

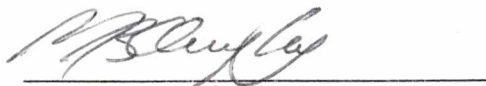
To the Graduate Council:

I am submitting herewith a thesis written by Charmaine S. Brickman entitled "Comparative Analysis of Flagellin Genes from Wild Type Fla⁺ and Non-motile Fla⁻ *Pseudomonas aeruginosa* Strains". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.



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We have read this thesis and
recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate
School

COMPARATIVE ANALYSIS OF FLAGELLIN GENES FROM
WILD TYPE FLA⁺ AND NON-MOTILE FLA⁻
P. AERUGINOSA STRAINS

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Charmaine Susan Brickman
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DEDICATION

This thesis is dedicated to my parents who taught me, not only the importance of education, but also the challenge of reaching and surpassing my goals.

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I will take with me many good memories of my time at the University of Tennessee. I am particularly grateful to my mentors Thomas Montie and Kimberly Kelly-Wintenberg for their endless encouragement, guidance, and endearing friendship. In addition to the required hard work, they made my graduate career a lot of fun. If I ever do continue with my education, I hope I am fortunate enough to work with such talented and generous people.

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ABSTRACT

The major structural protein of the *Pseudomonas aeruginosa* flagellum is the protein, flagellin. *Pseudomonas aeruginosa* strains express two types of flagellin proteins: b-type that have a molecular weight (M_r) of 53kDa and a more heterologous a-type ranging in M_r from 45 to 52kDa. The purpose of this research is two fold. It was of interest firstly, to make a comparative study of b-type genes and secondly, to test whether a PCR technique could be used to extend this comparison to Fla⁻ strains. To facilitate these objectives, a PCR technique was modified and employed to amplify most of the N- and C-terminus and all of the central variable region of the *Pseudomonas aeruginosa* flagellin genes. A PCR gene fragment size of 1.02kb corresponds to the flagellin a-type gene whereas an amplified gene fragment of 1.25kb corresponds to the flagellin b-type gene (Winstanley et al., 1996). To confirm and extend these correlations, we performed monoclonal antibody colony immunoblots on a number of motile Fla⁺ and non-motile Fla⁻ strains and isolated and sequenced amplified gene fragments of 4 Fla⁻ isolates and the wild type M2. The Fla⁻ phenotype exhibited absence of reaction with type specific monoclonal antibodies. These results suggest that little or no flagellin is expressed in these non-motile mutants or that a mutation in the *fliC* gene is located in the Mab reactive epitope in the flagellin protein.

The amino acid sequence results of strain M2 (Fla⁺, b-type) showed almost complete identity (99.7%) with the Fla⁺ b-type PA01, emphasizing the homologous nature of this group of flagellins. Variation in the b-type flagellins range from a single amino acid difference to a maximum of 5

amino acids in a Fla⁻ CF isolate. In analyzing the M2 protein sequence, we have found 3 potential sites for linear Mab epitopes in the b-type flagellin that are absent in the a-type flagellins.

The conserved nature of the b-type genes enabled one to rapidly detect mutations that might occur in the Fla⁻ gene sequences. Only one of the *fliC* gene sequences provided evidence which linked mutations in the structural gene to the Fla⁻ phenotype. A stop codon was found in the N-terminal region of CF isolate 448bb. Mutations at the regulatory level could explain the other flagellin deficiencies.

The a-type flagellin protein sequences exhibited less identity amongst *P. aeruginosa* strains. Results show evidence for protein sequence variation in the a-type flagellins. In strain PA103 (Fla⁻, a-type), there were structural differences which involved two distinct deletion regions and three additional substitution regions, in comparison to the wild type a-type strain PAK. These sequence variations corresponded precisely to those of another GenBank a-type *P. aeruginosa* CF isolate K701 (Fla⁺) to which the PA103 *fliC* gene shares a high degree of homology. Location of mutations in a-type flagellins of Fla⁻ strains is more difficult because of their decreased sequence homology compared to the b-types.

The PCR protocol described here, cannot only be used to identify and study the *fliC* gene and differentiate a- and b-type flagellins, but also it can be used to rapidly detect and type the *fliC* gene in Fla⁻ strains that do not react with monoclonal antibodies.

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CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa is characterized as a Gram negative, saprophytic rod that is widely distributed in soil, sewage, the mammalian gut and plants (Gilardi, 1985). It can utilize a variety of sugars, fatty acids, dicarboxylic and tricarboxylic acids, alcohols, glycols and aromatic compounds (Brock et al., 1984). This aerobic organism produces a characteristic blue-green pigment that is easily distinguished when the organism is grown on nutrient agar and can be an important identification factor in clinical isolation. *P. aeruginosa* is an important and highly virulent opportunistic pathogen and despite aggressive antimicrobial therapy, it is often associated with a high mortality rate in patients with burn wounds, organ transplants and cystic fibrosis (CF) (Doggett, 1979; Bodey et al., 1982,). *P. aeruginosa* possesses a single polar flagellum which is diagnostic and confers motility, chemotaxis and contributes to colonization and invasion in the early phase of infection in susceptible hosts (Montie et al., 1987).

Pathogenesis and Virulence

P. aeruginosa has a multifactorial pathogenesis due to the variety of toxins and enzymes that it produces (Liu, 1974). During the course of infection, several extracellular enzymes and toxins are expressed that contribute to the severity of infection. These virulence factors include

exotoxin A, exotoxin S, hemolysin, elastin, extracellular proteases, enterotoxin, leukocidic factor, and an alginate capsule (Lory and Tai, 1985; Nicas and Iglewski, 1986). Synthesis of these peptides is controlled at the transcriptional level in response to environmental conditions. For instance, alginate, exotoxin A, and hemolysin are regulated by osmolarity, availability of iron, and phosphate concentration, respectively (Bjorn et al., 1979; Nicas and Iglewski, 1986; DeVault et al., 1989). Thus, these and many other pathogens have developed a molecular regulatory system to regulate the expression of these virulence factors only when they are needed, such as in the establishment of infection (Nicas and Iglewski, 1986).

Both pili and flagella have been implicated in the pathogenesis of *P. aeruginosa* infection (McManus et al., 1980; Montie et al., 1982; Irvin et al., 1989). Previous data have indicated not only that flagella of *P. aeruginosa* contribute to virulence but also that flagellar protein serves as a protective antigen; thus the flagellum filaments can be a vaccine source for active or passive immunization (Holder et al., 1982; Drake and Montie, 1987; Landsperger et al., 1994).

The Flagellum Structure and Assembly

Possession of a single, polar flagellum, which imparts motility to the organism, is one of the stable, physiological traits used to characterize *Pseudomonas* species (Lanyi, 1970). Flagella consisting of flagellum filament, hook and basal body have been implicated as an invasive virulence factor in *P. aeruginosa* (Drake and Montie, 1988). In general,

flagella are anchored to the cell envelope via a basal body, which is connected to a hook structure and then extends into a long helical and flexible filament (Macnab, 1987). The hook is a curved rod structure that is composed of a single type of protein called hook protein (Iino, 1985). The hook provides attachment for the filament to the cell base and stability for the filament structure (Silverman and Simon, 1977; Iino, 1985). The second component of the flagellum, the basal body, is a multi-ring structure connected to the hook by a rod (Cohen-Bazire and London, 1967). The basal body is a key part of the flagellar motor allowing flagellar rotation. This rotation, either clockwise or counterclockwise, is powered by the flow of protons across the cytoplasmic membrane. (Jones and Aizawa, 1991). The flagellar filament is a helical thread typically 5-10 μ m in length with an outer diameter of 20nm (Jones and Aizawa, 1991). This filament is formed by the polymerization of a repeating flagellin protein (Macnab, 1987; Iino, 1985).

The bacterial flagellar filament can self-assemble in vitro from flagellin monomers. Assembly of flagellin in vivo occurs with the transport of synthesized flagellin monomers from the cytoplasm to the tips of the hooks where polymerization of flagellin begins filament (Iino, 1985; Jones and Aizawa, 1991). Accessory hook proteins are responsible for the conformational change that accompanies the monomers as they polymerize. In vitro and in vivo elongation of the flagellar filament occurs at the distal end of the filament (Iino, 1985; Jones and Aizawa, 1991).

Variation in flagellin size has been reported in *P. aeruginosa*, where two distinct flagellin protein types are found (Allison et al., 1985). Most strains of

P. aeruginosa express either type a or type b flagellin antigen; no antigen switching between type a and type b has been observed (Allison et al., 1985). These two types of flagellin antigen are distinguishable by molecular weight and reaction with type specific monoclonal and polyclonal antibodies (Landsperger et al., 1994; Drake and Montie, 1988; Montie and Anderson, 1988; Ansorg, 1978). Type b strains produce flagellin with a molecular weight of 53kDa; whereas type a strains were reported as more heterologous, producing flagellins with molecular weights ranging from 45kDa to 52kDa (Allison et al., 1985; Montie et al., 1982).

Analysis of flagellin sequence variation may give insight to possible functional roles for the unique central region, particularly in flagellum assembly, posttranslational modification and the mechanism of monoclonal antigenicity. Although the non-motile *P. aeruginosa* strains tested by Kelly-Wintenberg produced no detectable flagella, the nature of these mutations is not well characterized. Since flagellum synthesis and assembly is very complex, mutations which generate a Fla⁻ phenotype could involve a number of regulatory mutation not necessarily in the structural flagellin gene. It is known, however, that the conserved N- and C-terminal regions in both *Salmonella* and *Escherichia* are needed for assembly of flagella (Macnab, 1987). Kelly-Wintenberg (1991) restored motility to PA103 (Fla⁻) by introducing a cosmid carrying the flagellin b gene from PA01 (wild type) by tri-parental mating. The PA103 Fla⁺ conjugants produced flagellin protein that was not modified, as determined by amino acid analysis of the purified flagellin protein. There was an earlier suggestion that some of these non-motile strains were producing flagellin protein in either a monomeric form

or a polymerized form and secreting it in detectable amounts as determined by immunoblotting using polyclonal antibodies (Kelly-Wintenberg and Montie, 1989). Perhaps the location of the Fla⁻ mutation, if it is within the structural gene, may be in an area in which polymerization or assembly and secretion and not synthesis is affected.

Flagellum Genetics

The genetic make-up of *P. aeruginosa* flagella is thought to be similar to the genetic make-up of *E. coli* and *S. typhimurium* flagella (Iino et al., 1988). Flagella in prokaryotes are complex structures requiring the coordinate expression of over 50 genes, including *fliC* which codes for flagellin, the major repeating structural protein (Totten and Lory, 1990). There are a number of genes encoding products necessary to produce a functional flagellum including over 30 genes necessary for the synthesis and assembly (Tsuda and Iino, 1983b). In *P. aeruginosa*, genetic mapping has shown the flagellar genes to be in Regions I and II of the chromosome. Region I contains one motility and five flagella cistrons. Region II contains ten flagella and two chemotaxis genes (Tsuda and Iino, 1983a).

The *fliF* gene of *P. aeruginosa*, which is flanked by two other motility genes, *fliE* and *fliG*, is thought to be necessary for flagellar assembly and bacterial adherence to mucin (Arora et al., 1996). The *fliE*, *fliF* and *fliG* genes are transcribed in the same direction along the chromosome and perhaps part of a single operon (Arora et al., 1996). These genes code for three structural proteins, a basal body component, the membrane and

supramembrane (MS) ring, and the switch component of the flagellum structure, respectively (Macnab, 1992). In experiments performed by Arora et al. (1996), it was found that transposon inactivation of the *fliF* gene which resulted in mutants defective in both adherence and motility, could be restored by the addition of the *fliF* gene *in trans*.

Homologs of *P. aeruginosa* FliO are involved in the biosynthesis of flagella, but the function of FliO in this biosynthetic process remains unknown (Simpson et al., 1995). In *E. coli*, the *fliO* gene product is believed to be involved in the export of flagellar proteins (Malakooti et al., 1994). In experiments performed by Simpson et al. (1995), it was discovered that transposon insertions in the *fliO* gene created cells that were deficient in binding to eukaryotic cells or mucins and were non-motile. These researchers also found that the transposons in these mutants had a nonpolar effect, since the addition of the *P. aeruginosa* *fliO* gene *in trans* restored adherence to cells and mucins in the mutants along with complementing the motility defect. However, it is clear that flagellin itself is not the non-pilus adhesin in *fliC* or *fliA* and does not cause the loss of adherence.

Initial observations that σ^{54} (*rpoN*) is required for the expression of genes of nitrogen assimilation were followed by the discovery of a wide range of genes that are also transcribed by the σ^{54} -containing RNA polymerase such as bacterial adhesins and enzymes for amino acid biosynthesis (Gussin et al., 1986; Thony and Hennecke, 1989; Kustu et al., 1989; Jin et al., 1994). It has been shown that in *P. aeruginosa* the *rpoN* gene product is required for expression not only of pilin, but also of flagellin

(Ishimoto and Lory, 1989; Totten et al., 1990). Totten et al. (1990) found that in addition to glutamine synthetase, urease and pilin, flagellin is not expressed in the *rpoN* mutant.

