward primer for fragment 2 contained a *HindIII* restriction site and the reverse primer contained *PvuII* restriction site. Fragment 2 was 850 bp. Each fragment was gel purified

and cloned separately into the TOPO TA vector with blue/white selection. Fragment 1 was then digested, gel purified and ligated into the *Hind*III sites of the TOPO TA clone containing fragment 2. The resulting plasmid containing both fragments 1 and 2 was digested with *Pvu*II to separate the DNA from TOPO TA, and ligated into the *Sma*I site of the allelic exchange vector, pEX100T. The *Gm/gfp/FRT* cassette from pPS858 was then ligated into the *Hind*III site of the resulting plasmid, thereby interrupting the PA1167 gene. The resulting allelic exchange plasmid, containing the *Gm/gfp/FRT* cassette flanked by DNA upstream and downstream of PA1167 was labeled pSAD130.

A knockout mutant for PA1784 was conducted in a similar manner. The forward primer for fragment 1 was designed to yield a 2093 bp PCR product of DNA upstream of the PA1784 start codon. The PCR was used to incorporate a *Fsp*I and *Bam*HI restriction sites into the 5' and 3' ends of the PCR product. The resulting PCR fragment is ~2.1kb. The forward primer for fragment 2 contained a *Bam*HI restriction site and primes 47 bp upstream of the stop codon. The reverse primer primes 674 bp downstream of the stop codon and contains a *Fsp*I restriction site. The resulting PCR product produced a 700 bp fragment. As with the PA1167 knockout, the two PCR products were cloned into TOPO TA. Fragment one was digested and ligated into TOPO TA containing fragment 2 at the *Bam*HI restriction sites. This product was cloned into the *Sma*I restriction site of pEX100T, and the *Gm/gfp/FRT* cassette inserted between fragments 1 and 2 at the *Bam*HI site. The resulting plasmid was labeled pSAD132.

Allelic exchange on the PAO1, FRD1, FRD462 or FRD1153 genomes was carried out by mating pSAD130 and pSAD132 into these strains, and allowing

homologous recombination. Selections for recombinants were performed using PIA medium with 7% sucrose and 100 µg/ml gentamicin. The pEX100T vector contains *sacB* gene encoding levansucrase. This protein is lethal to cells when grown in the presence of sucrose. Therefore, clones that are gentamicin and sucrose resistant have undergone a double homologous recombination event, where the vector DNA is lost, but the gentamicin cassette was inserted into the chromosome. Confirmations of gene replacement of PA1167 and PA1784 were performed by PCR amplification of genomic DNA purified from the knockout mutants and from wild-type strains. The primers used to verify gene replacement were specific for PA1167 or PA1784. The correct sizes of the PCR products with gentamicin cassette were 1.8 kb and 1.72 kb for PA1167::Gm and PA1784::Gm respectively. Correct sizes of wild type PA1167 and PA1784 with these primers were 709 bp and 745 bp, respectively. The PCR reactions confirmed gene replacements.

A double mutant of both PA1167 and PA1784 was also constructed in *P. aeruginosa* PAO1. For these studies, plasmid pPS911 containing the Flp recombinase was introduced into *P. aeruginosa* PAO1167 (Δ 1167::Gm). The Flp recombinase excises the gentamicin cassette at the FRT sites, resulting in a gentamicin sensitive PA1167 mutant. Following removal of the gentamicin cassette, pSAD132 was mated into the resulting strain, and deletion mutation of PA1784 was constructed as described above. The resulting strains contained deletions of both PA1167 and PA1784. Double mutations were verified using PCR as described above.

Table 4.2. Oligonucleotides Used for Plasmid and Gene Knockouts

Primer description	Oligonucleotide	Fragment size
<u>PA1167 amplification</u>		
Forward with NcoI	GTCTCCATGGCTGACCTGAGTACCTGGAAC*	690 bp
Reverse with HindIII	GCGAAGCTTGCGGCGCAGACGCCTCGACCG*	
<u>PA1784 amplification</u>		
Forward with NcoI	CTCACCATGGTCGATCTCAGCACCTGGAAC*	740 bp
Reverse with HindIII	CGGAAGCTTGACAGGGCTGGGGCGGCGCG*	
<u>PA1167 knockout primers</u>		
Fragment 1		
Forward with PvuII	CTGCAGCTGTGCCCAGGAGCGTCGGT	1.1 kb
Reverse with HindIII	CAGAAGCTTATGCCGTCGGCGGTGCGC	
Fragment 2		
Forward with HindIII	CAGCAAGCTTCGCGTTAGCCATCAATG	2.1 kb
Reverse with PvuII	CGATCAGCTGTAAAGCCCTTCGCCGC	
<u>PA1784 knockout primer</u>		
Fragment 1		
Forward with FspI	GGCGCCTGCGCACTGGAAAACGGGAC	850 bp
Reverse with BamHI	AAGGATCCAGGTGCTGAGATCGATCATG	
Fragment 2		
Forward with BamHI	AGGGATCCGCGTGACCATCTACCACCTG	700 bp
Reverse with FspI	CAGCAGTGCCCTGCGCATAGCTGTTCAC	

*indicate primers used to verify gene knockouts of PA1167 and PA1784

Sequence Analysis

Amino acid sequence alignments were conducted on CLUSTALX (109).

