

To the Graduate Council:

I am submitting herewith a dissertation written by Kimberly Kelly Wintenberg entitled "Cloning and Isolation of the Flagellin Gene, and Characterization of the Posttranslational Modification of the Flagellin Protein in *Pseudomonas aeruginosa*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Microbiology.

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CLONING AND ISOLATION OF THE FLAGELLIN GENE, AND  
CHARACTERIZATION OF THE POSTTRANSLATIONAL  
MODIFICATION OF THE FLAGELLIN PROTEIN  
IN *PSEUDOMONAS AERUGINOSA*

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Kimberly Kelly-Wintenberg  
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## DEDICATION

This work is dedicated to my entire family. Without the love and support of my husband this work would not have come about. He is my inspiration. I also wish to dedicate this work to my mother and father who taught me at an early age that with hard work and persistence, I could accomplish anything. I appreciate their love and support during my enrollment in graduate school.

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## ABSTRACT

This dissertation was built around a central theme of developing a better understanding of the flagellin filament protein of *P. aeruginosa* by cloning the flagellin gene, and characterizing a posttranslational modification in the flagellin protein.

To detect the flagellin gene within a recombinant clone of the pLAFR1-PAO1 library, the recombinant clones were triparentally mated into *fla*<sup>-</sup> mutants and scored for their ability to complement the mutant. Motility spreading assays were performed determine if complementation had occurred, and confirmed by colony blot assays. Electron microscopy and amino acid analyses showed the structure and make-up of the flagella from the cells containing clones to be nearly identical with wild type flagella.

Following isolation of positive recombinant clones, two different approaches were employed to determine which region of the 25 kb PAO1 fragment was necessary for expression of the flagella. First, deletion mapping was utilized. The positive clones were further digested with restriction enzymes and religated and triparentally mated back into *P. aeruginosa fla*<sup>-</sup> cells, and complementation was scored. The *fla* gene is located within a 5.0 kb Xho I fragment. The second approach involved transposon mutagenesis with Tn501. The sites of the transposon insertions

was mapped by analyses of restriction digests. A gene replacement technique was utilized, and the loss of flagellar structure was characterized by motility spreading assays and colony blotting.

The other major portion of this research was identification and characterization of the posttranslational modification occurring in the flagellin filament of *P. aeruginosa*. Both a- and b-type purified flagellin from *P. aeruginosa* grown in radiolabeled phosphate were shown to be phosphorylated. Initial characterization of labeled flagellin was by autoradiograms of SDS-PAGE gels and immunoblots with anti-flagella antibody. Thin layer electrophoresis (TLE) of partial acid hydrolyzed flagella filaments revealed that the radioactivity was in phosphotyrosine. This finding was unusual because a phosphorylated tyrosine is not prevalent in prokaryotes. Intact flagella filaments have been shown to react specifically with antiphosphotyrosine monoclonal antibody. Also, the labeled filaments were susceptible to phosphatase, indicating a monophosphoester linkage. The biological role of these modifications remains unknown.

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PART I  
INTRODUCTION AND LITERATURE REVIEW.

## INTRODUCTION

*P. aeruginosa* is an aerobic, gram-negative ( G -) saprophytic rod, capable of growing on a number of different carbon sources. It was first characterized by Gessard in 1882, and its pathogenicity for animals first was described by Charrin in 1890 (Stanley, 1947; Bodey et al., 1983). The dimension of the organism varies from one to three microns (um) in length, and one-half to one um in width. *P. aeruginosa* is motile, and is characterized by a single polar flagellum.

Unlike most other human pathogens, *P. aeruginosa* is capable of causing diseases in a number of organisms including invertebrates, vertebrates, and plants. Fresh fruits and vegetables have been traced as vectors for colonization of hospitalized patients (Doggett, 1979). Recognition of *P. aeruginosa* as a human pathogen was slow, due to a lack of understanding of its mode of pathogenicity.

Today *P. aeruginosa* is an important pathogen for cystic fibrosis (CF) patients and immunocompromised hosts, including burn victims and patients undergoing chemotherapy, to name the more common. However, it is believed that 10 to 25% of healthy persons carry *P. aeruginosa* in their gut, and are asymptomatic. Therefore, *P. aeruginosa* is considered to be an opportunistic pathogen.

A factor common to immunocompromised patients, who are susceptible to a *P. aeruginosa* infection, is a break in the natural barriers that protect against invasion. The predominant isolate found in a burn wound is *P. aeruginosa*. Studies have shown that it colonizes over 60% of patients by the fifth day after burn injury, and *P. aeruginosa* septacemia becomes a major threat to these patients (Doggett, 1979).

A high percentage of infections in cancer patients are caused by *P. aeruginosa*. Typically this type of infection is found in patients with acute leukemias and other bone marrow diseases whose treatment of radiation and/or chemotherapy results in neutropenia (Froland, 1981).

A high incidence of *P. aeruginosa* infection is also seen in patients with CF, who are predisposed to pulmonary disease from infancy. *P. aeruginosa* is isolated from the lung of CF patients in different morphological forms: rough, smooth, and mucoid. During the course of the disease the mucoid form becomes the dominant morphological form isolated in the sputum of the terminal patient (Doggert, 1979).

*P. aeruginosa* is also an important pathogen in hospital-acquired pneumonias. It is considered the single most frequently isolated pathogen, followed by staphylococci (Scheld, and Mandell, 1991). It is frequently the cause of nosocomial urinary tract infections, although generally it is not the initial pathogen. It is well documented that persons undergoing surgical procedures, inhalation therapy, chemotherapy or urinary

catherization are predisposed to *P. aeruginosa* colonization and the resulting infection (Scheld, and Mandell, 1991).

*P. aeruginosa* is a very complex organism, producing many extracellular products including toxins, and cellular products including flagella. These products, many of which are considered virulence factors, aid in its ability to be highly invasive and to colonize. It is important to remember that the virulence of *P. aeruginosa* is multifactorial, and that a number of factors contribute to the cause of invasion and tissue destruction. The picture is more complicated by the fact *P. aeruginosa* causes a number of different infection processes, and the effect of a single virulence factor on the pathogenicity of one disease may be different than its effect in another disease (Nicas, and Iglewski, 1985).

Therefore it is appropriate to review a number of cellular and extracellular products associated with the pathogenicity of *P. aeruginosa* to help understand the complexity and virulence of the bacterium.