In cloning and sequencing the α -type flagellin gene from *P. aeruginosa* strain PAK, Totten et al. (1990) found that sequences 5' of the flagellin structural gene contain several potential RpoN-dependent promoters as well as a sequence resembling the flagellin gene promoter sequence recognized by RpoF (σ^{28}) and not RpoN (σ^{54}), that was utilized by RNA polymerase in transcribing the flagellin gene. Thus the flagellin gene promoter is not of the RpoN (σ^{54}) class, but instead the transcription of flagellin is initiated at a sequence similar to the promoter consensus sequence recognized by the σ^{28} class of sigma factors (*fliA/rpoF*).

The dependence on RpoN for transcription of flagellin results from its direct or indirect control of *flaR* expression, or its control of a gene encoding an uncharacterized transcriptional activator of the flagellin gene (*fliC*) (Starnbach and Lory, 1992). Starnbach and Lory (1992) found that insertional inactivation of the *fliA* gene (σ^{28}) with a gentamicin gene cassette rendered *P. aeruginosa* non-motile and unable to express flagellin. In addition, these researchers also found that motility in non-motile *E. coli fliA* mutants could be restored by introducing a plasmid carrying the *fliA* gene from *P. aeruginosa*. Controlled expression of flagellin genes by means of transcriptional factors and alternative sigma factors may represent a mechanism for regulating flagellum synthesis in response to

environmental stimuli or, alternatively for coordinating the expression of the many flagellum structural genes (Totten et al., 1990).

Two genes in *Pseudomonas aeruginosa*, *fleS* and *fleR*, which are located upstream from the *fliE* gene, were found to act as a two-component protein system and show high homology to 1) histidine kinases which in other two-component systems have been shown to regulate proteins and to 2) other regulatory proteins that bind to upstream activating elements to enhance genes with σ^{54} promoters, respectively (Ritchings et al., 1995). Evidence supports the *fleSR* system as a regulator of both flagellar synthesis and adhesion to mucin (Ritchings et al., 1995).

The N- and C-terminal regions of the flagellin gene (*fliC*) are conserved among most *Pseudomonas* species. These regions also exhibit homology with the N- and C-terminal flagellin regions of other bacteria, including *E. coli* and *S. typhimurium*; similarity among these genera ranged from 53-75% (Totten and Lory, 1990). In these genes, it is the central variable region that appears to be unique and antigenic for specific flagellin types among strains and species. A similar case is reported for *P. aeruginosa* (Totten and Lory, 1990). Both types have been cloned and protein sequence analysis has shown that variation is most apparent in the central region of the flagellin protein (Totten and Lory, 1990; N. Baker, unpublished, GenBank, accession U54775).

Cystic Fibrosis and *P. aeruginosa*

The disease cystic fibrosis is characterized by deficient epithelial Cl^- transport (Quinton, 1983). Problems with water movement result from mutations in a single gene whose protein product is CFTR, a membrane-spanning Cl^- ion-selective channel (Riordan et al., 1989). The opening and closing of the ion gates is regulated by a complex and novel mechanism involving cycles of protein phosphorylation and dephosphorylation and of ATP hydrolysis (Hwang et al., 1994; Gadsby and Nairn, 1994; Gunderson and Kopito, 1995). A single mutation that deletes phenylalanine 508 (ΔF508) accounts for two-thirds of all diagnosed cystic fibrosis, although hundreds of other disease-causing mutations have already been identified (Tsui, 1992).

P. aeruginosa has now emerged as the major pathogen associated with CF lung pathology (Hoiby, 1977). The nature of the disordered biochemistry that predisposes the patient to colonization by *P. aeruginosa* is not known (Davis and Di Sant'Agnese, 1980). The complex interaction between the host and pathogen in cystic fibrosis makes it difficult to separate the disease caused by the host and disease caused by the microorganism. Both flagellation and piliation have been shown to play a role in the pathogenesis by tissue invasion and attachment to the respiratory mucosa, respectively (Drake and Montie, 1988; Woods et al., 1980). The alginate exopolysaccharide produced by *P. aeruginosa* interferes with a number of host defense mechanisms and is especially important in the respiratory tract colonization of cystic fibrosis patients (Govan, 1988). Although the pathology of CF lung disease remains unclear, no doubt exists that it is the principal cause of death

(Wood et al., 1976). High antibody titer to *P. aeruginosa* in patients with advanced pulmonary involvement prevents developing systemic infections but cannot protect the lungs from progressive tissue damage to which the high levels of antibody may even contribute (Schiotz and Hioby, 1975).

Evidence now exists which suggests that certain physiological characteristics of *P. aeruginosa* such as motility, associated with its virulence in acute infections may, in fact, undergo alterations which enhance the state of chronic infection in the CF lung (Hancock et al., 1983; Penketh et al., 1983; Thomassen et al., 1979). Many isolates from chronically colonized patients lack motility and expression of flagellin; in a study of 1,030 *P. aeruginosa* CF isolates, 39% were found to be (Fla⁻) non-motile (Mahenthiralingam et al., 1994). Deficiency in the flagellation of *P. aeruginosa* strains isolated from CF patients has been observed in patients in poor clinical condition and these non-motile isolates demonstrated many avirulent properties (Luzar and Montie, 1985; Luzar et al., 1985). The presence or absence of a flagellum has been shown to be a stable, strain-dependent characteristic among CF strains (Pugashetti et al., 1983; Luzar et al., 1985). It is then reasonable to assume that bacterial motility in CF pathology may play a significant role in initial invasiveness, but be of less importance in maintaining a persistent infection in the lung. It is hypothesized that the non-motile phenotype may provide *P. aeruginosa* a survival advantage in chronic CF infection by enabling it to resist non-specific phagocytosis and conserve energy (Mahenthiralingam et al., 1994).

Posttranslational Modification and Antigenicity

PCR amplification using primers designed by Winstanley et al. (1996) produce amplified products of 1.25kb (b-type) and 1.02kb (a-type). Since a-type genes produce heterologous proteins of various molecular weights, the role of posttranslational modification seems apparent. Such modifications have been observed with other bacterial flagellins (Joys and Kim, 1978; Logan et al., 1989; Wieland et al., 1985; Doig et al., 1996). Additionally Kelly-Wintenberg et al. (1993) reported the presence of phosphorylated tyrosines on flagella in *P. aeruginosa* strains. South et al. (1994) found a putative 42kDa membrane kinase responsible for this phosphorylation. Cha et al. 1996 discovered that there was additional serine/threonine kinase activity in wild type PA01 strains. In strain PA103 Fla⁻, these authors found that there was no functional kinase activity; perhaps linking posttranslational modification with flagellum assembly. It is hypothesized that the membrane kinase may be a dual specific kinase for threonine/serine and tyrosine.

There is an additional question of the biological significance of posttranslational modification which is not completely understood. Posttranslational phosphorylation via a two component system (histidine kinase/protein regulator), is now recognized in bacteria as playing a vital role in physiological regulation (Hurley et al., 1996; Robertson et al., 1989) as well as in global control mechanisms (Deretic et al., 1989; Roychoudhury et al., 1992; Stock et al., 1989). Other recent evidence suggests it plays a significant role in the antigenicity of distinct species/strain specific epitopes

(Doig et al., 1996); thus taking part in the protective immune response. Doig et al. (1996) recently found that removal of glycosyl groups on the flagellins of *Campylobacter* spp. eliminated reactivity with monoclonal antibodies. Recent research on *Azospirillum* and archeal flagellins has revealed a number of unusual traits often including posttranslational modification such as glycosylation (Jarrell et al., 1996). Perhaps investigations of the posttranslational modification of different *P. aeruginosa* strains in particular Fla⁻ strains, can give some insight into the relationship between posttranslational modification and monoclonal antigenicity.

Statement of Purpose

Pseudomonas aeruginosa is a clinically important bacterium as it is an opportunistic pathogen for immunocompromised patients. The presence of a single polar flagellum which confers both motility and chemotaxis for *P. aeruginosa* has been indicated as a virulence factor contributing to the establishment of infection in susceptible hosts. Our laboratory has been studying the flagellin protein for many years with the intention of developing a flagellar vaccine.

This research directed towards a characterization of the *fliC* genes from various Fla⁺ and Fla⁻ *P. aeruginosa* strains.

The research reported in this thesis had four main objectives:

- 1) Confirmation that the 1.02kb and 1.25kb PCR amplified flagellin gene fragments correspond to the flagellin a gene and flagellin b gene, respectively, as defined by flagellin type specific monoclonal antibodies.
- 2) Determination if the clinical isolate M2, which is virulent in the burned mouse model, gives the same b-type flagellin sequence as PA01; which would confirm that there is a high degree of homology among these flagellin types.
- 3) Apply the PCR technique to determine if the non-motile phenotype is a result of mutations in the structural gene, particularly the variable region.
- 4) Using the same approach, determine if the Fla⁻ CF isolates exhibit mutations in the structural flagellin genes since CF isolates show a higher rate of mutations in general.

This research focused in part on isolation and analysis of flagellin gene sequences from a variety of *P. aeruginosa* strains. A number of CF isolates with varying degrees of motility were included in this study along with wild type Fla⁺ strains M2 and PA01 and two other non-motile (Fla⁻) strains, PA103 and AK1153. Investigations involved PCR amplification of most of the N- and C-terminal conserved regions and the central variable region in both flagellin a and b type genes; the latter region confers antigenicity to specific strains. Once isolated, selected genes were cloned and sequenced and the protein sequence was predicted. Comparative analyses of the predicted

protein sequences were used to associate any type of mutations in the flagellin gene of non-motile strains with the Fla⁻ phenotype. Additionally, protein analyses of the b-type flagellins gave incite into potential linear monoclonal epitopes for these flagellins.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains, Media and pPT218 Plasmid

The bacterial strains involved in this research include: M2 (Fla⁺ invasive burn strain), PA01 (Fla⁺), PA103 (Fla⁻), AK1153 (Fla⁻), and Fla⁺ CF strains 572b, 415gg, 402c, 676e, and Fla⁻ CF strains 448bb and 96e. *P. aeruginosa* strain M2, obtained from I.A. Holder (Shriners Burn Institute, University of Cincinnati) was originally isolated from the small intestine of a CF 1 mouse (Holder et al., 1982). The wild type strain PA01 and the Fla⁻ mutant AK1153 were supplied by A. Kropenski (Drake and Montie, 1987). *P. aeruginosa* strain PA103, a non-motile laboratory strain, was originally described by Liu (1966). The CF isolate K701 was a gift from C. Winstanley (University of Liverpool, UK). All other *P. aeruginosa* cystic fibrosis isolates were isolated from sputum (Thomassen et al., 1979). All strains were routinely cultured at 37°C in Luria-Bertani (LB) Broth (DIFCO Laboratories, Detroit, MI), 10% pancreatic digest of casein, 0.5% yeast extract, 0.5% NaCl. Bacterial cells used in the colony blots were grown on Luria agar, prepared with LB Broth and 1.6% agar. For the motility assays, the bacteria were grown on motility medium containing: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.3% agar. For the growth assays, the bacteria were grown in Mineral Salts Medium (MSM) containing: 4×10^{-2} M K₂HPO₄, 2.2×10^{-2} M KH₂PO₄, 7.5×10^{-3} M (NH₄)₂SO₄, 2.0×10^{-4} M MgSO₄, 6×10^{-6} M FeCl₃ with 4% sodium

succinate. The plasmid pPT218, is the pUC18 plasmid vector containing the cloned a-type flagellin gene (1.7kb fragment), from *P. aeruginosa* strain PAK (Totten and Lory, 1990). *E. coli* DH5 α cells harboring the pPT218 plasmid were a gift from S. Lory (University of Washington, Seattle, WA).

Electron Microscope Analysis

Electron microscopy was used to determine the flagellar phenotype of the various motile and non-motile strains. The strains analyzed were streaked onto plates containing LB agar and incubated overnight at 37°C. Following incubation, colonies were suspended in phosphate buffered saline (PBS, Sambrook et al., 1989). The bacterial suspension was applied to copper grids and negatively stained with 0.5% phosphotungstic acid (pH 7.0) and magnified 26,000-40,000X using a H-600 Hitachi transmission electron microscope.

Motility Assays

Motility swarm assays, based upon the method described by Adler (1966), were used to test the motility of the strains used in this research. Bacteria from overnight LB streak plates were stab-inoculated into the center of a 150mm petri plate containing 25ml of motility medium. The plates were incubated uninverted at 37°C and colony spreading was measured (in mm) at 6 and 24 hour periods. Plates were also checked for characteristic growth rings. Each assay was done in triplicate with the recorded migration

measurement values reflecting the average of the measurements for each strain.