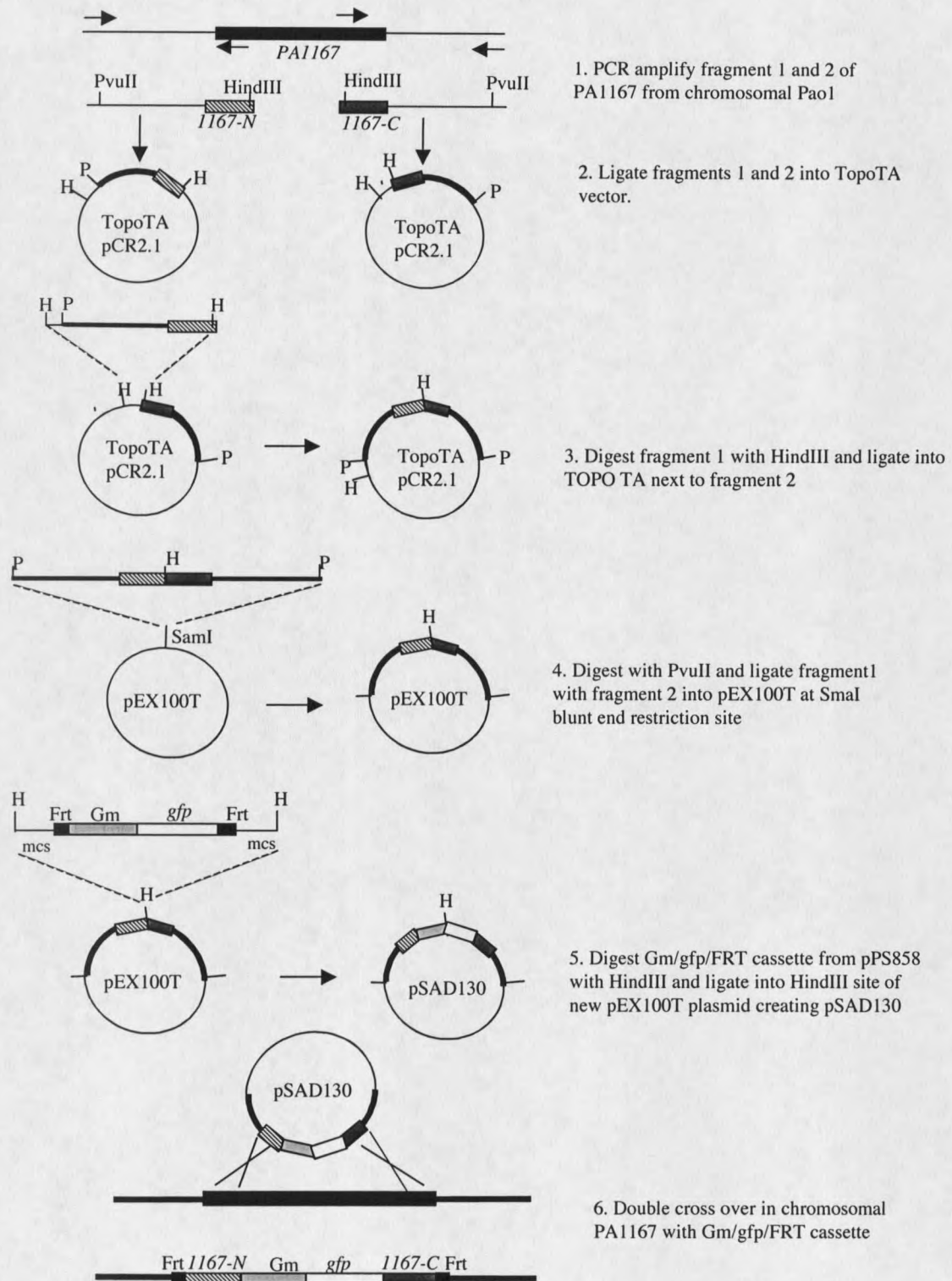


Figure 4.2. Construction of PA1167 knockout mutant with insertion of Gm/gfp/FRT cassette.

Alginate Growth Experiments

P. aeruginosa PAOI, and PAO1 containing selected plasmids, were grown in L broth overnight at 37°C with appropriate antibiotics. The cultures (100 µl) were inoculated into media 1 and media 2. Cultures were allowed to grow for up to six days at 37°C. OD readings at 600nm were read at 2, 4, at 6 days after transfer into the minimal media. The *P. aeruginosa* PAO1 deletion mutant strains were cultured overnight in LB and either streaked onto media 2 plates or transferred to 5ml of media 2 and incubated at 37°C for up to 6 days.

Results

Over-expression of PA1167 and PA1784 in Mucoïd Strains of *P. aeruginosa*

Amino acid sequence alignments were conducted with the PL-7 guluronate specific alginate lyases (Fig. 4.3). The alignments revealed that PA1167 and PA1784 contain two highly conserved motifs (RSELR and YFKAGXYXQ) found in the other PL-7 lyases. These conserved motifs suggest that PA1167 and PA1784 may belong to this family of guluronate specific alginate lyases.

```

PA1167          RFWVPVNGSHTRN-SEFFRSELRRETLSSG-----RPNWRYARA-----D
PA1784          VFVWVIDGSHTEd-STYHRETELREtQADG-----SLDNWYYWQA-----D
Sphingomonas  Al-II  AFWCPASGFKTTANTKYRSELRREMLDPDN-----HAVNWG-WQG-----T
Corynebacterium Aly-1 QFRAAVNGVTTSG-SGYRSELRREMTDGGE-----EKASWSATSG-----T
Streptomyces coelicolor QFRSAVNGVTTSG-SSYRSELRREMTSNGT-----KNASWSATSG-----T
Kleb. pneumoniae AlyY VFKAPISGAKTSKNTTYTRSELRREMLRKGDTSIATQGVSRNNWVLSAPLSEQKKAGGVD
Vibrio halioticoli Aly VFKAPNKAMTTPN-SKNRSELRREMLADYSS-----PKNNFTVASTKDAEKYG--AIG
* . . . * : .*:*** . .:

PA1167          VSVNG-----SALEQQLDPQWAYQGLYFKAGLYLQDNRGPSSEG--
PA1784          VRARSQDGE-----EDAYYQTLGSAWNDQLLYFKAGAYVNDNAGDSGEG--
Sphingomonas  Al-III  MTVNG--QT-----KTVDFVAKDAGWKDLKLYFKAGNYLQDRQADGSDTS-
Corynebacterium Aly-1 VYYNG-----VLQTTISHTSSGNYFKAGAYTQANCNSNSSPCSS
Streptomyces coelicolor AYYNG-----VLQTTIAHSASGNYFKAGGYTQANCGNSSPCSS
Kleb pneumoniae AlyY VTLMREGRPD-----VVKTVDMSKSGYSEAGQYLYFKAGVYVNCNKTGKPPDY--
Vibrio halioticoli Aly LTFFTKNPGQPNOEVKEFDVNLAKGHLKGDKYDQGYANDWNYFKAGNYNCNTGSAGCSNN
***** * : . . .

PA1167          -----GRATFSELRVSHQ---
PA1784          -----SRVTIYHLNtAHR---
Sphingomonas  Al-III  -----ALVKLYKLDVKHSS--
Corynebacterium aly-1 ----SNYGQVSLYKLQVTHS---
Streptomyces coelicolor ---SNYGQVSVYKLLVNHS---
Kleb pneumoniae AlyY - -----VQATFYRLKATHGAQR
Vibrio halioticoli Aly ---GLEAGDYAQVSYFKLNLDQ----
... * :