## VIRULENCE FACTORS AND POTENTIAL VIRULENCE FACTORS OF *PSEUDMONAS AERUGINOSA*

Exotoxins. *P. aeruginosa* is unique among G - organisms because it secretes a number of different proteins and other substances into the

medium (Woods, and Iglewski, 1983). In this aspect, *P. aeruginosa* is more related to gram positive (G +) bacteria where secretion occurs more often because of the loose makeup of peptidoglycan.

An extensively studied virulence factor is exotoxin A (Exo A), which is a heat labile protein. The protein is toxic because of its ability to inhibit protein synthesis in susceptible cells. Protein synthesis is inhibited by catalyzing the transfer of the adenosine 5' diphosphate ribosyl (ADRP) moiety of nicotinamide adenosine dinucleotide onto elongation factor two (EF2). This process has the same intracellular mechanism as diphtheria toxin (DT), however, the toxins have different receptors on susceptible cells (Iglewski, and Sadoff, 1979).

There is a large proportion of experimental evidence which suggests Exo A is a virulence factor of *P. aeruginosa*. First, Exo A is produced in experimental animal infections and in humans, in vivo (Cross et al., 1980). Second, working with different animal models and  $\text{tox}^-$  mutants, Exo A has been shown to be a virulence factor in infections of the cornea, lung and burn wounds (Woods, and Iglewski, 1983). Also, both passive and active immunization against Exo A protects animals from the lethal effects of pure protein. The protection of Exo A antibodies against active *P. aeruginosa* infections has not been demonstrated in humans, but the presence of antibody in human serum has been reported (Cross et al., 1980).

In addition to different receptors, there also seems to be a difference between DT and Exo A, in relation to their effects on the infection process. For instance, in CF patients, the toxemia phenomena produced by DT is not seen with Exo A, and CF patients may live years with high numbers of *P. aeruginosa* cells (Young, 1980).

*P. aeruginosa* produces another ADPR-transferase enzyme, exoenzyme S (Exo S) (Iglewski and Sadoff, 1979). Exo S does not modify EF-2, instead it modifies one or more different proteins present in eukaryotic cell extracts. Studies in a rat lung model with an isogenic mutant specifically deficient in Exo S demonstrated that Exo S may play a significant role in lung infections (Nicas et al., 1985). In addition, if the Exo S activity in vitro is suppressed by antibiotics, a decrease in lung pathology is seen in chronically infected rats with *P. aeruginosa* (Grimwood et al., 1988). Recently, it has been shown Exo S is an adhesion factor (Baker et al., 1991).

Other Extracellular Products. The majority of *P. aeruginosa* strains produce two proteases, alkaline protease (AP) and elastase (Ela) (Moriyama, and Tsuzuki, 1977). Both proteases are characterized by the possession of broad substrate specificities. Purified Ela and AP cleave and inactivate a number of important biological proteins such as fibrin, immunoglobulin G (IgG), immunoglobulin A (IgA), polymorphonuclear

neutrophils (PMNs), elastin, and lysozyme in susceptible cells (Doring et al., 1987). As in Exo A, the virulence of proteases varies according to the type of infection (Nicas, and Iglewski, 1985). The pathogenicity of proteases is now considered an “aggression activity”, because upon the addition of minute amounts of proteases, the virulence of a nonprotease-producing strain is increased in experimental models (Moriyama et al., 1989).

Phospholipase C is another extracellular product produced by *P. aeruginosa* that is considered a virulence factor. Phospholipase C is a lecithinase which liberates phosphorylcholine from lecithin (Berka, and Vasil, 1982). It is suggested, like Exo S in *P. aeruginosa* infections, that phospholipase C is more important in localized infections than in systemic ones. As described by Woods, (Woods, 1987) it has been demonstrated that phospholipase C plays a role in the colonization of airways in cystic fibrosis patients. It has also been suggested (Woods, 1987) that the presence of a lecithinase such as phospholipase C in the lungs of patients with *P. aeruginosa* pneumonia is a potential virulence factor, because the normal surfactant lining the lungs and preventing atelectasis, is lecithin (Woods, 1987).

More recently, iron siderophores have been considered as potential virulence factors. In the human body, most of the iron is located within the cells, in the form of ferritin, hemoglobin, and myoglobin. Any

extracellular iron is strongly bound to carrier proteins such as lactoferrin and transferrin that have association constants of  $10^{36}$  for iron (Cox, 1982). Bacteria, such as *P. aeruginosa* are capable of removing iron from the host in order to replicate, and therefore iron acquisition is considered a virulence factor.

Cellular components. The capsule exopolysaccharide, also termed slime, has long been considered a potential virulence factor. Alginate is the major component in this extracellular material which is secreted by mucoid strains of *P. aeruginosa*. After an initial infection of *P. aeruginosa* in CF patients, mucoid variants begin to emerge in vivo and become the predominant form isolated in the sputum (Williams, and Govan, 1973). The appearance of the mucoid phenotype typically translates into progressively worsening respiratory distress for the patient. Alginate appears to have antiphagocytic activities, an ability to interfere in the deposition of antibody, and to mediate adherence to human tracheal cells (Pier, 1989).

*P. aeruginosa* being a G - organism, possesses cell wall lipopolysaccharide (LPS), or endotoxin. The LPS of *P. aeruginosa* does not appear as toxic as LPS isolated from other *Enterobacteriaceae*. It has been generally accepted that LPS plays a role in the virulence of an organism by resisting the bactericidal action of complement in normal

human serum, and antibody towards it is highly protective in experimental models. LPS also plays a major role in the protection of the bacterial cell against opsonization and phagocytosis (Pitt, 1989).

Another potential virulence factor present on the surface of the organism are polar pili. Pili have been implicated in mediating adherence to buccal epithelial cells by the following results: nonpiliated strains do not adhere to epithelial cells; bacterial cells treated with protease have a greatly reduced ability to bind to epithelial cells; preincubation of purified pili with epithelial cells significantly decreases the adherence of intact organisms; and antibody to purified pili prevents the adherence of the organism to buccal epithelial cells (Ramphal et al., 1991). Woods has demonstrated that colonization of the respiratory tract of seriously ill patients by *P. aeruginosa* was accompanied in every case by in vivo adherence of the organism to epithelial cells of the upper respiratory tract (Woods, 1987).

Another important virulence factor and possible vaccine candidate is the flagellum of *P. aeruginosa*. To determine the contribution of motility and flagella to virulence, isogenic mutant strains (Fla<sup>-</sup>, Mon<sup>-</sup>) were used in a burned-mouse model (Montie et al., 1982; Holder, 1983), and compared to wild type cells in the same model. Besides the analogy to human burns, the burned-mouse model provides a sensitive means for elucidating invasive virulence factors (Holder, 1983).