Colony Blots

Colony blots were performed using either polyclonal or monoclonal antibodies to each flagellin type to determine if the type of flagellin protein produced by each strain correlated with the appropriate size of the PCR amplified gene product. Additionally, monoclonal colony blots were performed to test the non-motile strains for the production and secretion of flagellin protein. Colonies of each strain were stab inoculated 10 times onto a 150mm petri plate containing LB agar and incubated at 37°C for 16-20 hours. The *P. aeruginosa* colonies were then blotted onto a nitrocellulose membrane (BioRad Laboratories, Hercules, CA) and a colony blotting protocol was used to test colony antigenicity (Kelly-Wintenberg, 1991). Blotted membranes were incubated at 37°C for 2 hours in a PBS-1% gelatin solution (Sambrook et al., 1989) containing a 1:1000 dilution of either monoclonal or polyclonal antiserum raised against monomeric a- or b-type flagellin protein (Allison et al., 1985). After a two-hour incubation, the membrane disks were washed with PBS and reincubated at 37°C in a PBS-1% gelatin solution containing either goat anti-human IgG (monoclonal) or goat anti-rabbit IgG (polyclonal) horseradish peroxidase conjugate (BioRad). The disks were developed using BioRad HRP A development solution containing 4-chloro-1-naphthol and hydrogen peroxide (BioRad). The amount of antigen was qualitatively associated with the blue color intensity of the reaction. In experiments involving Fla⁻ strains and monoclonal

antibodies, the cells were chloroform lysed after blotting and prior to hybridization to test for any unsecreted flagellin protein. All of the samples were compared to both positive (wild type same flagellin type cells) and negative controls (wild type opposite flagellin type cells). Strain PA01 was used as a representative b-type flagellin and strain 5939 for the a-type flagellin.

Growth on Mineral Salts Medium with and without 0.2% L-Glutamine

To test the Fla⁻ strains for mutations in the *rpoN* gene (nitrogen regulator, σ^{54}), the Fla⁻ strains and Fla⁺ control strains PA01 and M2 were streaked for isolation on MSM (Mineral Salts Medium) both with and without the addition of 0.2% L-glutamine (Sigma Chemical Co., St. Louis, MO) as a nitrogen source. Fla⁺ strains PA01 and M2 were included as positive controls.

Polymerase Chain Reaction

As mentioned earlier, the primers developed by Winstanley et al. (1996) have been used to amplify most of the N- and C-terminal regions and all of the central variable regions of both the a- and b-type flagellin genes in *P. aeruginosa* extracts. Oligonucleotide primers GGCAGCTGGTTNGCCTG (CW45) specific for the C-terminus and GGCCTGCAGATCNCCAA (CW46) for N-terminus, were obtained from GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD). Target genomic DNA was prepared by removing cells from an overnight LB agar plate, suspending the cells in 50 μ l of 5mM EDTA

and boiling them for 5-10 minutes. The bacterial suspension was then centrifuged in a microcentrifuge for 2-3 minutes at 14,000 rpms. Amplifications were carried out in 50- μ l volumes containing 9 μ M of each primers, 2.5 units of Taq polymerase (Promega, Madison, WI), 1X Taq polymerase buffer, 100 μ M nucleotides (dATP, dCTP, dGTP, dTTP), 4mM MgCl₂, and 2 μ l of target DNA (see above for preparation). Amplifications were carried out using a GeneAmp PCR System 2400 Thermocycler (Perkin Elmer, Norwalk, CT) for 30 cycles consisting of 94°C (40s), 50°C (1 min) and 72°C (2 min) and an additional extension time of 10 minutes at 72°C after the 30 cycles were complete. After amplification, 5- μ l samples were subjected to electrophoresis (Sambrook et al., 1989) in a 1% agarose (Sigma) gel and stained with ethidium bromide (EtBr, 0.5 μ g/ml) to confirm the presence and size of amplified products.

In addition to the PCR protocol described above, a second protocol was designed in an attempt to amplify the entire flagellin gene from *P. aeruginosa*. This would allow the N- and C-terminal regions not amplified with the primers of Winstanley et al. (1996), to be cloned and sequenced. The same PCR conditions were used with this second protocol. However, new primers, specific for the non-coding regions upstream and downstream from the flagellin gene, were designed. The PAK flagellin gene in pPT218 was used as a template for these primers (Totten and Lory, 1990). In 1990, Totten and Lory sequenced the entire PAK gene in pPT218, including some upstream sequence containing potential promoter sites.

The first step in this project involved determining the downstream sequence in pPT218. This was accomplished by designing a 5' primer (CSN-10: CGGTGAGAACGTATCGGCTGG) to the C-terminal coding region and performing an automated sequencing run (see DNA Sequencing). After the downstream sequence was obtained, primers were designed for the N-terminal (CSN-1: CCTTGCGCGAACCCCTCCGACG) and C-terminal (CSC-2: CACGACGGATGCTCTGGG) non-coding regions. Using these primers and the same PCR conditions described earlier, DNA from PAK and other *P. aeruginosa* strains was amplified.

Isolation of Plasmid DNA

The pPT218 plasmid and flagellin clones in pGEM-T and pGEM-T easy vectors (Promega) were isolated using a alkaline lysis mini plasmid preparation protocol (Sambrook et al., 1989). A modification was added to this protocol involving the addition of 5M LiCl (Sigma) following the Solution III step. The first step involved centrifugation (high speed) of 3ml of an overnight culture of bacterial cells (grown with the appropriate antibiotic) in a 1.5ml microcentrifuge tube. The cells were resuspended in 100µl of Solution I (50mM glucose, 25mM Tris, 10mM EDTA) and then 200µl of Solution II (1% SDS, 0.2N NaOH) was added followed by immediate inversion the tube to facilitate cell lysis. Next 200µl of Solution III (7.5 M ammonium acetate) was added to the mixture again followed by inversion of the tube. Finally, 500µl of 5M LiCl was added before the tubes were centrifuged at high speed for 10 minutes. After centrifugation, 1 ml of the supernatant was removed and added to a microcentrifuge tube containing

600µl of isopropanol. Again the tube was centrifuged at top speed for 10 minutes. The supernatant was decanted and the DNA pellet was rinsed with 70% EtOH, dried and resuspended in dH₂O containing .2µg/µl RNase A (5' Prime-3' Prime, Boulder, CO).

Southern Hybridization

Southern hybridizations were used initially to confirm that the PCR products were flagellin gene fragments. PCR amplified DNA (1µg per sample) was resolved by electrophoresis (1% agarose in 1X TAE buffer) transferred to nylon membrane (Biotrans, ICN, Irvine, CA) in a 0.4N NaOH solution using a vacuum blotter. The DNA was cross-linked to the filters using short-wave UV light. The cloned a-type flagellin gene (1.7kb) in pPT218 (Totten and Lory, 1990) was PCR amplified from the plasmid using CW45 and CW46 (Winstanley et al., 1996) to give a 1.02kb product which was biotinylated (Nick Translation Kit, GIBCO BRL), purified (NucTrap Probe Purification Columns, Stratagene, LaJolla, CA) and used as a probe for the other flagellin PCR products. Prehybridization was carried out at 65°C for 2-4 hours in 6X SSC, 5X Denhardt's reagent, 0.5% SDS and 0.5mg/ml denatured and sheared salmon sperm DNA. Hybridization was carried out overnight at 65°C in the same solution with the addition of (0.1-0.5 µg/ml) denatured pPT218 probe. Filters were washed and the results visualized according to the BluGene Nonradioactive Nucleic Acid Detection Kit (GIBCO BRL).

Cloning and Expression of the Flagellin Genes in *E. coli* Cells

Using the pGEM-T or pGEM-T easy Vector Systems cloning kits (Promega), the PCR amplified flagellin gene fragments were ligated into plasmid vectors (pGEM-T or pGEM-T easy) and transformed into *E. coli* JM109 cells using a blue/white screening technique to find the transformants containing inserts. The cloning vectors pGEM-T/pGEM-T easy contain T7 and SP6 RNA Polymerase promoters which flank a multiple cloning region within the α -peptide coding region (*lacZ* gene) of the enzyme β -galactosidase. Insertional inactivation of the α -peptide allows recombinant clones to be identified by color screening on indicator plates. Prior to ligation, the PCR amplified DNA fragments were purified using the Wizard PCR Preps (Promega). After transformation, white colonies grown on LB agar with ampicillin/IPTG/X-Gal, represented transformants containing the cloned DNA insert. Plasmid DNA was isolated from selected white colonies and then digested with either restriction enzymes *Apa*I and *Sac*I (pGEM-T) or *Eco*RI (pGEM-T easy), flanking the insert site. The digested DNA was resolved by gel electrophoresis to confirm the presence of the flagellin gene inserts.

Preparation of Clones for Sequencing

The plasmid clones were reisolated using the 5M LiCl plasmid prep protocol (see Isolation of Plasmid DNA) followed by a purification procedure (Applied Biosystems, Inc.) which involved a chloroform extraction of the

DNA followed by precipitation using a 13% PEG₈₀₀₀ (polyethylene glycol) solution (Sigma) and 0.5M NaCl.

DNA Sequencing

The DNA from several of these flagellin clones was sent to the DNA Sequencing Laboratory (University of Tennessee, Knoxville) for sequence analysis. The clones were sequenced using a sequencing reaction kit by Perkin-Elmer which employs a cycle sequencing protocol. This protocol involves repeated primer extension reactions. Vertical polyacrylamide gels are used to resolve the products of the sequencing reactions. The ABI 373A sequencer employs a fine laser beam to excite the dye-labeled sequencing products as they migrate through the gel and past the laser. A photomultiplier tube detects and interprets the fluorescence reflected by each DNA band in each lane as one of four different colored lights: red, yellow, green, or blue. The peaks of fluorescence detected in each gel lane are digitized and the data is collected by a Macintosh computer.

Once a segment of DNA was sequenced, additional internal primers were designed and the process was repeated. From each sequencing reaction, about 400-500 nucleotides could be determined with high fidelity. However, the internal primers were chosen so that they produced overlapping fragments that could confirm the DNA sequence in ambiguous areas near the end of each sequencing run. Six reactions were necessary to sequence the 1.25kb b-type flagellin gene fragments and four were needed for the 1.02kb a-type flagellin gene fragments.

Computer Analysis of Flagellin DNA and Protein Sequences

After sequencing was completed, the flagellin DNA sequences were compared to the respective flagellin gene types (a or b) of other *P. aeruginosa* strains published in GenBank and other databases using the BLAST (tblastx) algorithm (Altschul et al., 1990). This algorithm of the National Center for Biotechnology Information (NCBI), takes a nucleotide sequence query, translates it in all six reading frames and then compares it to the six reading frame translations of a nucleotide sequence database. Based on information from the tblastx search, an appropriate protein sequence was determined for each strain. Using the computer program DNA* (Clustal alignment method), protein sequence comparisons were performed between different types of flagellins as well as between motile Fla⁺ and non-motile Fla⁻ strains. DNA* was also used for other protein analyses such as secondary structure predictions and hydrophilicity plots.

CHAPTER III

RESULTS

Electron Microscope Analysis and Motility Assays

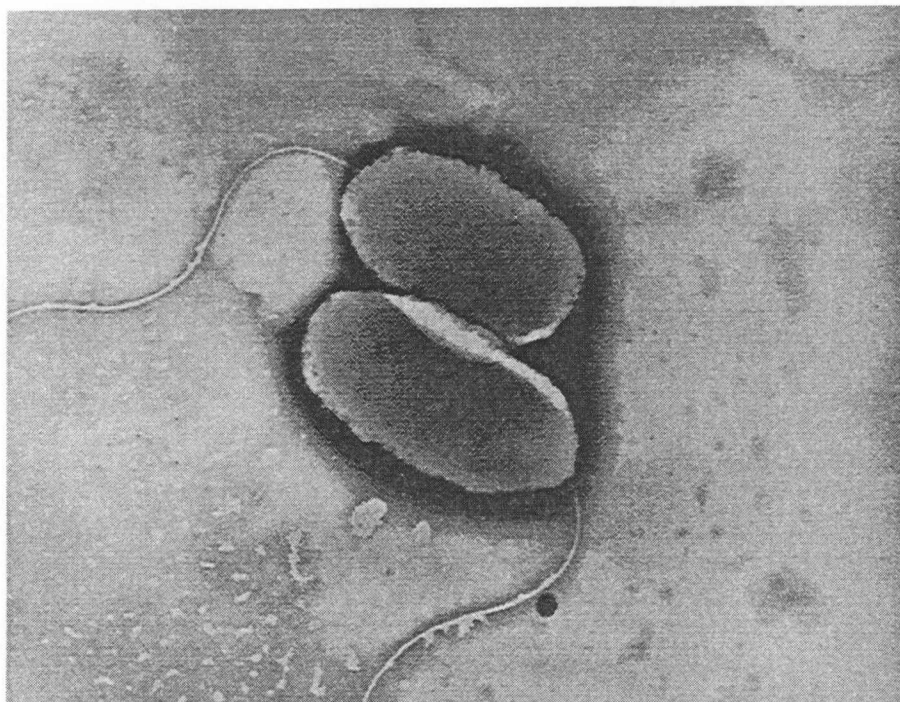
Listed in Table 1 are the results of the motility assays and the electron microscope (EM) findings for all the strains studied. The EM analysis confirmed the presence or absence of flagellum as previously determined by Luzar (1984). These results correlated with Luzar's, noting strains PA103, AK1153, CF96e, and CF448bb as the only Fla⁻ strains. Figures 1A and 1B show TEM (transmission electron microscope) pictures of representative Fla⁺ strain M2 and Fla⁻ strain 448bb, respectively. Motility assays were performed to confirm the flagellar phenotype determined by the EM analysis of the various strains, by measuring cell migration (in mm) in motility agar. A migration of 5mm or less is considered non-motile. Additionally, if a bacterium possesses a functional flagellum and is chemotactic, characteristic growth rings are visible on the plate after a period of time. Table 1 shows the Fla⁻ strains as having a migration at 24 hours of 4mm or less, confirming their status as non-motile strains. In addition, the Fla⁻ strains did not produce the growth rings characteristic for motile strains. Also notable, is the intermediate motility (significantly less than wild type) of three of the Fla⁺ CF isolates (402c, 415gg and 676e). Transmission electron micrographs of these isolates showed that not all the cells appear to be flagellated. During chronic infection, a high number of Fla⁻ isolates are observed which result

Table 1: Motility Assays and Flagellar Phenotype of Wild Type Fla⁺ and Non-motile Fla⁻ *P. aeruginosa* Strains.