```

Figure 4.3. Amino acid sequence alignment of PL-7 guluronate lyases and the putative lyases of *P. aeruginosa* PA1167 and PA1784. Conserved motifs are boxed. Sequences were aligned by CLUSTAL X.

To test this hypothesis, PA1167 and PA1784 were cloned into the IPTG-inducible expression vector pMF54 and mated into FRD1, FRD462 and FRD1153. These strains were chosen because they overproduce different types of alginate. FRD1 produces a wild type alginate with M/G blocks and O-acetyl groups on the mannuronate residues. FRD462 produces an acetylated polymannuronate alginate, and FRD1153 produces a non-acetylated M/G alginate. It was our goal to determine if over expression of these putative lyases would degrade the endogenously produced alginate and cause non-mucoid phenotypes in these strains. These strains were streaked onto PIA plates containing Cb with and without the inducer, 1mM IPTG. FRD1 with overexpressed PA1167 was non-mucoid upon IPTG-induction, but mucoid without IPTG induction (Fig 4.4A). These

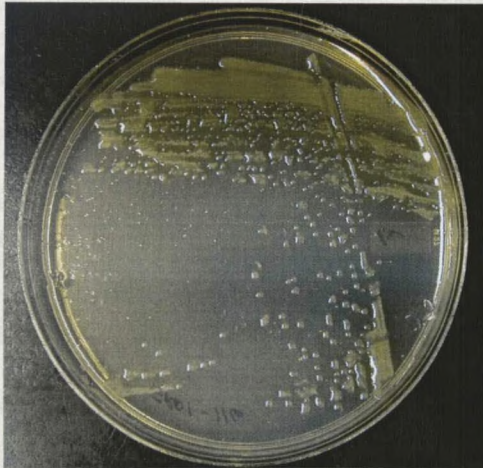
results suggested that the product of the PA1167 gene degrades alginate produced by FRD1. Similarly, FRD1153 with overexpressed PA1167 was non-mucoid when induced and mucoid when not induced (Fig 4.4B), suggesting that the product of PA1167 is functional against both acetylated and non-acetylated alginates. FRD462 did not show any difference between the PA1167 induced or non-induced cultures (Fig 4.5A). Since these putative lyases resemble guluronate specific alginate-lyases it was expected that overexpression in FRD462 would not affect the mucoid phenotype. Therefore, these results indicate that PA1167 is an alginate lyase that is specific for guluronates.

To determine if non-mucoidy in the PA1167 overexpressed strains was in fact due to the expression of PA1167 and not due to some other unrelated factor the IPTG-induced cultures were transferred to medium without IPTG. In these cultures, the mucoid phenotype was restored (Fig 4.4C). These results support PA1167 as an alginate lyase that is capable of degrading acetylated and non-acetylated M/G alginate.

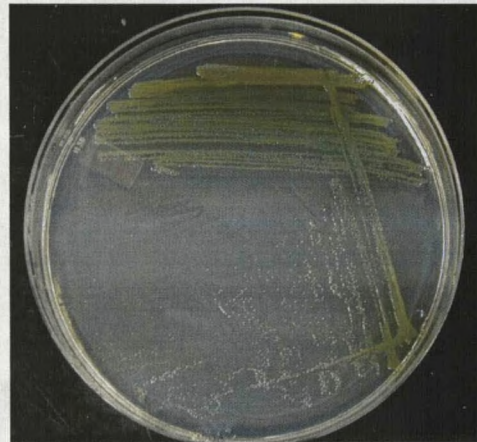
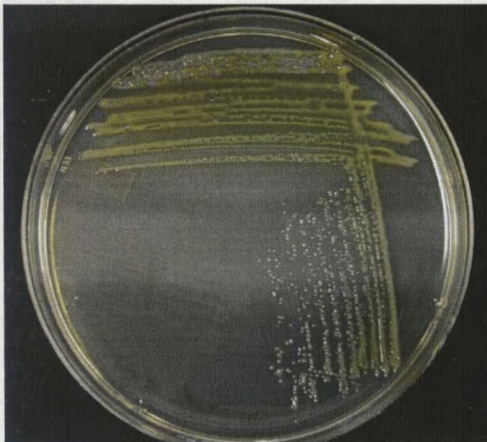
Induction of PA1784 in FRD1 and FRD1153, on the other hand, did not show any significant difference between mucoidy (Fig 4.6). Both cultures grew at a slower rate with PA1784 expression, suggesting that overexpression of this gene was toxic to the cells. These data do not support PA1784 as an alginate lyase.