Upon comparison of the wild type and mutant strains in the burned-mouse model, a considerable loss of virulence was observed in the mutants. These losses in virulence coincided with a loss in the flagellar organelle or its capacity to function properly. Decreases in virulence were seen when these mutants were compared to wild type strains by various routes of inoculation including; intraperitoneal or topical, and in a scalded-mouse model (Drake, and Montie, 1987; Drake, and Montie, 1988).

Results from tissue colonization experiments has shown that motility was required for rapid invasion from the burn-skin colonization site to the blood and internal organs sites such as the liver (Montie et al., 1987). The capacity of motile cells to move rapidly into the bloodstream is probably a factor which aids in surpassing the capability of the animal immune system. These observations further imply that some chemoattractant gradient must be involved in this directional movement. Certain evidence also suggests that movement from other sites such as the peritoneum or perhaps from an intestinal colonization site through the bloodstream also requires motility (Montie et al., 1987).

It is interesting that motility in *P. aeruginosa* may also be important in early lung colonization in CF patients; however, selective pressures in the lung may provide a different environment where motility and/or

chemotaxis as well as other invasive virulence factors become less important after a chronic infection occurs (Luzar et al., 1985).

Recently it has been shown in this laboratory that CF patients make large amounts of antibody to *P. aeruginosa* flagella filament protein (Anderson et al., 1989). These results suggest that at an earlier stage of infection, flagellated cells are important. These results are consistent with data that showed isolates from CF patients with early infections tended to exhibit normal motility, whereas in isolates from chronic infections, the presence of flagella and motility was decreased severely (70% of the rough isolates were Fla<sup>-</sup>). In a recent report, Nelson (Nelson et al., 1990) has shown that CF lung mucin likely serves as a chemoattractant for *P. aeruginosa*. These data taken together, indicate that flagella and motility are important components to be considered in the understanding of virulence and pathogenesis of *P. aeruginosa* in cystic fibrosis.

Work in this laboratory has involved the characterization of the flagella in *P. aeruginosa*. Preparations of flagella were made by shearing the flagella filament with a blender. The filament was isolated by differential centrifugation and purified by molecular sieving. Characterizations of different *P. aeruginosa* strains showed they contained a major flagellin protein subunit that was specific to a given strain (Allison et al., 1985). Based upon the molecular weight, the flagellin have been designated either a-type or b-type (Ansorg, 1978; Lanyi, 1970). The b-type flagellins are

homogeneous and therefore all have a molecular weight of 53,000. The a-types are heterogeneous and showed flagellins ranging from 45 to 52 kilodaltons (kDa) (Allison et al., 1985). The a-type flagellins all contain a common subtype a<sub>0</sub> antigen or various combinations of the subtypes a<sub>1</sub>, a<sub>2</sub>, a<sub>3</sub>, and a<sub>4</sub> (Ansorg, 1978). The closeness of molecular weights in the a-types may relate to some antigenic commonality.

Some years ago, this laboratory proposed that flagella antigen (FLAg) would be a good candidate as a protective antigen. It has been shown that FLAg was protective both as an actively administered immunogen (Montie et al., 1987), and more recently in passive studies in compromised animals (Drake, and Montie, 1987). The flagella is well exposed on the surface of the cell, the protein is highly antigenic and stable with respect to immunogenicity, and it exhibits cross-reactivity. At the present time both antigen and antibody are being evaluated by industry for clinical applications.

## STRUCTURE OF FLAGELLA

Flagella are responsible for movement and chemotaxis of the cell. Bacterial flagella are different from eukaryotic flagella in several ways discussed below. A bacterial flagellum is passively driven by a motor; it

does no chemomechanical work. Unlike eukaryotic flagella, the motion of a prokaryotic flagellum is rotation, not propulsion. The flagellar motor can be switched so that the movement generated is either counterclockwise (CCW) or clockwise (CW). In a prokaryotic flagellum, the energy is thought to be generated by a proton motive force, not ATP driven. The filaments of prokaryotic cells are composed of a single protein to several proteins (Vogel et al., 1991), while eukaryotic filaments are composed of a large number of different proteins. Lastly, the filaments in prokaryotes are much smaller (20  $\mu\text{m}$ ) than eukaryotic flagella, which are generally ten times greater (Macnab, 1987).

The flagella from *P. aeruginosa* cells, as in other bacterial species, are composed of three parts, the filament, the hook, and the basal body. These components are located within the cell, spanning the envelope, and outside of the cell. The majority of components found within the envelope or outside the cell are thought to be exported by a flagellum-specific pathway. These proteins (rod proteins, hook protein, hook associated proteins, and flagellin) travel up the hollow core of the assembling flagellum and are added at the tip (Homma et al., 1990; Kuwajima et al., 1989). Only the outer rings (P and L) (see basal body section) are thought to be exported by a signal-peptide dependent pathway (Jones et al., 1990).

The Filament. The filament is the external portion of the flagella, visible by light microscopy. The filament is composed of thousands of copies of a single protein, flagellin. Although recently it has been indicated in *E. coli*, there are several other proteins associated with the filament (Vogel et al., 1991). On a mass basis, it represents the major component of the flagella. The length of the filament varies, however, it is generally no longer than 20  $\mu\text{m}$ . The filament has a constant diameter of 20 nanometers (nm) throughout its length.

Flagellin sequences in some instances exhibit an unusual characteristic of posttranslational modifications. In *E. coli* and *S. typhimurium*, flagellin filaments contain methylated lysine residues (Ambler, and Rees, 1959). The precise function of this modification is unknown, since mutants without this modification function normally.

Isolated flagellar filaments are easily disassociated by heat and acid. Under proper conditions, polymerization into a filament occurs spontaneously, with the resulting filament showing the same characteristics as the filament protein of the intact organelle (Asukura, 1970). It has been demonstrated in *E. coli*, *S. typhimurium* and other bacteria, that flagella increase in length by flagellin subunits traveling the entire length of the existing filament and are incorporated at the distal end (Emerson et al., 1970). This process is very orderly since these flagella proteins must be added in the correct amounts and order. The most

reasonable hypothesis is that the subunits pass down the length of the filament through a central cavity and are added at the tip. There is some experimental evidence to support this model, and it has been generally accepted (Silverman, and Simon, 1977). The shape of the filament is dependent upon conditions in the medium and upon the primary structure of the flagellin subunit (Iino et al., 1974). In addition to shape, the structure of the flagellin subunit is responsible for the antigenicity.