STRAIN	FLAGELLUM ^a	MOTILITY IN AGAR ^b	
		6 HOUR	24 HOUR
M2 Control	+	8	39
PA01 Control	+	9	35
PA103	-	2	3
AK1153	-	2	4
CF572b	+	12	39
CF415gg	+	3	9
CF402c	+	4	21
CF676e	+	1	14
CF96e	-	2	2
CF448bb	-	2	3

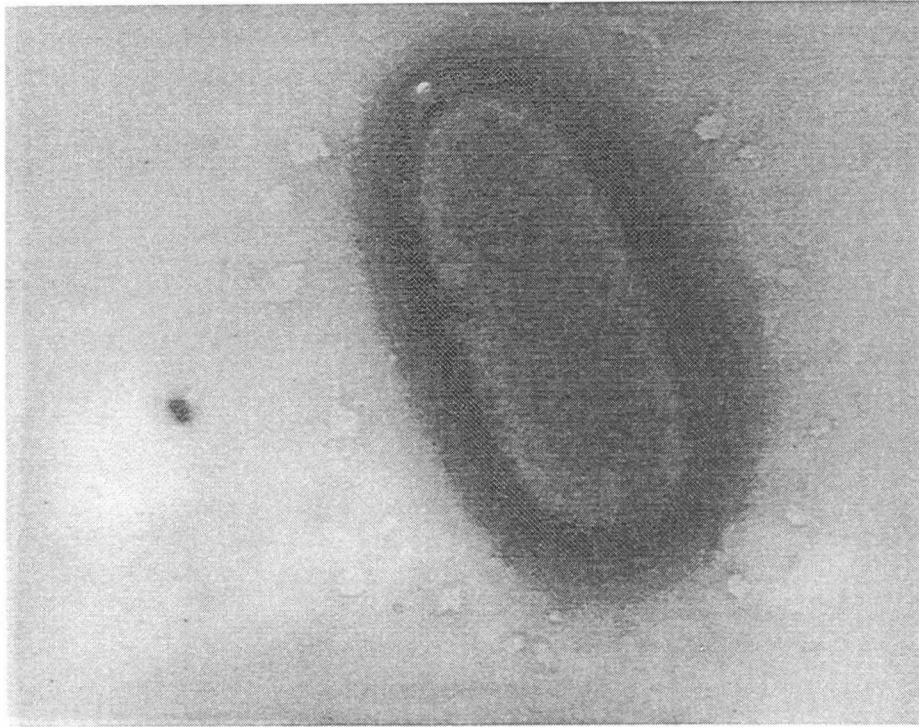
^a Presence of flagellum as determined by electron microscopy.

^b Motility in agar was measured in millimeters.



A) Transmission Electron Micrograph of Motile *P. aeruginosa* Strain M2. Cells were taken from an overnight LB plate, suspended in PBS buffer and stained with 0.5% phosphotungstic acid. Magnification: 26,000X.

Figure 1: Transmission Electron Micrographs of Motile and Non-motile *P. aeruginosa* Strains.



B) Transmission Electron Micrograph of Non-motile *P. aeruginosa* Strain AK1153. Cells were taken from an overnight LB plate, suspended in PBS buffer and stained with 0.5% phosphotungstic acid. Magnification: 40,000X.

Figure 1: Continued

from increased mutations due to the unusual environment in the CF lung (Luzar et al., 1985; Mahenthiralingam et al., 1994). Perhaps there is some type of mutation in the functioning of the flagellum in these isolates.

During routine culturing, it was noted that strain PA103 has an unusual colony morphology when grown on LB agar. This strain appears to spread on the agar in comparison to the other *Fla⁻* strains which exhibit a more compact and restricted growth. The colony morphology of PA103 also differs from motile *P. aeruginosa* strains which produce a smoother, glossier colony growth. The polar pili in *P. aeruginosa* are responsible for a novel mode of flagellum independent surface translocation known as twitching motility (Bradley, 1980; Henrichsen, 1983). Very recently, PA103 has been reported to exhibit this twitching motility when grown on certain solid media covered with polyethylene (Whitchurch et al., 1997). The biosynthesis of type 4 pili, responsible for this type of motility, is regulated by a number of genes including the group of genes *pilGHIJK*, (homologous to the chemotaxis genes of enteric bacteria) as well as the alternate sigma factor RpoN and the transcriptional activator PilR (Ishimoto and Lory, 1989; Ishimoto and Lory, 1992; Darzins, 1994). Our laboratory has previously shown that the production of polar pili can be induced by growing specific strains on media containing deoxycholate (unpublished data). The transmission electron micrograph of PA103 in Figure 2 shows the polar pili responsible for this twitching motility (Bradley, 1980; Henrichsen, 1983). Interestingly, EM analysis of the other *Fla⁻* strains show no evidence for pilin production on any of these strains (Figure 1B). Perhaps a regulatory mutation is responsible for the uninduced and constitutive pili production in PA103.



Figure 2: Transmission Electron Micrograph of Non-motile *P. aeruginosa* Strain PA103. Cells were taken from an overnight LB plate, suspended in PBS buffer and stained with 0.5% phosphotungstic acid. Magnification: 26,000X.

Colony Blotting

In Table 2, two Fla⁺ (M2 and PA01) and two Fla⁻ (AK1153 and PA103) strains were tested for their reactions with monoclonal and polyclonal antibodies. PCR flagellin gene sizes were also included in Table 2, as a reference to the flagellin type of each strain. The interpretation of the colony blot reactions is illustrated in Figure 3. The amount of antigen was qualitatively associated with the blue color intensity of the reaction and the reaction was given one of four designation +, ++, +++ and no plus (no reaction). Monoclonal and polyclonal reactions of M2 and PA01 were in accordance with the b-type flagellin designation; with a negligible a-type reaction. The weak reaction, one + sign, associated with the polyclonal a-type reaction for M2 can probably be attributed to non-specific cross-reactivity commonly seen with polyclonal antibodies. Colony blots were performed with lysed and unlysed cells to confirm that the lack of monoclonal antibody (Mab) reaction was not due to little or no secretion of flagellin protein, but to the lack of expressed flagellin. Since Fla⁻ strains do not possess an assembled flagellum, it is not likely that a significant amount of flagellin would be secreted even if flagellin expression were at wild type levels. Neither Fla⁻ strain in Table 2 reacted with the monoclonal antibodies indicating that very little or no flagellin protein was expressed. The weak polyclonal reaction with these Fla⁻ strains can probably be attributed to either non-specific cross-reactivity or to the presence of a small amount of expressed flagellin. There is the possibility that some Fla⁻ strains are expressing protein, but it is folding differently, since it is not polymerizing, so that the Mab epitope site is not exposed. Immunoblotting with concentrated extracellular flagellin

Table 2: Colony Blot and PCR Fragment Size Analysis of Wild Type Fla⁺ and Non-motile Fla⁻ *P. aeruginosa* Strains.

STRAIN	REACTION WITH MAB ^a	REACTION WITH POLY-CLONAL AB	PCR FLAGELLIN GENE SIZE ^b (kb)
M2 (Fla ⁺)	b-type +++ a-type -	b-type +++ a-type +	1.25
PA01 (Fla ⁺)	b-type +++ a-type -	b-type +++ a-type -	1.25
AK1153 (Fla ⁻)	none	b-type ++ a-type +	1.25
PA103 (Fla ⁻)	none	b-type + a-type -	1.02

a Colony blots were performed on the strains using human anti a-type and anti b-type flagellin monoclonal antibodies. The colonies of the Fla⁻ strains were chloroform lysed prior to the blotting procedure.

b The flagellin genes were amplified by PCR using the primers CW45 and CW46 designed by Winstanley et al. (1996).

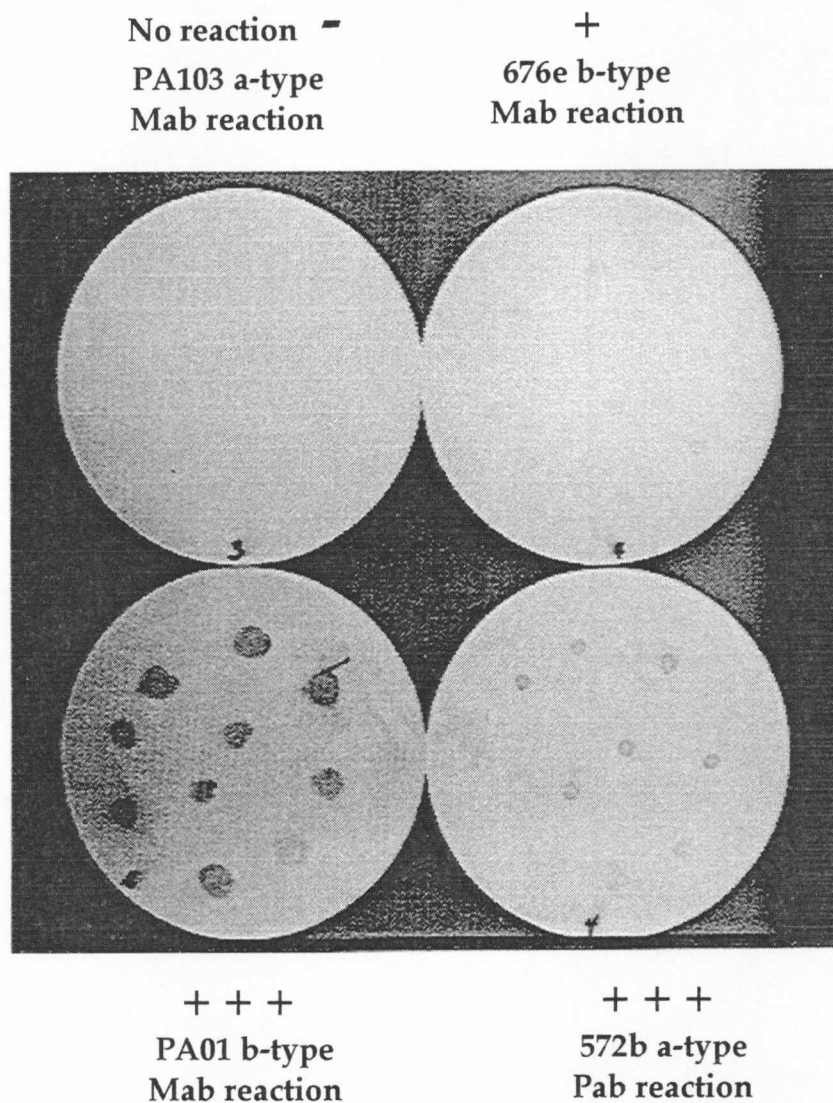


Figure 3: Interpretation of Colony Blot Reactions. The amount of antigen was qualitatively associated with the blue color intensity of the reaction. The reactions were rated from a negative sign for no reaction to a designation of "+++" for a strong wild type reaction.

protein may help to clarify this discrepancy. Due to the absence of monoclonal reactivity and lack of polyclonal specificity, colony blotting cannot serve as a reliable confirmation for flagellin typing of non-motile strains, although it could be used to differentiate these strains from Fla⁺ strains. Similar tests were performed on the Fla⁺ and Fla⁻ CF isolates with the results listed in Table 3. Again the monoclonal antibody reactions correlated with the appropriate flagellin type PCR gene fragment size for the motile Fla⁺ isolates. All the Fla⁺ CF isolates except CF572b, produced a PCR product of 1.25kb and had b-type monoclonal reactions. However, the CF isolates described as exhibiting intermediate motility appeared to have a weaker Mab reaction than that of the wild type CF572b (also refer to M2 and PA01 from Table 2). Electron microscope evaluations with several of these isolates (data not shown) showed that they contained a number of unflagellated cells and therefore the lower concentration of total flagellin in the reaction would yield a weaker reaction.