PIA/CbPIA/Cb/IPTG

A. FRD1-pSAD116



B. FRD1153-pSAD116



C. FRD1153-pSAD116 recovery of mucoid phenotype



Figure 4.4. Mucooid phenotype in FRD1 and FRD1153 with overexpression of PA1167. FRD1 (A), FRD1153 (B) with and without overexpression of pSAD116 (PA1167) Induction occurred with 1mM IPTG. (C) recovery of mucooid phenotype in FRD1153 with pSAD116 after removal from IPTG media.

Chromosomal Deletions of PA1167 and PA1784 in Mucoid Strains

Knockout mutants with the *Gm/gfp/FRT* cassette were made to study the mucoid phenotype in the absence of PA1167 or PA1784. Repeated attempts to delete PA1167 from strain FRD1 were not successful, perhaps due to the lack of homologous DNA on the FRD1 genome. Wolfgang et al. (289) indicated that CF isolates do contain the PA1167 gene, although strain FRD1 was not tested. We were unable to PCR amplify the gene PA1167 from FRD1. Deletion mutants in strain PAO1 were successful. Knockout mutants of PA1784 were obtained in strains FRD462, FRD1 and FRD1153. In FRD462 and FRD1 the mutant cells appear no different from their isogenic parent strains. However, in FRD1153 the alginate visually appeared more fluid than the parent strain. The reason for this reduced viscosity in the mutant strain is not clear at this time.

Growth of PAO1 with Alginate as a Sole Carbon Source

Sphingomonas A1-II alginate lyase is able to degrade alginate intracellularly and use alginate as a sole carbon source. To test whether *P. aeruginosa* could grow with alginate as a sole carbon source we overexpressed the PA1167 and PA1784 genes in *P. aeruginosa* PAO1. In addition, we produced knockout mutants of these genes to determine if growth on alginate would be hindered in mutant strains. PAO1 or PAO containing the plasmids pSAD116 (PA1167), pSAD118 (PA1784) and pMF54 (vector control) were grown in alginate media 1 or alginate media 2 containing difference carbon sources or no carbon source. Glycerol and glutamate are readily utilized by *P. aeruginosa* as a carbon source, and therefore, these carbon sources were used as a positive control. Alginate growth media contained 0.1% alginate or 0.1% hydrolyzed

alginate that contains shorter polymers. An OD measurement was made at 600nm after 2, 4, and 6 days of growth. Table 4.3 lists the OD readings for each of these strains. A small amount of growth in all strains occurred on all media types. The media with glycerol and glutamate have much higher growth yields than media lacking these carbon sources.

Growth in media with alginate and media lacking a carbon source were similar, indicating that *P. aeruginosa* does not grow with alginate as a sole carbon source.

Likewise, growth on media with alginate was not observed in PAO overexpressing the putative alginate PA1167, or PA1784, from plasmids pSAD116 or pSAD118, again suggesting that expression of these putative alginate lyases is not sufficient for growth of *P. aeruginosa* on alginate as a sole carbon source.

Deletion mutants PA1167, PA1784, as well as, the PA1167 and PA1784 double mutant also showed no difference in growth on alginate media, indicating that these genes do not play a role in alginate utilization by *P. aeruginosa* PAO1.

Table 4.3. Growth of PAO1 with alginate as a carbon source

Strain/ Plasmid	Days incubated	Media type 1			Media type 2		
		2 days	4 days	6 days	2 days	4 days	6 days
PAO1	Glycerol and glutamate	0.199	0.220	0.207	0.528	0.552	0.533
	No carbon	0.030	0.070	0.068	0.033	0.062	0.071
	0.1% alginate	0.059	0.093	0.087	0.037	0.065	0.065
	0.1% alginate hydrolyzed	0.031	0.070	0.069	0.036	0.060	0.074
PAO1/ pMF54	Glycerol and glutamate	0.182	0.210	0.199	0.521	0.503	0.427
	No carbon	0.031	0.060	0.064	0.034	0.065	0.068
	0.1% alginate	0.029	0.061	0.064	0.030	0.058	0.061
	0.1% alginate hydrolyzed	0.027	0.067	0.068	0.029	0.059	0.061
PAO1/ pSAD116	Glycerol and glutamate	0.199	0.206	0.198	0.365	0.418	0.444
	No carbon	0.032	0.069	0.068	0.031	0.062	0.067
	0.1% alginate	0.032	0.062	0.065	0.036	0.067	0.063
	0.1% alginate hydrolyzed	0.031	0.066	0.069	0.042	0.070	0.098
PAO1/ pSAD118	Glycerol and glutamate	0.166	0.187	0.186	0.367	0.436	0.435
	No carbon	0.029	0.069	0.068	0.030	0.065	0.070
	0.1% alginate	0.031	0.062	0.059	0.027	0.067	0.056
	0.1% alginate hydrolyzed	0.034	0.066	0.067	0.021	0.060	0.060

OD readings at 600nm of broth cultures testing growth in different carbon sources; 0.09mM sodium glutamate plus 0.5mM glycerol, 0.1% alginate *Macrocystis pyrifera*, 0.1% hydrolyzed alginate, or no carbon. Media1 and Media 2 are identical except Media 2 contains 1mg/ml of $(\text{NH}_4)_2\text{SO}_4$. pSAD116 contains PA1167, pSAD118 contains PA1784, and pMF 54 is the control vector with no gene inserted.