Flagellin and flagellar filaments have been characterized as potent antigens (Macnab, 1987). Based upon genetic mapping, sequence analysis, and peptide sequence analysis, it is now accepted that the amino (N) and carboxyl (C) termini of flagellin from different bacteria including *P. aeruginosa* are highly conserved (Totten, and Lory, 1990). The central region is considered to be the antigenic variable region, and this variability does not interfere with the assembly of the filament. The antigenic region of the protein is hydrophilic and is the exposed portion of the molecule. Flagellins exhibit a wide range of antigenicity, even within one species. This is probably because of the fact that the flagellar filament constitutes a much greater antigenic challenge to the host organism, and that the bacterium has, therefore, developed the potential for antigenic variability in response (Macnab, 1987).

Hook. The filament is connected to the cell at the base of the flagellum by the hook. In *E. coli* and *S. typhimurium*, the hook protein has an apparent Mr of 42000 (Wagenknecht et al., 1982). It is believed that the hook acts as a universal joint between the filament and cell to allow efficient rotational motion to the rest of the structure. In addition, the presence of the hook structure on the filament stabilizes its structure. The hook protein is more stable than flagellin protein, so organic solvents and acidic conditions are used to separate the hook protein from the flagellin protein (Silverman, and Simon, 1977). Antisera have been prepared against different hook proteins. Upon comparison, the antisera show a wide range of cross-reactivity with other hook antigens (Kagawa et al., 1973), but antihook serum does not cross react with flagellar filaments (Silverman, and Simon, 1977).

Basal Body. The hook is connected to a complex structure embedded in the cell surface called the basal body (Cohen-Bazire, and London, 1967). The basal body is composed of a rod joined to the hook, and a set of four rings as visualized by electron microscopy. The outer two rings, termed L and P, are spaced apart but are linked by a cylindrical wall. The two inner rings, termed S and M appear to be connected. In a G - cell, the outermost ring, the L ring is associated with the LPS, and the P ring is associated with the peptidoglycan layer.

The S and M rings appear to be associated with the inner membrane. The M ring is directly in contact with the membrane and the S ring is immediately above it (DePamphilis, and Adler, 1971). The L and P rings in conjunction with the cylindrical wall are thought to provide a bushing through which the rod passes. The M ring is postulated to act as a mounting plate for motor rotation and switching components (Macnab, 1987).

## GENETICS

There are several types of mutants defective in flagellar structure and function. These include: a) nonflagellated or partially flagellated mutants (Fla<sup>-</sup>), b) motility mutants (Mot<sup>-</sup>) in which no detectable morphological defects are seen in the flagella, yet it does not rotate, c) flagellin mutants (Hag<sup>-</sup>) which have flagella that are altered in configuration or antigenically, or are lacking flagellin altogether. For *P. aeruginosa* the Hag<sup>-</sup> type of mutants have been described as Mon<sup>-</sup> to represent mutations in the monomeric filament (Wintenberg et al., 1989), d) chemotaxis mutants (Che<sup>-</sup>) which possess a rotating flagellum, but one which is biased for either CCW or CW rotation (Yamaguchi et al., 1986).

Genetics of *E. coli* and *S. typhimurium* Flagella Genes. There are approximately 35 genes involved in flagella assembly, either one (*E. coli*) or two (*S. typhimurium*) flagellin genes and two mot genes (Iino et al., 1988). In *P. aeruginosa*, the genetic makeup is thought to be similar to these models (Tsuda, and Iino, 1983b). The flagella and motility-linked genes are highly clustered on the chromosome in all genera studied (Manab, 1987). For a number of genes, products have been determined and are associated with flagellar structure and function (Macnab, 1987). Approximately 17 genes have been identified to which no function has been attributed.

In *E. coli* and *S. typhimurium*, there is a large cluster that occurs at about 24 minutes and contains 12 genes. This is termed Region I. Most of the basal body genes are located in this region. There are two other clusters termed region II and region III, occurring at about 42 minutes.

Regulation of the Flagella Genes. As discussed previously, all genes related to motility exist within specific operons. This system of operons represents a regulon. In *E. coli* and *S. typhimurium* cells, all of the flagella associated operons are under control of a master regulatory operon consisting of two gene products. This operon is positively regulated by a cyclic AMP/CAP protein complex. Negative regulation is thought to involve the structural protein when it is not synthesized or

incorporated into the filament. These specific operons are thought to be present in *P. aeruginosa*, however, it is not presently understood what the positive control is, since it is probably not regulated by a cyclic AMP/CAP protein complex.

Genetic Makeup of *Pseudomonas aeruginosa* Flagellar Genes. In *P. aeruginosa*, genetic mapping performed by Tsuda and Iino (Tsuda, and Iino, 1983a) has shown the flagellar genes of this organism are clustered into two distinct regions. These regions are termed Region I and Region II, and are located in the very late region of the linkage map of the chromosome (Tsuda, and Iino, 1983b). One motility (*mot*) and five flagella cistrons are identified in Region I, and ten flagella and two chemotaxis genes are identified in Region II. By using multiflagellate mutants and a sequence coding for mercury resistance in Tn501, Tsuda has determined the order of one chemotaxis and 14 flagella genes (Tsuda, and Iino, 1983b). In *P. aeruginosa*, genes coding for related functions usually do not show the close linkage characteristics of *Enterobacteriaceae*. This clustering of the flagellar genes suggests that expression of each *fla* cistron appears to be coordinately regulated, constituting operons. It is apparent that little is known about the genetic makeup and organization of the *P. aeruginosa* flagella. It is an important first step to clone the flagellin gene.

## POSTTRANSLATIONAL MODIFICATIONS OF FLAGELLA

Interest in investigating possible posttranslational modifications of flagellin occurred after a block at N-terminal amino acid ten was detected several years ago. A common posttranslational modification in both eukaryotes and prokaryotes is phosphorylation. Certain observations led us to investigate if *P. aeruginosa* flagella and, in particular, flagellin, is phosphorylated. In *Campylobacter* flagella, phosphorylation of filaments has been suggested by Logan (Logan et al., 1989). It is believed the *Campylobacter coli* flagellin protein probably contains modified serine residues generated by phosphorylation. In *Campylobacter*, the proposed phosphorylated serines occur with a specific region containing two nine amino acid repeating peptides separated by 34 unique amino acids (Logan et al., 1989).

In addition, there are several different posttranslational modifications reported for flagella and pili (Macnab, 1987). In a major class (type IV) of pillin, the N terminus component is modified by N-methyl phenylalanine. This is known to occur in *P. aeruginosa*.