Growth on Mineral Salts Medium with and without 0.2% L-Glutamine

Since *rpoN* (σ^{54}) mutants are defective in the expression of glutamine synthetase and thus unable to use arginine, glutamate, histidine or proline as a sole nitrogen source (Totten et al., 1990), glutamine must be supplied in mineral salts medium in order for *rpoN* mutants to grow. Therefore, if a bacterial strain that does not grow on MSM without glutamine, can grow on MSM supplemented with glutamine, then it can be assumed that the supplemented medium is rescuing an *rpoN* mutant. All the strains tested (PA01 and M2 as controls and the four non-motile strains) grew on the

Table 3: Colony Blot and PCR Fragment Size Analysis of Fla⁺ and Fla⁻ *P. aeruginosa* Cystic Fibrosis Isolates.

STRAIN	REACTION WITH MAB ^a	REACTION WITH POLY- CLONAL AB	PCR FLAGELLIN GENE SIZE ^b (kb)
572b (Fla ⁺)	b-type - a-type +++	b-type + a-type ++	1.02
96e (Fla ⁻)	none	b-type + a-type -	1.25
448bb (Fla ⁻)	none	b-type ++ a-type +	1.25
415gg (Fla ⁺)	b-type ++ a-type -	b-type ++ a-type -	1.25
402c (Fla ⁺)	b-type ++ a-type -	b-type ++ a-type -	1.25
676e (Fla ⁺)	b-type + a-type -	b-type + a-type -	1.25

a Colony blots were performed on the strains using human anti a-type and anti b-type flagellin monoclonal antibodies. The colonies of the Fla⁻ strains were chloroform lysed prior to the blotting procedure.

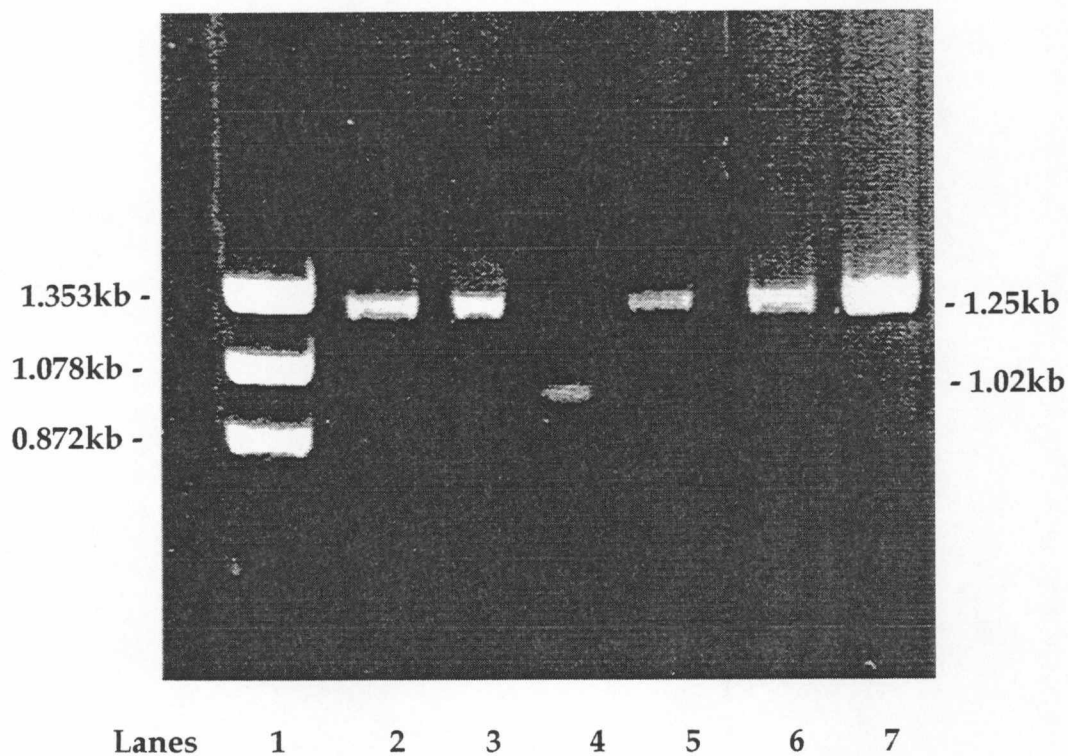
b The flagellin genes were amplified by PCR using the primers CW45 and CW46 designed by Winstanley et al. (1996).

plates both with and without the addition of 0.2% L-glutamine. These positive growth results suggested that a mutation in the *rpoN* regulatory gene is not responsible for the Fla⁻ phenotype of these non-motile strains.

Many *Pseudomonas* species are known for their production of a blue green pigment, usually visible when the organism has been streaked onto an agar plate. A number of the strains tested (PA01, M2, AK1153, CF96e), produced this pigment when streaked onto the MSM agar plates. However, it was observed that the particular color of the pigment was different between the 2 types of MSM plates. The media containing glutamine produced a brighter, more fluorescent green whereas the MSM plates without glutamine produced the typical blue green pigment.

Polymerase Chain Reaction

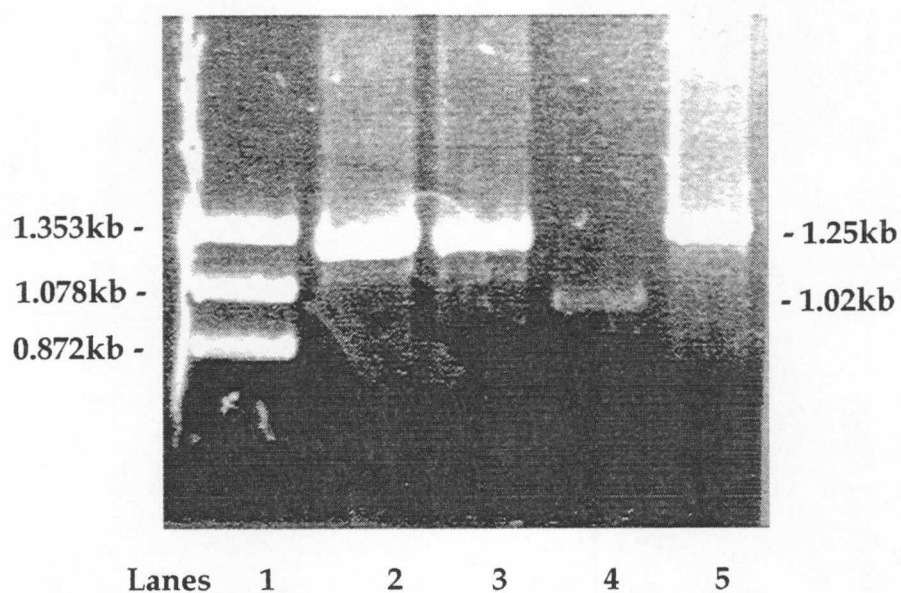
PCR amplification of the flagellin genes produced a 1.02kb fragment corresponding to the a-type flagellin gene and a 1.25kb fragment corresponding to the b-type flagellin gene. Agarose gel electrophoresis analyses of these PCR products are represented in Figures 4A and 4B. ϕ X174 174/*Hae*III DNA markers (Promega) were used as a molecular size reference. Eight of the ten strains tested produced 1.25kb PCR products (b-type) and the remaining two strains (PA103 and CF572b) produced 1.02kb products (a-type). In Figure 4A, electrophoresis data is shown for amplified flagellin genes from the motile strains and in Figure 4B similar data is shown for the non-motile strains. PCR amplification of PA103 gives a gene size of 1.02kb which



A) Agarose Gel Electrophoresis of PCR Amplified Flagellin Genes from Motile *P. aeruginosa* Strains.

In lanes: 1) Φ X / *Hae* III DNA markers, 2) PA01, 3) M2, 4) CF572b, 5) CF415gg, 6) CF402c, 7) CF676e.

Figure 4: Agarose Gel Electrophoresis of PCR Amplified Flagellin Genes from Motile and Non-motile *P. aeruginosa* Strains.



B) Agarose Gel Electrophoresis of PCR Amplified Flagellin Genes from Non-motile *P. aeruginosa* Strains.

In lanes: 1) ϕ X / *Hae* III DNA markers, 2) CF448bb, 3) CF96e, 4) PA103, 5) AK1153.

Figure 4: Continued

corresponds to the flagellin a gene. This is an interesting finding since PA103 was previously suspected to produce a b-type flagellin protein as shown by polyclonal immunoblots performed by Kelly-Wintenberg (1991).

The second PCR protocol was not as successful as the first. The PAK flagellin gene fragment generated with CSN-1 and CSC-2 is estimated at about 1.4kb. However, these primers failed to amplify the flagellin gene in strain M2 or any other wild type b-type strain. The most likely explanation of this result is that the downstream non-coding DNA, is not well conserved between a and b-type strains.

Southern Hybridizations

Southern hybridization experiments using Totten and Lory's a gene probe from pPT218, confirmed that the PCR products, generated using primers CW45 and CW46, were indeed *P. aeruginosa* flagellin genes. The a-type flagellin gene fragments migrated to 1.02kb and the b-type flagellin gene fragments migrated to 1.25kb (see Figures 4A and 4B). Results for the cloned non-motile strains are represented in Figure 5. Genomic *E. coli* DNA was used as a negative control. In Figure 5, the a-gene probe reacted with the all four flagellin gene fragments.

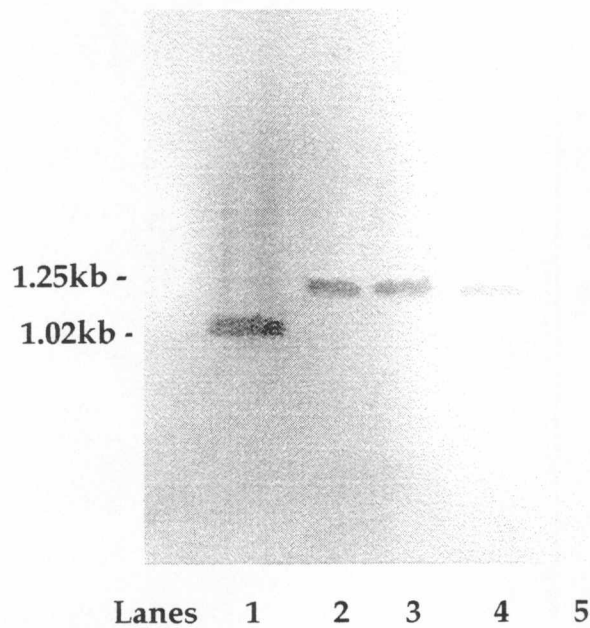


Figure 5: Southern Hybridization of pPT218 to Amplified *P. aeruginosa* Flagellin Genes. In lanes: 1) PA103, 2) AK1153, 3) CF96e, 4) CF448b, and 5) *E. coli* DNA as a negative control.

Flagellin DNA and Protein Sequence Analysis

The flagellin genes cloned and sequenced for this project are listed in Table 4. After sequencing, the nucleotide sequence for each strain was analyzed using the tblastx algorithm of the BLAST DNA search computer program. First, the DNA was translated into protein using the computer program DNA*. Various flagellin sequences were aligned so that protein sequence comparisons could be made between a and b-type flagellin from both motile and non-motile strains to elucidate the role that primary protein structure plays in expression of flagellin and assembly of a functional flagellum.

The b-type flagellin genes code for proteins that are 488 amino acids in length, whereas the a-type flagellin genes code for 391 amino acids. The PCR amplified gene fragments cloned represent 90% of the flagellin protein. The missing regions include 46 N-terminal amino acids and 8 C-terminal amino acids. The NCBI database search revealed the sequenced flagellin genes to have the highest homology with other a and b-type flagellins from *P. aeruginosa*.

In Figure 6, the protein sequence of strain M2 was compared to the sequences of strains PA01 and PAK. M2 and PA01 are wild type strains which produce b-type flagellins; whereas the wild type strain PAK produces a-types flagellins. Within the 434 amino acid sequence of the amplified M2 flagellin fragment, there is only a single conservative amino acid difference. The alanine at amino acid 377 in PA01, is changed to a glycine in M2. This

Table 4: List of Cloned *P. aeruginosa* Flagellin Genes.