Discussion

Family PL-7 of the polysaccharide lyases consists of the guluronate-specific alginate lyases. Two putative alginate lyases, PA1167 and PA1784, that group in the PL-

7 family, were identified on the sequenced *P. aeruginosa* genome. These putative lyases contain two highly conserved motifs found in all known guluronate-specific alginate lyases, suggesting that these genes may encode alginate lyases. To test this hypothesis, these proteins were overexpressed in mucoid strains of *P. aeruginosa*. The results indicate that PA1167 does act as a lyase *in vivo* and is capable of degrading both acetylated and non-acetylated alginate. Interestingly, knockout mutants of PA1167 were never obtained in the FRD mucoid strains, but could be obtained in strain PAO1. The presence of PA1167 was shown to exist in other mucoid CF isolates (288). However a PCR product of PA1167 was never obtained for FRD1 suggesting that this gene may not be present on the FRD1 genome. Deletion mutations of the mannuronate-specific alginate lyase, *algL*, in strain FRD1 are lethal to the cells (182). Therefore, a deletion mutation of PA1167 in mucoid strains may also be lethal, and therefore, it may not be possible to delete this gene in FRD1. The mutation is not lethal in nonmucoid strains, since we were able to delete this gene in *P. aeruginosa* PAO1.

Overexpression of PA1784 in the FRD strains does not appear to have any effect on mucoid phenotype. These data suggest that this enzyme may not be an alginate lyase. However, the unusual alginate viscosity in FRD486:: Δ 1784 (FRD1153 with Δ 1784) suggests that PA1784 may play a role in alginate production, but perhaps not as a lyase. PA1784 is up regulated in PAO1 biofilms and is regulated by quorum sensing (242). Its role may not be essential in alginate production or degradation, but rather in biofilm formation or dispersion. Further research is required to characterize the role of this protein in biofilm formation or in alginate lyase activity. The PA1167 gene was not

reported to be quorum sensing regulated (242). Further investigation is required to determine its role in cellular processes, such as the conditions under which it is expressed, its role in alginate production and degradation, and its role in the biofilm phenotype.

To begin to understand the function of these proteins an *in vitro* assay with these proteins would be invaluable in confirming these enzymes as alginate lyases. This, however, requires adequate expression and at least a crude extract with sufficient concentrations of the putative lyases. Initial experiments were attempted with overexpression of both PA1167 and PA1784 in *E. coli*. However, in cytoplasmic extracts, no bands corresponding to these proteins could be seen compared to the vector control strain. *In vitro* assays with crude extracts from cells overexpressing PA1167 and PA1784 did not show alginate lyase activity *in vitro* (data not shown)(26). However, it is likely that these enzymes are secreted from the cells as is the case with the Aly alginate lyase from *Klebsiella aerogenes*, and therefore would be found in the culture supernatant. Future studies regarding these putative lyases as extracellular alginate lyases are being conducted in collaboration with the lab of Dr. Matt Parsek and the University of Iowa.

Utilization of alginate as a sole carbon source has been described in several bacterial species (169, 289). Our results suggest that *P. aeruginosa* is not able to use alginate as a sole carbon source and indicate that the putative lyases PA1167 and PA1784 have alternative roles.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The original goals of this research were to identify and characterize protein domains that interact with polysaccharides. In particular, this research was designed to characterize enzymes involved in the structural modification of the *P. aeruginosa* extracellular polysaccharide and virulence factor, alginate. Through these studies, predictive structural models and active domain studies were used to characterize the alginate C-5 epimerase, AlgG, and the alginate O-acetylase, AlgJ. This research also provided preliminary evidence that in addition to the alginate lyase, AlgL, at least one additional alginate lyase, PA1167, is encoded on the *P. aeruginosa* genome.

Results described in Chapter 2 provide evidence that AlgG likely folds as a parallel right handed beta helix (RH β H). The known RH β H proteins consist of sugar hydrolases and lyases that degrade long chain linear polysaccharides. The evidence described here suggests that in addition to sugar hydrolases, AlgG, a mannuronan epimerase, falls into this class of proteins. Evidence supporting AlgG as a RH β H include the amino acid sequence repeats in the C-terminal domain of the protein. Each repeat is predicted to contain three β -sheets separated by three turn regions. The repeating sequences in AlgG contain a pattern with the consensus sequence is I.X.Z.X.(+/-).(+/-).(S/A/V).(+/-).(+/-).(3Z).X.N.(4X).N.(3X).G. The repeats of AlgG have a predicted secondary structure similar to repeats found in RH β H proteins, whose structures have

been determined by X-ray crystallography studies. These structural similarities between AlgG and other RH β H proteins are apparent even though these proteins have little primary sequence homology. Within the RH β H proteins, the turn regions often contain asparagine residues that stack in the folded structure. Asparagine stacking is important in structural stability and maintenance of the helical fold of RH β H proteins (120). In addition, the β -sheets contain aliphatic and aromatic residues that are important in stacking interactions and maintenance of the protein structure (120). The repeats of AlgG model as a RH β H and provide further evidence that AlgG contains a core β -helical structure at its C-terminus that may accommodate a long chain linear polysaccharide.

Site directed mutagenesis studies described here provide support for the RH β H model of AlgG. Mutations of amino acids N362 and N367 disrupt both the epimerization and polymerization activities of AlgG. These residues often appear in turn 2 and turn 3 of the repeats and stack in the AlgG β -helix model. The results suggest that these residues are involved in asparagine stacking and protein structural stability. Site directed mutagenesis indicated that the DPHD motif of AlgG is important for epimerase activity, but not essential for polymerization of the alginate precursor. This suggests that these mutants have likely maintained their structural integrity, since the mutant proteins complement the polymerization defect in FRD1200. However, these residues are likely important either in catalysis or in the interactions of AlgG with polymannuronate. The structural predictions of AlgG provide support for these amino acids being important for catalysis or for substrate binding. In RH β H proteins, the polymer lies across the PB1 regions that form the bottom of the shallow groove in this structure (3, 143, 232,