Most phosphoproteins that have been characterized are those from *E. coli* (Cozzzone, 1984). In *E. coli* K-12, 130 different phosphoproteins have been identified by analyzing the effects of protein phosphorylation in various culture conditions including growth on different carbon-sources

in either exponential or stationary phase, amino acid starvation, or heat shock (Cortay et al., 1986b). Analyses of various subcellular preparations have shown that most of the phosphoproteins are located in the cytoplasm; none of them are within the ribosomes or nucleoids, and only three are associated with membranes (Cortay et al., 1986b).

General Characteristics of Phosphorylation. In proteins, the phosphorylated amino acid residues are classified into three groups. The first group is generated by the phosphorylation of the hydroxyamino acids; serine, threonine, and tyrosine, and are acid stable. These are termed O-monophosphoesters, and serine and threonine but not tyrosine are susceptible to cleavage by alkali. The second class is formed by the phosphorylation of the basic amino acids arginine, histidine, and lysine. These are termed phosphoramidates and are stable in alkali, but hydrolyzed by hydroxylamine and acid. The last group, acylphosphates, is produced by the phosphorylation of the acidic amino acids, aspartic acid and glutamic acid. They are extremely labile to acidic and basic conditions, and are degraded by hydroxylamine and reductive cleavage (Martensen, 1984).

Inside of cells, two types of intracellular phosphoproteins have been identified; proteins that are phosphorylated by protein kinases and enzymes that are intermediately phosphorylated, usually at the active sites

(Knowles, 1980). Phosphorylation of histidine or aspartate reflects a requirement for rapid response and turnover in cytoplasmic regulator proteins, whereas serine, threonine or tyrosine modifications are more stable, reflecting their longer half-life functions in surface signaling and enzyme modulation (Stock et al., 1990).

In prokaryotes, as in eukaryotes, the common assay to identify phosphoamino acids is by the use of partial acid hydrolysis with HCl. Typically only O-monophosphoesters have been studied because proteins containing N-phosphates or acyl phosphates are acid labile, and are rapidly degraded before they are identified (Martensen, 1984).

Generally, phosphorylation by protein kinases takes place on the hydroxyamino acids (Roach, 1984), while phosphorylated intermediates in enzymatic mechanisms are either phosphoramidates or acyl phosphates (Huebner, and Matthews, 1985; Fox, and Roseman, 1986). In past reports, errors in the characterization of phosphoamino acids, especially phosphotyrosine have been made when phosphoryl groups act as bridges in phosphodiester linkages to nucleotide protein. The majority of phosphotyrosine found in prokaryotes is thought to be generated by partial acid hydrolysis of proteins to which a nucleotide or nucleic acid is linked via a phosphodiester bond to the phenolic hydroxyl of a tyrosine residue (Foster et al., 1989). In enteric bacteria, no tyrosine protein kinase activity has been isolated (Foster et al., 1989).

If a phosphoprotein containing phosphotyrosine or phosphoserine or phosphothreonine is isolated from cells grown in the presence of labeled Pi, it is important to characterize the type of linkage. To demonstrate the presence of an O-phosphomonoester linkage as opposed to a phosphodiester linkage, the labeled samples are treated with phosphatase and characterized by the release of labeled Pi. Failure to release Pi would indicate the presence of a phosphodiester linkage. This type of linkage is typically confirmed with snake venom nuclease, which removes labeled nucleotide ligated through a 5'-phosphodiester to tyrosine (Martenson, 1984).

The total amount of phosphoamino acids in prokaryotes is much lower than in eukaryotic organisms. In phosphoproteins of *E. coli* cells radiolabeled in vivo, two-dimensional gels have shown 96% of the acid-stable amino acids are phosphoserine residues. The rest are phosphothreonine and phosphotyrosine (Cortay et al., 1986a). Not only is phosphoserine the most common phosphoamino acid in proteins in *E. coli* cells, but also in other bacterial species. Although it does not occur frequently, phosphotyrosine has been identified in other bacteria.

In *E. coli* the protein PP40 is phosphorylated exclusively at tyrosine. This protein is associated with the membrane/ribosome fraction of the cell, but it is not a constituent of either. Instead, it seems to be located at the interface (Cortay et al., 1986b). Phosphotyrosine has also been

identified in proteins of *S. typhimurium* (Cortay et al., 1986a), *Rhodospirillum rubrum* (Holuique et al., 1985), *Clostridium thermohydrosulfuricum* (Londesborough, 1986), *Acinetobacter calcoaceticus*, *Arthrobacter globiformis*, and *Pseudomonas fluorescens* (Cozzone, 1988).

In eukaryotic systems, tyrosine phosphate is well characterized. It is involved in a number of metabolic processes such as viral transformation, growth control, and regulatory events (Hunter, and Cooper, 1985). Several hormone receptors such as insulin and epidermal growth factor, a variety of oncogene proteins, and tubulin function and motility are all modified by phosphorylation of tyrosine. It is thought that tyrosine phosphorylation must have appeared during the evolution of unicellular eukaryotes, and generally would not be present in prokaryotes, while nucleotide posttranslational modification in prokaryotes commonly occurs at tyrosine (Foster et al., 1989). However there are examples in the literature where tyrosine is phosphorylated in prokaryotes (Pairoba, and Vallejos, 1989; Wintenberg et al., 1990). Recently, Vallejos and associates have shown evidence of tyrosine kinase activity in *Rhodospirillum rubrum* (Vallejos et al., 1985).

Biological Significance of Bacterial Phosphorylation. It has been shown that protein kinases and phosphoprotein phosphatases exist within

prokaryotic cells (Cozzzone, 1988). It is likely that, as in eukaryotes, reversible phosphorylation represents a regulatory device for controlling cell functions.

A well characterized example of a reversible phosphorylation mechanism is isocitrate dehydrogenase (IDH). When acetate serves as the carbon source in *E. coli* cells, IDH activity decreases sharply, and at the same time the enzyme is phosphorylated. This modification allows part of the carbon flux to be diverted from the Krebs cycle, at the point of isocitrate, through the glyoxylate cycle. This modification provides energy for the cell and four-carbon intermediate structures for biosynthesis. However, when cells are cultured on glucose and the glyoxylate cycle is not employed, IDH is not phosphorylated, and is fully active (Nimmo, 1984). Therefore, it appears that phosphorylation controls the carbon flux between the Krebs cycle and glyoxylate cycle. It has recently been reported in *E. coli* cells, two enzymes of the glyoxylate bypass are phosphorylated at histidine (Robertson et al., 1987). A similar mechanism has been described in *S. typhimurium* (Maloy, and Wilson, 1987).