PLASMID CLONE	STRAIN
pBT7	M2
pBT1	PA103
pBT5	CF448bb
pBT44	CF96e
pBT8	AK1153

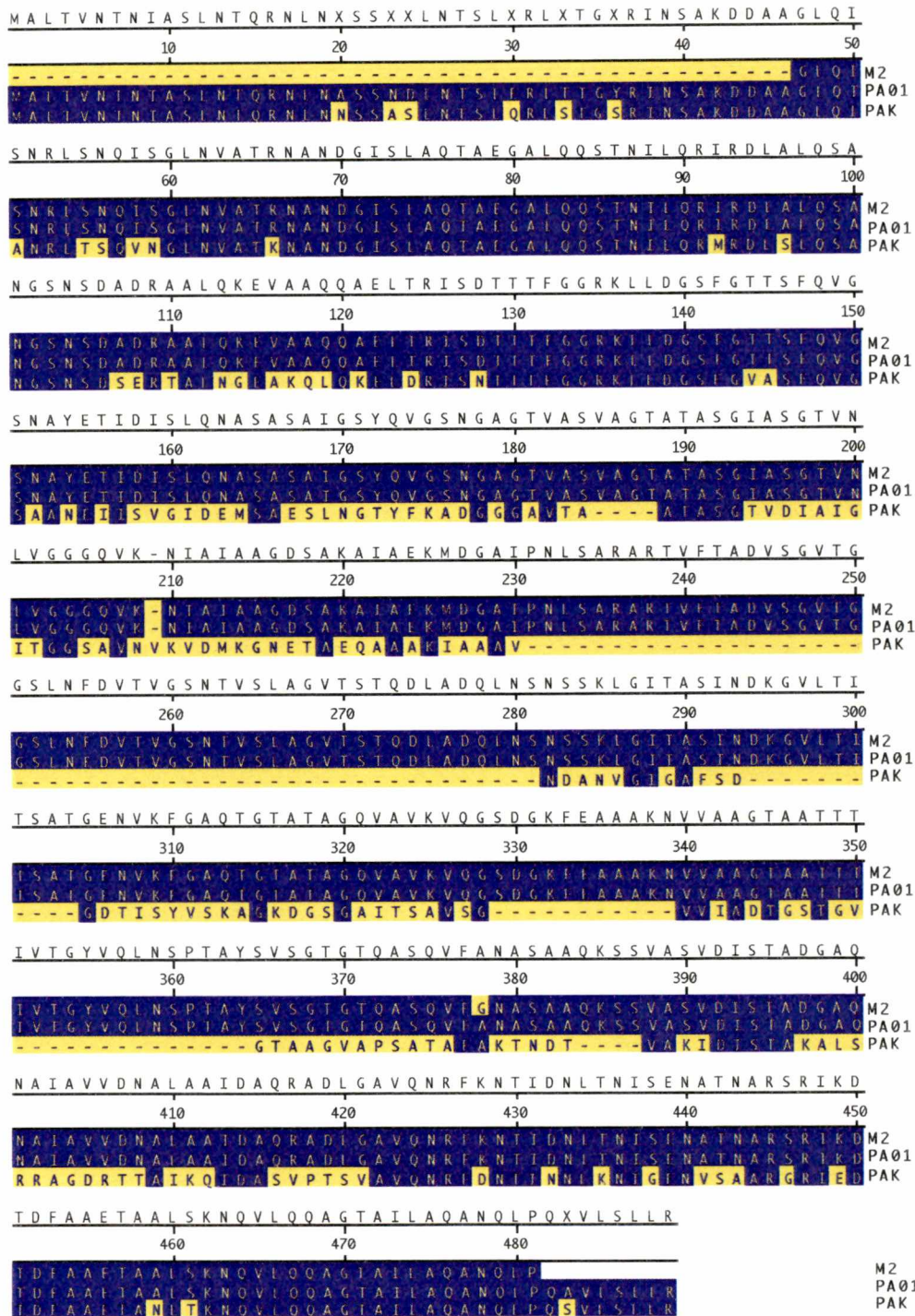


Figure 6: Protein Sequence Comparison Between Wild Type Flagellin b-type Strains M2 and PA01 and Wild Type a-type Strain PAK.

finding definitively demonstrates and confirms the homologous nature of these flagellins. We have also cloned and sequenced the flagellin gene from PA01 and found our sequence data to match that of the PA01 flagellin sequence entered into GenBank by Neil Baker (accession U54775). The highly conserved nature of the b-type *fliC* genes has also been confirmed by Spangenberg et al. (1996). In a study that involved eight *P. aeruginosa* strains that encode a b-type flagellin, these researchers found that the flagellin amino acid sequence was identical in all eight strains. The amino acid sequences of strains PAK and M2 were compared in Figure 6 and showed a marked decrease in the degree of identity (42%). This value seems unusually low since it was reported that pairwise sequence comparisons of a- and b-type flagellins of *P. aeruginosa* gave a 74% identity of the nucleotide sequences and a 63-65% identity of the amino acid sequences (Spangenberg et al., 1996). The sequence variation between flagellin types, predominately in the central region, explains why there is no cross reactivity with the monoclonal antibodies for each type.

In Figure 7, the flagellin sequences of the non-motile CF isolates 448bb and 96e were compared with the amino acid sequences of wild type strains M2 and PA01. Protein sequence analysis of isolate 448bb illustrates that there is a stop codon at amino acid 84 (T-A-G at nucleotides 230-232) which is responsible for the Fla⁻ phenotype of this particular CF isolate. Yet despite the stop codon in the 448bb flagellin sequence, this protein shares 99% identity with the wild types with 4 additional amino acid differences. The protein sequence of 96e shares 100% identity with the wild type flagellin from M2. Interestingly the three clones, M2, 448bb and 96e, all share a

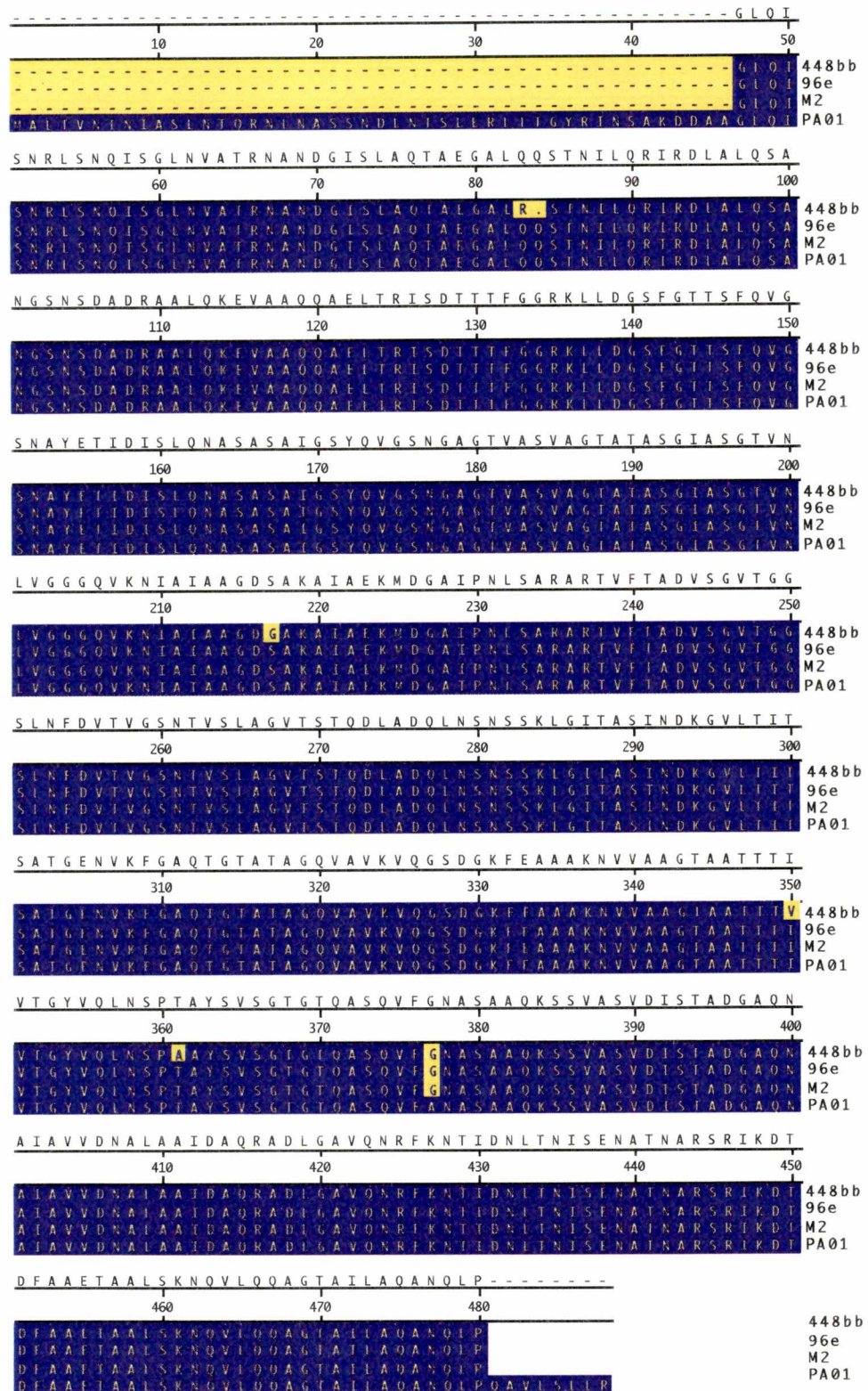


Figure 7: Protein Sequence Comparison Between Non-motile Flagellin b-type Strains CF448bb and CF96e and Wild Type b-type Strains M2 and PA01.

common amino acid difference, the alanine present in PA01 at position 377, is changed to a glycine in M2.

The sequence comparison in Figure 8 again confirmed the current theories that the b-type flagellins are highly homologous and that the flagellin b gene (*fliC*) is highly conserved. The Fla⁻ b-type strain AK1153 exhibited 99% homology with both PA01 and M2 flagellins. Although, there may be a potential mutation in the flagellin regions not sequenced, the central variable region seemed to be well conserved. As one of its 3 amino acid differences, the AK1153 flagellin also contained the same amino acid difference as the other clones in comparison to strain PA01: A377G. Assuming that the N- and C-terminal regions not sequenced are not mutated, one or more regulatory mutations are most likely responsible for the Fla⁻ phenotype of AK1153.

Strain PA103 has been used by a number of laboratories to study virulence and virulence determinants. In a burn model, PA103 is several logs less virulent compared to the Fla⁺ strains tested (Craven and Montie, 1981; Holder et al., 1982). We were interested to determine the state of the flagellin gene in this strain. In Figure 9, the comparison of a-type protein sequences from non-motile strain PA103 (337 amino acids) and motile strain CF isolate CFK701 (also isolated using the CW primers) and PAK illustrates some interesting findings. The amino acid sequences of PA103 and CFK701 showed 96% identity with 13 amino acid differences. However, the protein comparison of non-motile a-type strain PA103 and motile a-type strain PAK showed lower homology with only 87% identity.

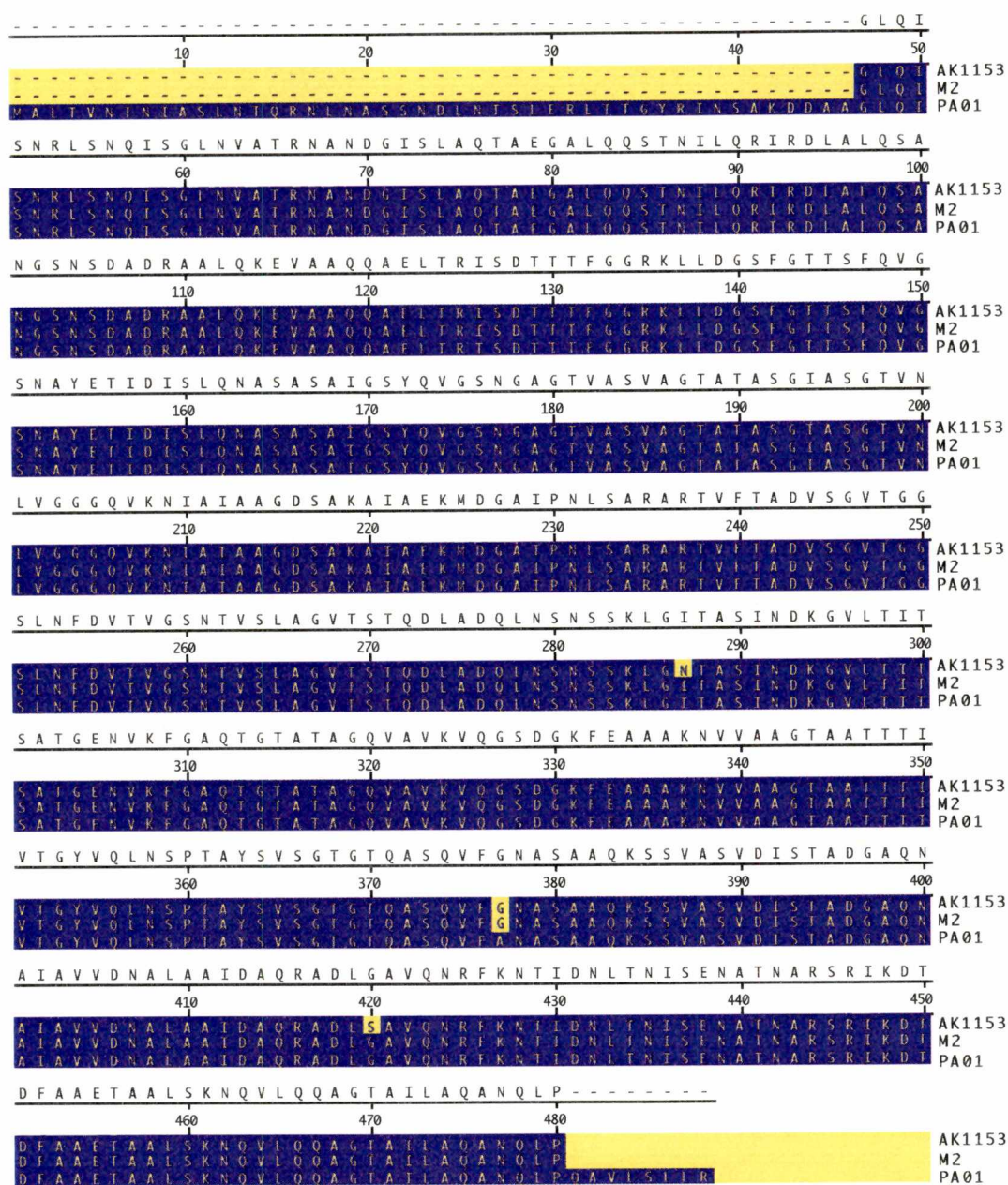


Figure 8: Protein Sequence Comparison Between Non-motile Flagellin b-type Strain AK1153 and Wild Type b-type Strains M2 and PA01.