248). Frequently in RH β H proteins, the catalytic activity occurs in the center of the β -helix where active residues are located on PB1 or in the loops adjacent to the groove (3, 136, 143, 222, 230, 232, 248, 262). The presence of DPHD in the center of the helix and on PB1 supports the proposal that this motif is a catalytic center for epimerization. Work by Svanem et al. (270) indicated that the equivalent residue to D324 in AlgE7, the extracellular epimerase from *A. vinelandii*, was also important for enzyme activity. Aspartate residues are essential as reactive residues in β -elimination reactions. If the proposed mechanism for epimerization mimics that of β -elimination, aspartate residues in AlgG may also be essential as reactive residues in epimerization (77). The location of the mutations in the RH β H model and the effects of these mutations on protein function are consistent with the RH β H fold and with the DPHD motif being a catalytic center. The characterization of AlgG as a RH β H provides functional importance of the β -helical fold in protein binding to long chain polysaccharides.

Cellulases, as well as several other carbohydrate binding proteins, such as chitinases and xylanases, have separate catalytic and carbohydrate binding domains (274). Several cellulose-binding domains have been characterized, purified and crystallized (139). Even though the cellulose binding domains are variable, the residues that interact with the polymer are highly conserved. These residues are also conserved in chitin binding motifs and xylan binding motifs (271). Proteins that interact with long chain polysaccharides, including the cellulose binding proteins, have flat or shallow grooves composed of β -sheets, and either fold into helical structures such as the RH β H, or as a β -sandwich structure. The long chain polysaccharides bind to these grooves (3,

30, 55, 113, 248, 275, 276). In cellobiohydrolase I of *Trichoderma reesei*, at least seven sugar residues bind in the long shallow groove. The AlgE4 of *A. vinelandii* may also accommodate at least seven uronic acid residues (55, 99). Since the β -helical region of AlgG is likely similar to this region of the *A. vinelandii* epimerase, then AlgG may bind multiple uronic acid residues in its shallow groove, and epimerize approximately every other mannuronic acid residue as the alginate polymer moves through the cell periplasm.

The amino acid sequences among carbohydrate binding proteins including the RH β H proteins, do not have high primary sequence identity. In addition, these proteins do not have small linear sequences of amino acids that interact with the polymer. Instead, the important amino acids are spread throughout the polypeptide sequence, and the polysaccharide binding domains form following the three-dimensional folding of the protein. Therefore, this phenomenon does not support the use of peptide phage display as a useful tool for recognizing linear protein motifs that interact with polysaccharides. In retrospect, phage display was not useful in identifying polysaccharide binding residues in AlgG or AlgJ. On the other hand, not all polysaccharide binding proteins have flat or shallow grooves. The α/α barrel of A1-III mannuronate specific alginate lyase of *Sphingomonas* sp. forms a barrel with a deep cleft that folds around the polymer (296). Phage display may have been useful in identifying short motifs that may exist in proteins with these structures. Other studies using monoclonal antibodies to specific polysaccharides demonstrated that phage display is useful for the identification of peptides that mimic carbohydrates (210).

Predicting AlgG as a RH β H put this protein in a structural class dominated by enzymes that are responsible for polysaccharide degradation. Similar repeats that have been classified as the CASH domain are found in surface layer protein B of *Methanosarcina* sp., the A modules of the extracellular C-5 epimerases of *A. vinelandii*, and GP-1 coat protein of *Ectocarpus siliculosus* (33). These proteins are not involved in polysaccharide degradation. The presence of similar repeats in these proteins suggests that they may also fold as RH β H. This structure appears to be a general binding structure for long chain linear polysaccharides rather than one evolved specifically for the degradation of polysaccharides. The two different mechanisms for cleaving glycosidic bonds in RH β H proteins, either acid/base hydrolysis or β -elimination, further supports this hypothesis. Similarly, β -elimination observed in both the RH β H proteins and in the α/α barrel of A1-III also supports the hypothesis that these proteins fold based on polysaccharide structure. Interestingly, the mechanism for epimerization has been suggested to be similar to that of β -elimination (77), suggesting that the RH β H protein structure involved in long-chain polysaccharide binding has been maintained through evolution. However, the RH β H structure is not the only fold that binds polysaccharides, since AlgL, a lyase that binds M/G alginate has an α/α barrel fold.

Crystallization of AlgG is paramount for confirming the RH β H fold, and confirming the importance of N362 and N367 in asparagine stacking. Crystallization with polymannuronate will also show which amino acids are required to bind to the polymer, and should verify the importance of the DPHD motif in catalysis. This will also provide better hypotheses for the function of the N-terminal α -helical portion of AlgG,

which to date has not been characterized. The N-terminal domain may be important in protein stability, or in protein-protein interactions with other members of the alginate biosynthetic scaffold. Other RH β H proteins, such as the tailspike protein of phage P22, are involved in protein/protein interactions and form protein/protein complexes. The protein/protein interactions that occur in the tailspike occur in two different domains. This protein contains an amino-terminal domain that binds to the phage head and folds into a dome-like structure containing two anti-parallel β -sheets. The β -helical domains interact with β -helical domains of individual tailspike proteins, forming a homotrimer (245, 263). The putative RH β H of the surface layer protein B of *Methanosarcina* sp. often have multiple domains, and it has been suggested that these domains are important in protein/protein interactions involved in the intercellular interactions of these Archaea (124). The N-terminal region of AlgG may also be involved in protein/protein interactions, since deletions of this region from AlgG were not able to complement the alginate polymerization defect of strain FRD1200. Studies addressing the structure and importance of the α -helical N-terminal region of AlgG, as well as, studies on protein/protein interactions of AlgG with other alginate biosynthetic proteins will be useful in testing the alginate biosynthetic scaffold hypothesis, and will provide a better understanding of alginate biosynthesis in *P. aeruginosa*.