Another example of phosphorylation in prokaryotes is in the mechanism of sugar uptake. There are five distinct mechanisms involved in the uptake of external sugars characterized thus far (Postma, and Lengeler, 1985). In G<sup>+</sup> organisms, a regulatory mechanism has been

proposed which responds to changes in energy levels and metabolic concentrations of ATP, sugar phosphates and/or inorganic phosphate. Sugar intake is generated by changing the direction and rate of sugar transport. This response appears to be accomplished by the ATP-dependent phosphorylation of one of the PTS proteins, which is a phosphate carrier protein of the phosphotransferase system (Deutscher, and Saier, 1983).

It is also believed that phosphorylation is involved in the control of bacterial photosynthesis. This has been suggested in the organism *Halobacterium halobium* which uses retinal-containing protein pigments for both photoenergy and photosensory transduction (Spudich, and Stoeckenius, 1980). In the purple photosynthetic bacterium *Rhodospirillum rubrum* phosphorylation is also thought to occur. Two apoproteins of the light harvesting complexes have been detected in both in vivo and in vitro phosphorylation assays. The phosphorylation appears to be reversible, indicating a regulatory function (Pairoba, and Vallejos, 1989). The phosphoamino acids identified are phosphoserine, phosphothreonine, and phosphotyrosine. These authors have identified a protein kinase as tyrosine kinase, and have partially purified it. However, this work apparently has not been repeated in other laboratories (Kaplan, S., personal communication).

Transient protein phosphorylation is an important feature in bacteria sensing and adaptive responses to a wide array of environmental signals including changes in chemical concentrations, viscosity, light, osmolarity, and temperature. Genetic and biochemical approaches have shown that stimulus-response coupling involves two families of signal transduction proteins in which pairs of proteins transduce environmental signals into metabolic changes by regulation of transcription by the second component (Stock et al., 1989). Members of this group are characterized by the presence of a conserved amino acid sequence spanning about 250 amino acids near the C-terminus of the first protein and about 120 amino acids near the N-terminus of the second protein. Examples of the diverse processes controlled by the two component regulatory systems include chemotaxis, nitrogen assimilation, and outer membrane proteins.

The first component of the two component family is a protein kinase that phosphorylates itself by catalyzing the transfer of the gamma phosphate of ATP to a histidine residue. The phosphoryl group is then transferred to an aspartate residue of the second component that is referred to as the regulator. This phosphorylation modifies the activity of the regulator. Finally, a specific phosphatase activity may reset the system restoring the regulator to its unphosphorylated state (Bourret et al., 1991).

Enteric bacteria regulate the expression of nitrogen-regulated (Ntr) genes by responding to changes in the ratios of 2-ketoglutarate and

glutamine (Stock et al., 1989). The ratio of these compounds is constantly relayed to the modulator protein, NtrB (NRII), which controls the activity of the effector NtrC (NRI). NtrB is a protein kinase that catalyzes the phosphorylation of NtrC. Upon phosphorylation, NtrC is a transcriptional activator at nitrogen-regulated promoters (Ninfa et al., 1988).

In the bacterial chemotaxis system, the modulator protein CheA is a protein kinase that catalyzes the phosphorylation of two chemotaxis proteins. First, Che Y, which interacts with the flagellar motor to control the cell's swimming, and Che B, a methylesterase which controls receptor methylation. In both systems, the phosphorylation reactions proceed in a mechanism similar to the Ntr system. In a recent paper, Ninfa et al., demonstrated that the protein kinases regulating chemotaxis and transcription of nitrogen-regulated genes have cross-specificities. In other words, Che A can phosphorylate NtrC, and NtrB can phosphorylate Che Y. (Ninfa et al., 1988). It has been implicated that the sensory transduction in the Ntr and Che systems involves a common protein phosphotransfer mechanism. Also, the conserved C-terminal sequences between the different modulatory proteins reflect conserved protein kinase function, and the conserved N-terminal sequences between effector proteins reflect conserved phosphoacceptor activities.

In recent years, it has been firmly established that phosphorylation occurs in prokaryotes as well as eukaryotes. This modification seems to be of ancient evolutionary origin, like a number of other posttranslational modifications such as acetylation, methylation, or ADP ribosylation which are all universal.

### AIMS OF DISSERTATION WORK

The research discussed in this work was built around a central theme of developing a better understanding of the flagellin filament protein of *P. aeruginosa*. Currently little is known about the biosynthesis, secretion, and assembly of flagella in *P. aeruginosa*. To investigate these aspects, two approaches were taken. First, a molecular approach, whereby the b-type flagellin structural gene was isolated and cloned. Second, a biochemical approach was taken to identify the posttranslational modification of flagella by phosphorylation. This work has been extended with the ultimate aim of explaining a biological role for tyrosine phosphorylation since it is an uncommon event in prokaryotes.

To isolate the flagella structural gene of *P. aeruginosa*, a clone bank of strain PAO1 was constructed in the cosmid vector pLAFR1. To detect the flagellin gene within a recombinant clone of the library, the recombinant

clones were triparentally mated into *fla*<sup>-</sup> mutants and scored for their ability to complement the mutant (Wintenberg-Kelly, and Montie, 1989). The transformation of large recombinant plasmids in *Pseudomonas* is difficult unless a conjugative helper plasmid is employed. To determine if complementation has occurred, motility assays were performed, these results were confirmed by colony blot assays with monoclonal and polyclonal flagellar antisera.

Following isolation of positive recombinant clones, two different approaches were employed to determine which region of the 25 kb PAO1 fragment was necessary for expression of the flagella. The first approach was deletion mapping. The positive clones were further digested with restriction enzymes and religated and triparentally mated back into *P. aeruginosa fl*<sup>-</sup> cells, and complementation was scored. The second approach involved transposon mutagenesis with Tn501. The sites of the transposon insertions were mapped by analyses of restriction digests. To confirm these results, a gene replacement technique was utilized in which the clones containing the Tn501 insertions in the *fla* region were mated into wild-type PAO1 cells, and the loss of flagellar structure was characterized.

The second major portion of this research was identification and characterization of the posttranslational modification occurring in the flagellin filament of *P. aeruginosa* (Wintenberg-Kelly et al., 1990). It

appears that the only hydroxyamino acid residue that is phosphorylated is tyrosine. This finding is unusual because tyrosine phosphorylation is not prevalent in prokaryotes.