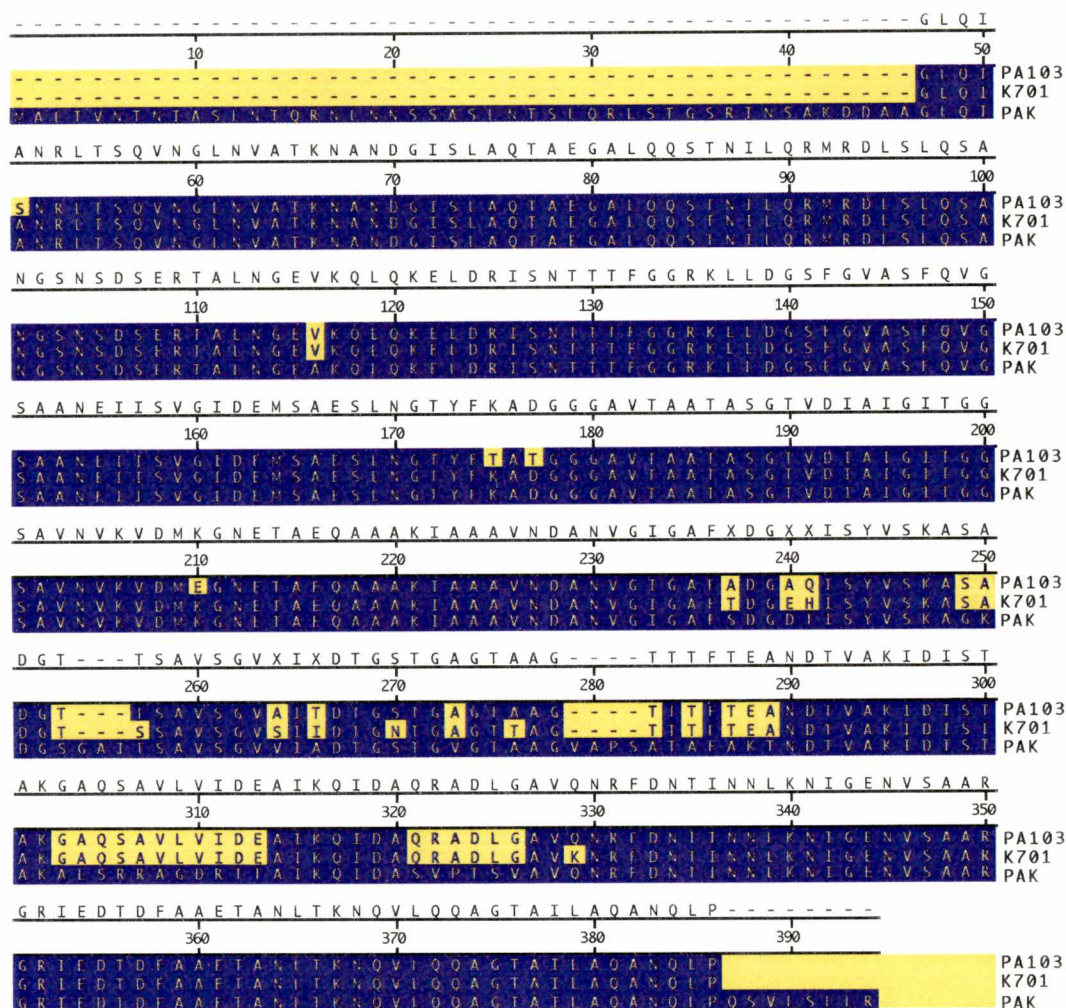


Figure 9: Protein Sequence Comparison Between Non-motile Flagellin a-type Strain PA103 and Wild Type a-type Strains CFK701 and PAK.

This is an interesting finding since in a study of 12 a-type flagellin sequences, Spangenberg et al. (1996) found 96-98% identity in both their nucleotide and amino acid sequences. It was initially hypothesized that the amino acid sequence variation in the flagellin of the non-motile strain PA103 in comparison to PAK, would explain the Fla⁻ phenotype of this strain. However, in considering that the CF isolate K701 is motile (as confirmed with motility assays and electron microscopy, data not shown) and shares a high degree of sequence identity with strain PA103, this hypothesis seemed unlikely. It was therefore tentatively concluded that the amino acid sequence variation in the a-type flagellin of PA103, is not a structural mutation responsible for the Fla⁻ phenotype, but an example of the inherent heterogeneity of these a-type flagellins.

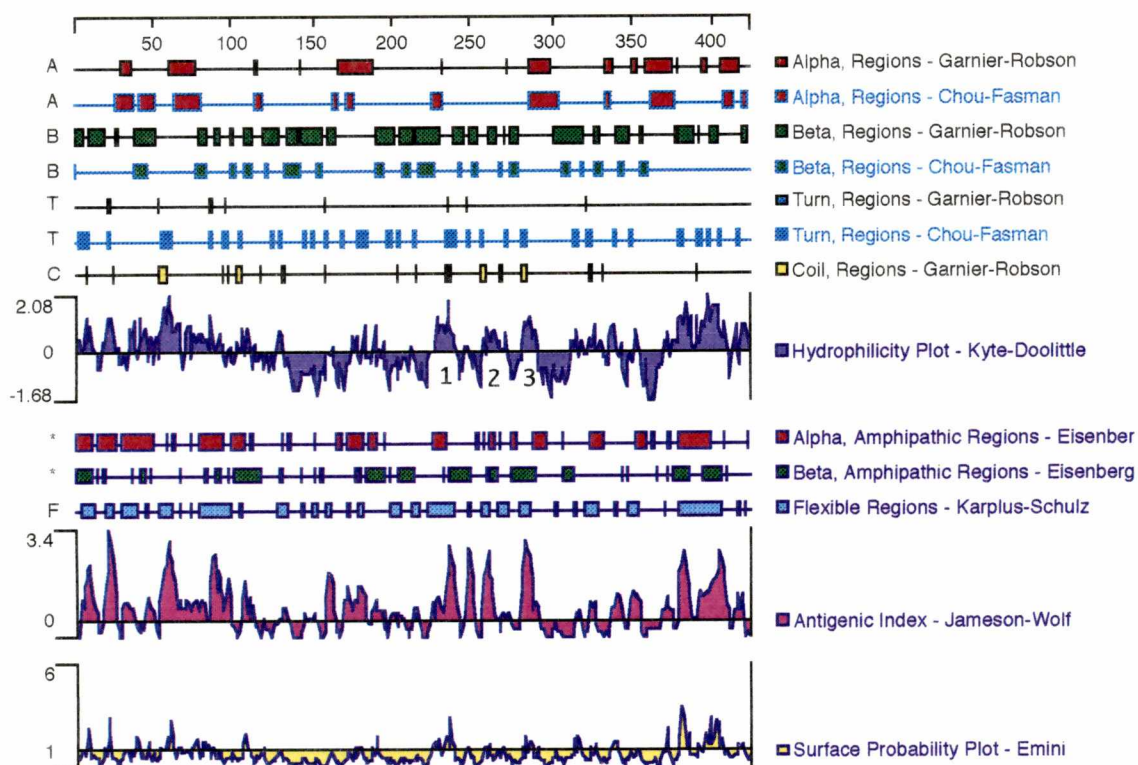
Potential Linear Epitopes for the b-type Flagellins

In considering the degree of protein sequence variation between the a and b-type flagellins, it is not surprising to find that the b-type monoclonal antibody does not cross react with the a-type flagellins. Therefore, if there are any linear epitopes in the b-type flagellin, these potential Mab sites should be absent in the a-type flagellin protein sequences.

Cyanogen bromide (CNBr) cleaves proteins at methionines. CNBr cleavage of the b-type flagellin from strain PA01 yields 2 cleavage fragments with apparent molecular weights of approximately 23.6kDa (225 amino acids) and 28kDa (263 amino acids) (Nichols and Montie, unpublished data). Western blot analysis of the PA01 CNBr fragments with anti b-type

monoclonal antibodies showed a positive reaction with the higher molecular weight fragment. According to the amino acid sequence of the PA01 flagellin, the larger CNBr cleavage fragment is located at the C-terminal end of the protein.

Considering the conserved nature of the N- and C-terminal regions of flagellin proteins among the a and b-types, any monoclonal antibody epitope sites will probably be found in the variable region. In analyzing a hydrophilicity plot of the M2 protein fragment in Figure 10, it was noted that there are 3 hydrophilic regions within the variable region (C-terminal end) of the M2 flagellin, ranging in size from 11 to 13 amino acids, that align with the antigenic index and thus are potential candidates for linear monoclonal epitope sites. These three hydrophilic regions are estimated at amino acid positions: 1) 273-286, 2) 304-315, 3) 327-337, in the full size b-type flagellin protein and at amino acid positions: 1) 227-240, 2) 258-269, 3) 281-291, in the M2 flagellin fragment in Figure 10. The Chou-Fasman analysis predicts turn and alpha regions for the first epitope and no specific predictions for the second epitope. In these same predictions, the third epitope, like the first, is suggested to be a combination of turn and alpha regions. The Garnier-Robson predictions show epitope 1 as a combination of alpha, beta and turn regions and epitope 2 as a combination of beta and coil regions. In similar predictions, epitope 3 is shown to be a combination of alpha and coil regions. In analyzing Figure 11, it is apparent that these potential epitopes sites are almost completely absent in the a-type flagellins of strains CFK701 and PAK. However, there may be a number of additional a-type flagellin protein sequences not yet determined that may disqualify one or all of these



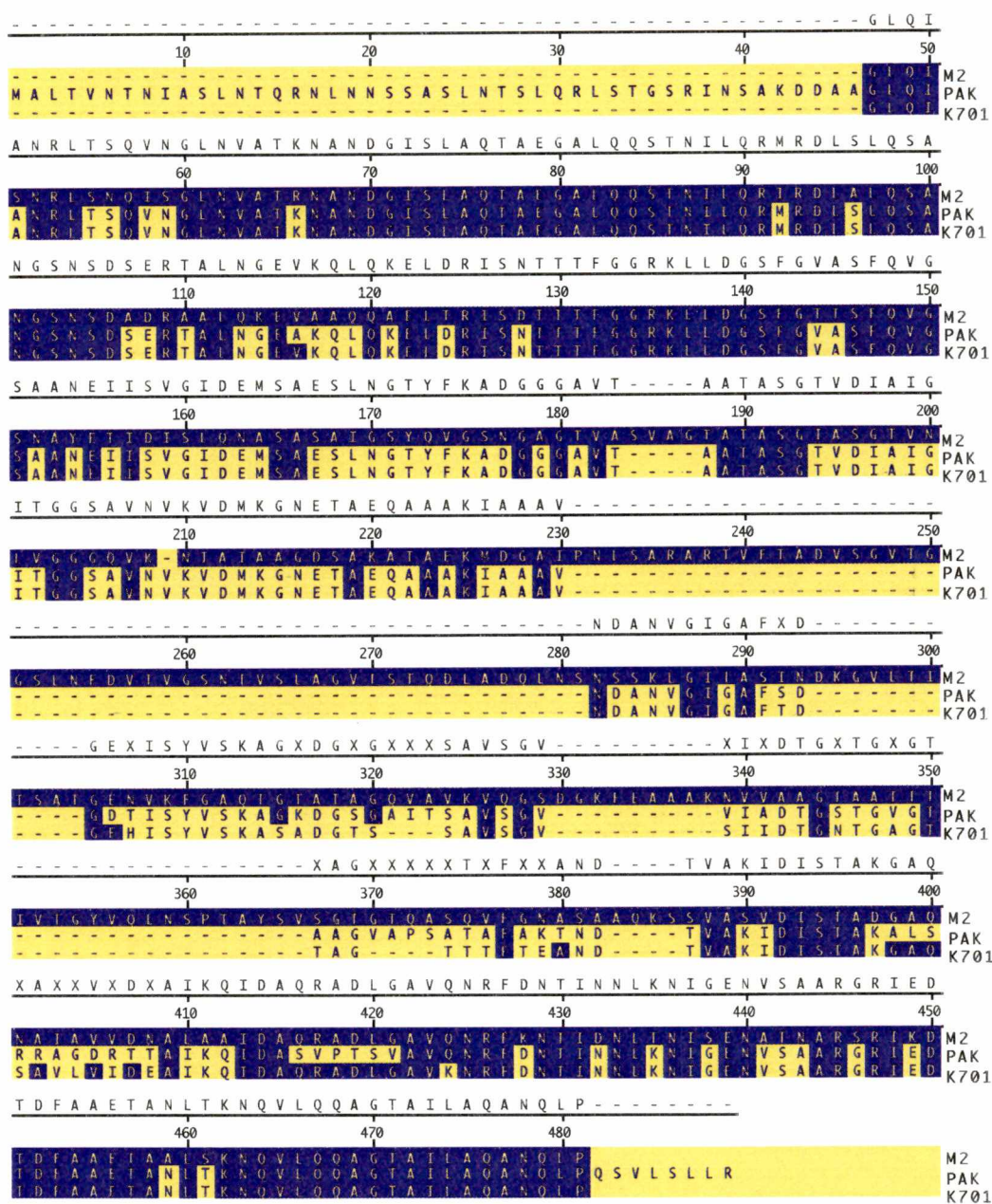


Figure 11: Protein Sequence Comparison Between Wild Type Flagellin b-type Strain M2 and Wild Type a-type Strains PAK and CFK701.

potential epitope sites. Epitope mapping assays with synthetic peptides and b-type flagellin monoclonal antibodies may provide conclusive evidence to support the hypothesis of a linear epitope for the b-type flagellins.