The RH β H structure has not yet been identified in higher eukaryotes. Therefore, this structure could be a good candidate to target with drug therapies and for treatment of *P. aeruginosa* chronic lung infections. However, the epimerases involved in synthesis of dermatan sulfate and heparin sulfate may resemble the RH β H fold. Hydrophobic plots of

dermatan sulfate epimerase of *Drosophila* resemble plots of AlgG, suggesting that these proteins may have a similar fold. Likewise, the CASH domain has been identified in some human proteins (33, 279), suggesting that they may fold as a RH β H. Therefore, research on these other proteins is necessary before the utilization of this protein structure as a good therapeutic target.

In addition to the characterization of AlgG, other alginate modifying processes were investigated in this research. These studies included two putative alginate lyases encoded by the *P. aeruginosa* PA1167 and PA1784 genes. Even though these predicted protein sequences are similar, these experiments indicate that only PA1167 acts as an alginate lyase. These studies also demonstrate that unlike *Sphingomonas* sp. A1, *P. aeruginosa* is not able to use alginate as a sole carbon source. Therefore, the PA1167 alginate lyase likely plays another role in the metabolism of *P. aeruginosa*. The plasmid-based expression constructs and the deletion mutations of PA1167 and PA1784 produced here will provide useful tools for characterizing the function of these proteins. The expression of PA1784 during biofilm formation and under the control of quorum sensing molecules raises several questions about its functional role in biofilm development. It would be reasonable to think that it acts as an alginate lyase and is responsible for the detachment of planktonic cells from a biofilm. However, the evidence presented here does not support this hypothesis, since the product of PA1784 does not appear to be an alginate lyase, under the conditions tested here. On the other hand, PA1167 is shown here to act as an alginate lyase, but is not induced by quorum sensing. This raises the intriguing questions, under what conditions is PA1167 expressed, and what role does it

play for the survival of these bacteria? Previously, AlgL was the only alginate lyase thought to be present in *P. aeruginosa*. The presence of one and possibly two additional alginate lyases in *P. aeruginosa* has opened a wide variety of questions, including those on the role of these enzymes in biofilm formation or maintenance. If these putative lyases are involved in breakdown of alginate and help maintain the biofilm structure, the system might be exploited to enhance alginate degradation as a therapy for CF patients infected with mucoid strains of *P. aeruginosa*.

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APPENDIX

Amino Acid letter representations*Aliphatic:*

G-glycine
A-alanine
V-valine
L-leucine
I-isoleucine
P-proline

Aromatic:

F-phenylalanine
Y-tyrosine
W-tryptophan

Sulfur containing:

M-methionine
C-cystine

Alcohols:

S-serine
T-threonine

Acids:

D-aspartate
E-glutamate

Bases:

H-histidine
K-lysine
R-arginine

Amides:

N-asparagine
Q-glutamine

Phage sequences

M/M alginate conjugated to epoxy sepharose

First set of sequences

1 VEAKGHKKK	17 GKDWMTLTA	33 RTQGTGMA
2 QRRKSIDAS	18 DHPRSTERC	34 HGAPRAPHK
3 LPSRA	19 RTVQWDMHL	35 EAHRAAFSL
4 KDVTKRDI	20 SEHSFRNGW	36 EAVE
5 VVDAEHPRG	21 DWKYGFTPP	37 GQGVHQALW
6 RPPGDDRI	22 QTTWRDHTS	38 RNLEMMRS
7 NAAPQDRAV	23 DPKETA AKV	39 ESGNTG
8 SQLGRNVWF	24 AAFEKHRKE	40 SPEKDVSLR
9 ASAGLGSKG	25 KGALTGSRQ	41 GWARRKLF
10 TPGTFNLQK	26 LFHSISLC	42 SGMFGQSGD
11 ERDDREPSV	27 GHRDLTTVN	43 KHGQVMIPV
12 HAGASERKS	28 RGGLRNWQK	44 DSYDYWKFS
13 FTAGWRQQP	29 AKEARGMRG	45 IQGYVHRTV
14 VFAASPDGK	30 GGTVWQEHP	46 DRGRTMEAP
15 RCSEVCSGC	31 KTLRHTEGG	47 VETDIVRRM
16 MMKDRYLKL	32 FRSVARRLK	48 DWSNFVKLP

49 SRDRSSDS
 50 DNSQSLANH
 51 RVLNFTQVD
 52 VEDRIFRRG
 53 TGGWKNQPL
 54 FKPLYNFNA
 55 TKEGVYNVM
 56 HGGQGALWK

57 RDGGVDASC
 58 GVPGEGAGR
 59 HSRTQNYIS
 60 QAAMHKNWL
 61 NLWDGANGG
 62 DSKTLARRL
 63 AREDFVVLG
 64 GAKGAKAMV

65 AQSTRSVPW
 66 FTGSGLGLS
 67 FQDSSGSIG
 68 GGSNVNAEV
 69 ETDTRARR
 70 KGVGILAGG

Second set divided into NaCl elution and Lyase elution

NaCl

1 GKVKKEKPI
 2 ANRWDGGPS
 3 DQKSNAARL
 4 EMMMRSQLE
 5 AGEKGGLSK
 6 AETERSPTA
 7 DVQALALQH
 8 NVHGVNAKK
 9 SQVDGSNKE
 10 GWKAKKPPK
 11 ETRMVPQAG

12 RLGEMSRLI
 13 SSMKHNTWTG
 14 GLNQ

Lyase

15 YRNNISIMS
 16 GFDGQHART
 17 TRFLNSDRP
 18 QIGKTAPKS
 19 EMTGRSDKY
 20 RLSYNDRTA
 21 MKSSVKDYK

22 ARASWDEQG
 23 GGPQQKMRA
 24 AKVPEVTPG
 25 VQIHRAANA
 26 TERQRPTGD
 27 HYSNQFSHM
 28 QQQGSRHES
 29 SHNQIKRGL
 30 EQVGKVPKA
 31 VTGTTMYKE
 32 SRTVQNLST