Both a- and b-type purified flagellin from *P. aeruginosa* grown in radiolabeled phosphate were shown to be phosphorylated. Initial characterization of labeled flagellin was by detection on autoradiograms of SDS-PAGE gels and immunoblots with anti-flagella antibody. Thin layer electrophoresis (TLE) of a partial acid hydrolysate flagella filaments revealed that the radioactivity was in phosphotyrosine, and three phosphopeptides containing phosphotyrosine. Intact flagella filaments have been shown to react specifically with antiphosphotyrosine monoclonal antibody. Also, the labeled filaments were susceptible to phosphatase, indicating a monophosphoester linkage. The biological role of these modifications remains to be elucidated. However, some ideas are presented in Part VI, based upon the recent experimental evidence.

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PART II

DETECTION OF NONPOLYMERIZED FLAGELLAR FILAMENT  
PROTEIN IN FLA<sup>-</sup> MUTANT STRAINS OF *PSEUDOMONAS*  
*AERUGINOSA*.

## INTRODUCTION

In order to clone the b-type flagella filament structural gene, flagellin, it must first be located. This can be accomplished using a *P. aeruginosa* strain containing a mutation in the flagellin gene region. There are a number of genes encoding products necessary to produce a functional flagella, including over 30 genes necessary for synthesis and assembly (Tsuda, and Iino, 1983b). Of the many genes necessary for complete assembly of the apparatus, a large fraction are necessary for any detectable flagellar structure (Suzuki, and Komeda, 1981). Defective genes for later flagella components of the basal body, for the hook, and for the filament can, however, permit production of partial flagellar structures.

Therefore, in order to characterize the nature of *P. aeruginosa* Fla<sup>-</sup> mutants and isolate a *fla*<sup>-</sup> mutant, a colony blot assay was developed utilizing LPS-free, absorbed antibody directed against the flagellin protein (Montie et al., 1987) and goat anti-rabbit immunoglobulin G peroxidase conjugate. This assay provides a method for easy detection of the monomeric flagellin protein in both Fla<sup>-</sup> mutants and flagella-containing strains of *Pseudomonas aeruginosa*. This assay was able to

detect less than eight nanograms (ng) of purified flagellin protein spotted directly on nitrocellulose.

The colony blot assay was derived from a similar procedure used by Nicas (Nicas et al., 1985). In that study, CF clinical isolates were tested for the presence of exoenzyme S, an ADP-ribosyl transferase enzyme. Essentially this technique is a modified Enzyme Linked Immunosorbent Assay (ELISA) which utilizes nitrocellulose membranes rather than a plastic microtiter plate (Montie, and Anderson, 1988).

The major difference in the two methods is that, unlike the ELISA, the colony blot technique allows the use of whole colonies instead of antigen. Also, unlike an ELISA, blotting of colonies onto nitrocellulose is immediate; there is no overnight incubation to allow the protein to stick to the ELISA plate. Results from a colony blotting assay can be read visually, no special or expensive equipment is necessary. A brief explanation of the colony blot procedure follows. Colonies are incubated on agar plates and the colonies are blotted onto washed nitrocellulose disks. After blocking, the disks are incubated with the anti-flagellin antibody, followed by goat antirabbit IgG peroxidase conjugate. Strains which are Fla<sup>+</sup> (containing flagellin, the monomeric protein), turn yellow upon development with a orthophenylenediamine /H<sub>2</sub>O<sub>2</sub> solution.

In this study, a number of *Pseudomonas aeruginosa* laboratory strains were examined to characterize the nature of their Fla<sup>-</sup> phenotype. The colony blot technique described here was a fundamental part of this study.

## MATERIALS AND METHODS

Bacteria and Media. The bacteria used in this study are listed in Table II-1, along with their flagellar phenotypes. The MT strains were a gift from Drs. M. Tsuda and T. Iino (Tsuda, and Iino, 1983a, b). The wild type strain PAO1 and its corresponding Fla<sup>-</sup> mutants, AK1147, AK1152, AK1153 were graciously supplied by A. Kropenski (Drake, and Montie, 1987). All of the cystic fibrosis (CF) isolates were from sputum and supplied by M.J. Thomassen (Luzar et al., 1985a). Bacteria were routinely cultured in Luria broth (LB) (10% pancreatic digest of casein, 0.5% yeast extract, 0.5% NaCl). Cells for colony blot assays were prepared on Luria agar (LA) which was prepared with LB and 1.6% agar. Cells for motility assays were grown in Luria broth overnight and inoculated on medium described below. For isolation of flagella protein from supernatants of *P. aeruginosa* cells, bacteria were grown in

Table II-1. The presence or absence of polymerized flagella of various *Pseudomonas aeruginosa* strains used in this study.

| <u>Strain</u>                   | <u>Flagella</u> <sup>a</sup> | <u>Strain</u>                        | <u>Flagella</u> |
|---------------------------------|------------------------------|--------------------------------------|-----------------|
| <u>CF Isolates</u> <sup>b</sup> |                              | <u>MT Strains</u> <sup>d</sup>       |                 |
| 66g                             | -                            | M10                                  | +               |
| 86f                             | +                            | MT354                                | -               |
| 96e                             | -                            | MT519                                | +               |
| 155c                            | -                            | MT1007                               | -               |
| 402c                            | +                            | MT1008                               | +               |
| 409c                            | +                            | MT1032                               | -               |
| 414ii                           | -                            | MT1058                               | -               |
| 414nn                           | -                            | MT1100                               | +               |
| 414rr                           | -                            | MT1129                               | -               |
| 423mm                           | -                            | MT1200                               | +               |
| 437e2                           | +                            | MT1200-80                            | +               |
| 448bb                           | -                            | MT1211                               | -               |
| 448hh                           | -                            | MT1220                               | -               |
| <u>AK strains</u> <sup>c</sup>  |                              | <u>Other strains</u> <sup>e, f</sup> |                 |
| AK1147                          | -                            | PAO1                                 | +               |
| AK1152                          | -                            | PA103                                | -               |
| AK1153                          | -                            | PA10329                              | -               |

<sup>a</sup>The presence or absence of flagella was determined by motility assays and light microscopy.

<sup>b</sup>Cystic Fibrosis clinical isolates

<sup>c</sup>A. Kropenski laboratory strains

<sup>d</sup>M. Tsuda laboratory strains

<sup>e</sup>Strains from our laboratory

<sup>f</sup>PAO1, a positive control; *P. fluorescens*, a negative control, in all blotting experiments.

Mineral Salts Medium which contained:  $4 \times 10^{-2}$  M  $K_2HPO_4$ ,  $2.2 \times 10^{-2}$  M  $KH_2PO_4$ ,  $7.5 \times 10^{-3}$  M  $(NH_4)_2SO_4$ ,  $2.0 \times 10^{-4}$  M  $MgSO_4$ ,  $6 \times 10^{-6}$  M  $FeCl_3$  with 0.4% sodium succinate (Allison et al., 1985).