It is important to note however, that there are other areas of the M2 fragment on both sides of these hydrophilic regions (C-terminal to the Met at position 179 in the M2 fragment) that are both antigenic and hydrophilic, however the length of these regions is much smaller at 2-5 amino acids and a number of these regions are located very close if not within the conserved C-terminal end of the protein. These regions are more likely to be associated with a three dimensional epitope site. According to Figure 11, the conserved N-terminal region appears to be about the first 150 amino acids whereas the C-terminal conserved region is shorter at only about 70 amino acids.

CHAPTER IV

DISCUSSION

Isolation of Flagellin Genes

The research reported here demonstrates a correlation relating PCR product size, gene size and antibody reaction to flagellin type. Furthermore, the flagellin type designation of Fla⁻ strains by PCR fragment gene size was confirmed with sequence analysis. Although PCR flagellin gene fragments do not represent the entire coding sequence, the missing terminal sequences are in regions that are not only highly conserved, but also where no significant variation in length is apparent even when flagellin genes from very different bacterial species are aligned (Winstanley et al., 1996). In agreement with the data shown here, it has recently been reported that both a and b-type flagellins share nearly identical N- and C-terminal sequences where as the central region is variable in size and primary structure (Spangenberg et al., 1996). As noted earlier, the conserved N-terminal region in these flagellins appears to be about the first 150 amino acids whereas the C-terminal conserved region is shorter at only about 70 amino acids.

Comparative Antigenic Analysis of Fla⁺ and Fla⁻ Strains

There was an earlier suggestion that some of the examined non-motile strains were producing flagellin protein in either a monomeric or a polymerized form and secreting it into the supernatant in detectable amounts as determined by colony blotting using polyclonal antibodies (Kelly-Wintenberg et al., 1989). Subsequently however, it was hypothesized that non-motile strains CF96e and CF448bb do not produce detectable amounts monomeric flagellin protein (Kelly-Wintenberg et al., 1991). It is unlikely that the other two non-motile strains PA103 and AK1153 produce flagellin protein considering their lack of reaction with the monoclonal antibodies. Monoclonal colony blots both in lysed and unlysed cells of the Fla⁻ strains showed no reactivity indicating the absence of antigenic protein both inside and outside the bacteria. These results suggest that 1) the flagellin protein is not being synthesized or 2) the protein is both expressed and secreted but it is folding differently, perhaps due to a gene sequence mutation, so that the Mab reactive epitope is not exposed. However, no further experimentation was performed to explore this latter hypothesis. The absence of antigen reactive material in lysed cells suggested that the flagellin protein is not being synthesized in the Fla⁻ strains due to a transcriptional or translational mutation or to a mutation in another type of gene such as regulatory.

Comparative Analysis of Flagellin Protein Sequences

When the flagellin protein sequence of wild type b-type strain M2 was compared with the amino acid sequence of Fla⁺ b-type strain PA01, this comparison showed only a single amino acid difference between the two types, thus supporting the concept of the homologous nature of these flagellins. Protein sequence comparison of the non-motile b-type flagellins from strains CF448bb, CF96e and AK1153 to the motile wild type strains M2 and PA01, showed these proteins as also having a high degree of identity with only up to 4 amino acid differences (despite the presence of a stop codon in the N-terminal region of CF448bb).

Evidence was found both in this research and in that of other researchers, to confirm the variation in the a-type flagellin sequences. The concept of a central variable region in flagellins appears to be only applicable to the a-type flagellins, especially in comparison to the b-types, considering the high degree of sequence identity throughout the amino acid sequence of the b-type flagellins (Spangenberg et al., 1996; Brimer et al., 1997). This seems to be reflected in the differential response of a-type flagellins to a-type monoclonal antibodies (Landsperger et al., 1993; Montie et al., 1996). Both *P. aeruginosa* motile strains CFK701 and PAK produce a-type flagellins that reacted with a-type flagellin monoclonal antibody, yet their flagellins share only 87% identity. Spangenberg et al. (1996) noted that in comparison to the b-type flagellins, the a-types show a significantly greater amount of DNA sequence variation which could be partially responsible for the differences in molecular weights. Phosphorylation and other types of posttranslational

modification have been reported to contribute to the molecular weight discrepancies in flagellins (Kelly-Wintenberg et al., 1993). Additionally, evidence was found for glycosylation on a number of a-type flagellins which seem to be a contributing factor to the molecular weight variations (Brimer et al., 1997). However, no glycosylation was found on b-type strain M2.

Mutations Responsible for the Fla⁻ Phenotype

The fact that flagellin genes could be amplified in Fla⁻ strains confirmed that the structural gene is present, however it does not rule out the prospect that the structural gene is mutated nor that the amino acid sequence is altered. This concept stresses the importance of gene and protein sequencing in understanding and analyzing mutant phenotypes such as the Fla⁻ phenotype seen in non-motile *P. aeruginosa*. Although there may be a potential mutation in the portion of the b-type flagellin proteins not sequenced, the N- and C-terminal regions and central regions seemed to be well conserved. The homogeneity among the b-type *fliC* genes provides a reliable profile for comparison of shifts in sequence that may represent significant mutations.

Comparison of both of the conserved regions and these unique central regions of the flagellin proteins of mutant Fla⁻ strains with wild type flagellin proteins does not seem to consistently link mutations in this central area to the assembly of the flagellin protein into a functional flagellum in *P. aeruginosa*. Protein sequence analyses of the b-type flagellins illustrated the high degree of identity among these flagellins, regardless of

the Fla⁻ phenotype of the non-motile strains. Other than the stop codon in the N-terminal region of Fla⁻ CF isolate 448bb, no other evidence for mutations in the structural gene of this non-motile strain, was found.

The discrimination between a-type sequences that are different due to mutations as opposed to heterogeneity, is unclear. As stated earlier, the flagellin protein sequence of the non-motile strain PA103 shares 96% homology with the motile strain CFK701. However when compared to the wild type PAK, the PA103 flagellin shows a lower degree of homology (87%). It was concluded that the protein structure variation in the PA103 flagellin as compared to the PAK flagellin, does not result in mutations responsible for the Fla⁻ phenotype of this strain. Thus these areas of sequence variation in the PA103 flagellin, mostly present in the CFK701 flagellin, are not needed for flagellum biosynthesis and assembly. The origin of the mutation responsible for the Fla⁻ phenotype of PA103, may involve posttranslational modification of the flagellin protein, since it was reported that this strain lacks the putative membrane kinase found in wild type strains (Cha et al., 1996). It has been shown that a-type flagellins exhibit a higher degree of heterogeneity, not only in molecular weight, but also in DNA sequence as confirmed by a variety of RFLP's (restriction fragment length polymorphisms) found with the a-type flagellin genes (Winstanley et al., 1996). Thus protein sequence comparison of a non-motile a-type strain to a wild type a-type strain becomes more complicated. Although it would seem logical to conclude that any conserved sequences within the N- and C-terminal and central variable regions of a-type flagellins may be required to produce a Fla⁺ phenotype. Investigations involving site-directed

mutagenesis of these conserved areas in the central region of the a-type flagellins may give insight as to the importance of specific conserved sequences in the assembly of a functional flagellum.

Since flagella synthesis and assembly is very complex, mutations which generate a Fla⁻ phenotype could involve a number of regulatory, biosynthesis and assembly genes as well as additional structure genes other than the *fliC* gene. At least in the b-type strains examined here, it appears that the flagellin gene *fliC* tends to be conserved and less prone to mutations. There are a large number of genes involved in the regulation and biosynthesis of flagellin such as the *rpoN*, *fliA*(*rpoF*), *fliO*, *fliF*, *fleS* and *fleR* genes. Mutations in any one of these regulatory genes results in a Fla⁻ phenotype (Totten et al., 1990; Starnbach and Lory, 1992; Simpson et al., 1995; Arora et al., 1996 and Ritchings et al., 1995). However, the MSM growth assays using a glutamine requirement as a selective marker for *rpoN*⁻ strains, suggest that an *rpoN* (σ^{54}) regulatory gene mutation, is probably not responsible for the Fla⁻ phenotype of the strains in this study.

The environmental conditions in the CF lung during chronic infections cause high levels of mutations in *P. aeruginosa* isolates such as the loss of motility (Luzar et al., 1985; Mahenthiralingam et al., 1994). Therefore, the protein sequences of two non-motile CF isolates were analyzed to identify any common sequence mutations or "hot spots". In the CF isolate 448bb, evidence was found linking a mutation (stop codon in the structural *fliC* gene) to the lack of motility of this CF isolate, acquired from a patient with chronic infection. The stop codon in the flagellin gene sequence of CF448bb,

truncates the flagellin of this isolate, resulting in the loss of the capacity to assemble a functional flagellum filament. However, the amino acid sequence of the flagellin b gene from 96e, which shares complete identity with the wild type M2 flagellin, shows no evidence for mutations in the structural gene.

To investigate the variety of mutations that result in a Fla⁻ phenotype for the CF strains specifically, additional strains would need to be examined to verify if a preponderance of structural or regulatory gene mutations exists. Since flagellar biosynthesis may be controlled at several levels, mutations at the regulatory level may be more effective in adaptation of *P. aeruginosa* to survival in CF lung infections. It would also explain the coordinate down regulation of several invasive virulence factors, such toxins and LPS, which is observed in such isolates (Hancock et al., 1983; Penketh et al., 1983; Thomassen et al., 1979).

Mahenthiralingam et al. (1994) reported that partial complementation of motility in non-motile *P. aeruginosa* isolates was achieved by introduction of extra copies of the *rpoN* locus carried on plasmid pPT212, indicating that the alternate sigma factor, RpoN, may be involved in the coordinate regulation of virulence factors during CF infection. During chronic infection, a high number of Fla⁻ isolates are observed which result from increased mutations due to the unusual environment in the CF lung (Mahenthiralingam et al., 1994). Since flagella are required for non-specific phagocytosis, it was hypothesized in their findings that bacteria demonstrating the Fla⁻ phenotype were resistant to non-specific

phagocytosis by macrophages, thereby increasing their survival in the CF lung. The bacteria may repress the expression of flagellin since motility is not an efficient motor function for *P. aeruginosa* in the highly mucoid environment in the CF lung.

The Use of PCR in Clinical Isolation of *P. aeruginosa*

The PCR technique designed by Winstanley et al. (1996) has been ascertained to be a rapid and accurate flagellin typing method for *P. aeruginosa* strains and we have confirmed this by antibody typing. In addition, flagellin genes are rapidly isolated which facilitates comparison of gene sequence. Additional a-types were analyzed in our laboratory by Brimer et al. (1997). The use of specific monoclonal antibodies was shown here to be a reliable method for differentiating Fla⁺ and Fla⁻ *P. aeruginosa* strains and for typing the Fla⁺ *P. aeruginosa* strains. The PCR protocol used to amplify the flagellin genes can not only be used to identify and study the *fliC* gene in Fla⁻ *P. aeruginosa* strains, but it could also be used to detect Fla⁻ isolates in the sputum of CF patients that do not react with flagellar monoclonal antibody and thus may be more difficult to identify. The use of restriction fragment length polymorphisms (RFLP) of flagellin DNA could provide additional data for distinguishing specific strains with respect to epidemiological outbreaks (Winstanley et al., 1996).

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VITA

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