M/G alginate conjugated to epoxy sepharose beads

1. VEAQKQPRR
 2. VEAKKRTAA
 3. VEAKKVRDS
 4. VQAKKQKNS
 5. VLAKKGKLT
 6. VYAKKTRTA
 7. GKKTPKIPV
 8. VWGAKTHKK
 9. KVPKIKPKV
 10. SLAKVKKPK
 11. ALAKKGPKP
 12. ADAKTRKPK 2x
 13. VKKPPKVKG
 14. GWKAKKPPK 2x
 15. GKVKKEKPI 2x
 16. KAKPPKGHG

17. AKGHKAPRK
 18. AKIPRHKKP
 20. GKLQKRPK
 21. GKSPKRPK
 22. MKSPKKPRH
 23. EKPSRKPSK
 24. KPVKKLPKP
 25. HRPKKPDKM
 26. IHAKKPKPK 2x
 27. GWAKPKAPK
 28. KAPKRAPKE
 29. KPVKKLHKR
 30. KPSKMMHVK
 31. KPPKTRPVL
 32. CLAKRQPKR

M/G alginate Lyase elution

- | | | |
|-----------------|------------------|-----------------|
| 1. AVSYVAKTG | 18. LNTTYVETQ | 35. ELKHWFDRE |
| 2. GNGEANLLL | 19. SMSISLRV | 36. VNNGKHPAV |
| 3. SVEMRHRVW x2 | 20. EERWSHGRS | 37. NQHVRWAK |
| 4. VNNGKHPAV | 21. EKCCSVQKA | 38. KAAYPEPRF |
| 5. TWWTAGMSG | 22. SNTGGPGRW | 39. DTRWVSKHG |
| 6. YEKAGEIDR | 23. GNLLSHSSV | 40. GWKNYWRTA |
| 7. KTLQGIAHS | 24. ESARKPLLT X2 | 41. IKMITGGTS |
| 8. SITNAVTS | 25. AARIGHSLF | 42. KSMLSLQTK |
| 9. GQKVHVNG | 26. HSFSKRPYP | 43. LSYGAVNDA |
| 10. GWSAKYIRQ | 27. INDQRSPRK | 44. TKPTFDNGA |
| 11. VSQGVKQV | 28. WGAWGGKRS | 45. GTVITSRQV |
| 12. TLKARQSGF | 29. YNAKLMPLN | 46. GLFNGVRQG |
| 13. SGVMFRTIP | 30. GWWGWSGKG | 47. KMKVPRPI x2 |
| 14. GIISKRPKG | 31. EHRIKMETR x2 | 48. QTMAGPRWG |
| 15. LPGKPDGRQ | 32. WNVGASKQA | 49. TLHWESSPL |
| 16. HMKGCCRPK | 33. NGGAFAVRV x2 | 50. SPRIDQKIS |
| 17. TAPRFSSTA | 34. SKAGKEIHR | |

Controls: naked epoxy sepharose beads

- | | | |
|----------------|------------------|---------------|
| 1. IHAKPKPK 3x | 6. GINKPKPK | 11. TGRHLVGTS |
| 2. VLGKIKPK | 7. VMAKKLPK | 12. GKALKVQTI |
| 3. AVGKMKPK | 8. VIAKIKPK | 13. LEKHPSES |
| 4. SLAKVKKPK | 9. VHAKKDKWR 15x | 14. RVVVDKYYE |
| 5. GSLRNIVRG | 10. IHAKPKPK 3x | |

M/M alginate no epoxy sepharose beads

With on NaCl elution

- | | | |
|---------------|---------------|---------------|
| 1 ILS-short | 15. SNWTTSQRH | 29. LELDRTWKQ |
| 2 SKSLLLHPG | 16. KPDVEGGAL | 30. HEKGQNGGD |
| 3 TMRLVEVRR | 17. EGMADRRPG | 31. GFANGAGQP |
| 4 LSPLDRITK | 18. KSSGDDPRH | 32. KGGSVGMRQ |
| 5 GAQLLGRVN | 19. LDQRRTYPL | 33. SMDGYSSI |
| 6 MQKDYPWAG | 20. DVGLLWQPG | 34. KPKMSRAVE |
| 7 GAQSKMLRG | 21. AAWGNRALY | 35. KLKHMKDVV |
| 8 RIGGQGLEK | 22. RTLPTRFES | 36. LVAGSKGKH |
| 9 ASGHQTPAK | 23. GSVSKEADR | 37. GLQLQGWHR |
| 10 GTRGSFLHQ | 24. SLGIRRASL | 38. VPVHKKGTK |
| 11. GDNGARRNL | 25. NMGMGWPNP | 39. KVAVMTGHR |
| 12. DRAQLQLSK | 26. GWGGSLSA | 40. GHHQRCLMS |
| 13. LDRTGVGVA | 27. AYMERKAKM | 41. EWRDIASKW |
| 14. IIKKMEDSL | 28. KKMDTATHR | |

Controls: Just empty columns

1. DAWPVLHSA
2. QVEDRGLAL
3. EERRDGAEP
4. RDYIRDARG
5. ERNTVVNGL
6. MGIGTALAA
7. VVGRTFKAA
8. KWTHDNASV
9. SMQFIGDKP
10. DEHRYKALV
11. AVGRAEASP
12. KYAADDKRG
13. LVVPFYSHS
14. ISRPGAFGG
15. FGRIAKEPH
16. VIYNQRN
17. RLWHMNFRR
18. WNIGQADSV
19. EAPGDWLAL

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