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

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 Algl, 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 (RHβH) and bind to long chain linear polysaccharides. AlgG was predicted to fold as a RHβH by the 3D-PSSM structural prediction program. RHβH 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

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and is ready for submission to the College of Graduate Studies.

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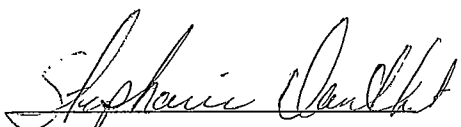
Signature 

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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.

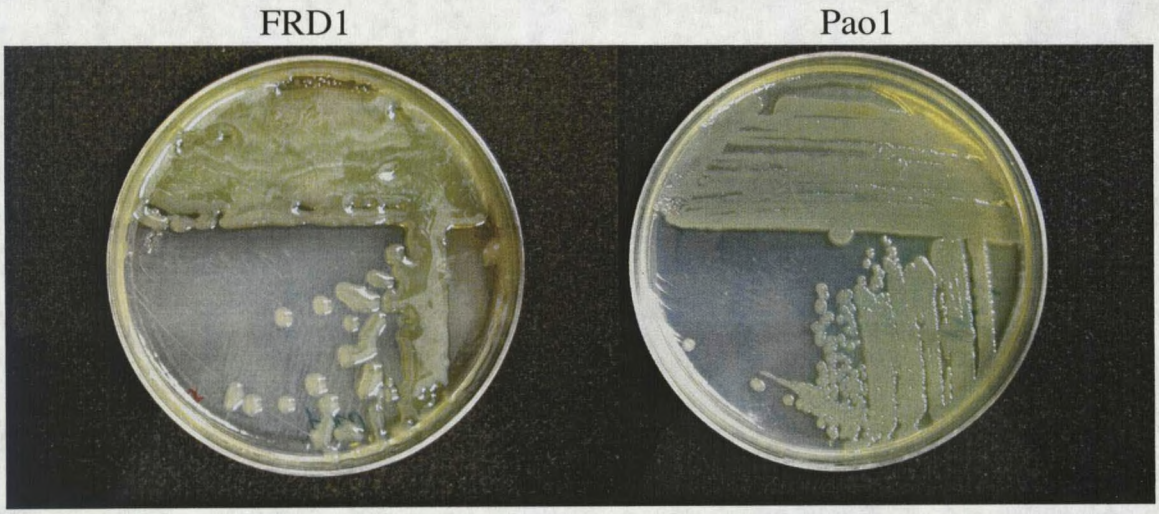

Figure 1.1. Mucoid CF isolate FRD1 (alginate overproducing strain) and non-mucoid burn wound isolate PAO1

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 (RHβH) and bind to long chain linear polysaccharides. AlgG was predicted to fold as a RHβH by the 3D-PSSM structural prediction program. RHβH 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 (ΔF508) (133, 303). This mutation occurs in 70% of patients and over 50% of patients are homozygous for this mutation. The frequency of ΔF508 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 ΔF508 (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 Na^+/ 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^-. Loss of this transport affects cytosolic pH (216). Inadequate acidification of cells leads to under sialylation of glycoproteins, namely gangliotetraosylcerimide, 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 TNFα and NO_3^- were lower in malnourished mice and that TNFα 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 TNFα (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 TNFα and NfκB (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 lumin, 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.