Motility Assays. To determine if the various *P. aeruginosa* strains tested were motile, the bacteria were stab-inoculated into the center of a 50 mm petri plate containing 10 ml of motility medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.3% agar). The plates were incubated cell side up at 37°C and examined over a period of 24 h for colony spreading and distinctive growth rings, a characteristic of motile cells. The colony diameter was measured with vernier calipers. Each assay was done in triplicate, and the values given represent an average of these three values.

Colony Blot Assays of Fla<sup>+</sup> and Fla<sup>-</sup> Cells. Colonies of the various strains from Luria agar plates were stab-inoculated on 150 mm petri plates containing Luria agar and incubated at 37°C for 20-24 h. The nitrocellulose (Schleicher and Schuell, Inc. Keene, NH) was carefully cut into disks of a smaller diameter than the petri plate (140 mm), washed in a solution of 0.2% sodium dodecyl sulfate (SDS) in phosphate buffered saline (PBS: 14 mM  $NaH_2PO_4$ , 36 mM  $Na_2HPO_4$ , 28 mM NaCl) and

sterilized by autoclaving. The *P. aeruginosa* colonies were blotted onto the sterile nitrocellulose disks and blocked for 30 min at 37°C using 3% gelatin-PBS solution). After washing with PBS to remove any colony debris, the nitrocellulose disks were placed in a 1% gelatin-PBS solution containing the LPS free, absorbed polyclonal antiserum diluted (1:1000) directed against monomeric flagellin protein. The disks were incubated at 37°C for two h with gentle shaking. After repeated washings with PBS to remove any antibody not bound to antigen, the nitrocellulose disks were placed in a 1% gelatin-PBS solution containing the secondary antibody, goat anti-rabbit IgG conjugated to peroxidase, diluted 1:10000.

Following an one h incubation at 37°C with gentle shaking, and washing with PBS; the disks were developed using a substrate solution of orthophenylenediamine (1mg/ml) and H<sub>2</sub>O<sub>2</sub> (0.03%) in Tris-buffered saline (TBS), pH 7.4 (10 mM Tris, pH 7.4, 0.9% NaCl). The reaction was stopped by repeated washings with H<sub>2</sub>O. The disks were stored between sheets of filter paper in the dark. Upon development, any colony which produced flagellin antigen was yellow and was considered positive. The amount of antigen was qualitatively associated with the color intensity of the reaction. Each of the samples were compared to known positive and negative controls. In certain experiments, the cells were lysed with chloroform after blotting. This was accomplished by placing the

nitrocellulose disks in a glass chamber which had been previously equilibrated with chloroform. The disks were then incubated for 30 min.

Preparation of Supernatant Samples. The different strains of bacteria were inoculated from slants into 5 ml of sterile MSM containing 0.4% succinate and incubated at 37°C overnight. After growth, these cells were inoculated into 500 ml of fresh MSM with 0.4% succinate and incubated at 37°C for 20-24 h in a gyratory shaker water bath. The cells were harvested by centrifugation (8000 x g) for 25 min. The supernatants were retained following centrifugation and dialyzed in 1 mM phosphate buffer, pH 7.5 for two days with frequent buffer changes. The supernatant samples were concentrated by lyophilization. Immediately, following centrifugation, the supernatant was filtered through 0.45  $\mu$ m filters to remove any remaining cells. In later experiments this was found to be unnecessary, since no viable cells were detected in the supernatant after centrifugation.

Polyacrylamide Gel Electrophoresis. Lyophilized supernatant samples and molecular weight markers were suspended in 0.5 M Tris-Cl buffer (pH 6.8) with 2.5% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue. Samples were heated at 100°C for five minutes

in a heat block. SDS-polyacrylamide gel electrophoresis (PAGE) was conducted with either a 10% homogeneous acrylamide gel or with a 10-15% gradient gel. The gradient gels used were precast gels for the Phast system.

The 10% acrylamide separation gel was poured in a thin (0.75 mm) vertical slab unit (Hoeffer Scientific, San Francisco, CA). It was composed of 10% acrylamide/NN'-methylene-bis-acrylamide (30:0.8), and 0.1% SDS in 0.375 M Tris-Cl (pH 8.8). The stacking gel consisted of 5% acrylamide, 0.1% SDS in 0.125 M Tris-Cl (pH 6.8). Following de-aeration, gels were polymerized with 10% ammonium persulfate and tetramethylethylenediamine (TEMED). The supernatant samples (20-50  $\mu$ l) were loaded into the gels with a Hamilton syringe and electrophoresis was carried out at constant current of 20 mA per gel. The running buffer was 0.025 M Tris base, 0.19 M glycine, and 3.5 mM SDS.

The Phast system Phastgel (Pharmacia, Piscataway, NJ) was composed of a continuous 10 to 15% acrylamide gradient in 0.112 M acetate and 0.112 M Tris-Cl (pH 6.4). Each gel was approximately 0.45 mm thick, with a 32 mm separation zone. Phastgel SDS buffer strips were composed of 0.2 M glycine, 0.2 M Tris-Cl, and 0.55% SDS (pH 7.5) with 2% agarose. Samples and molecular weight markers (1-2  $\mu$ l) were added with an applicator, and electrophoresis was carried out at 10 mA per gel.

Following electrophoresis, protein bands were visible when gels were stained using Wrays Silver Stain (Wray et al., 1981) or were blotted onto nitrocellulose for immunoblots blots. The Wrays Protein Silver stain for the Phast system included several initial steps in 50% ethanol/10% acetic acid and 10% ethanol/5% acetic acid. Gels were then sensitized with 8.3% glutardaldehyde, and washed with water. Gels were stained in 0.25% (w/v) silver nitrate and developed in 2.5% sodium carbonate with 400 microliters (ul) of formaldehyde per liter of solution. Development was stopped with 5% acetic acid.

Immunoblotting of Supernatant Samples. Following electrophoresis, the proteins were transferred to prepared nitrocellulose by diffusion for two hours at 37°C or with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio Rad Labs, Richmond, CA) according to manufacturers instructions. The sandwich for the diffusion method was prepared as follows. Several layers of Whatman 3M paper were placed on a glass plate. The Phast gel in which the proteins were separated by electrophoresis was placed on top. The gel was flooded with a 3% bovine serum albumin (BSA)-TBS solution. The nitrocellulose was placed carefully on the gel, and the air bubbles were removed with a roller. To complete the sandwich, the nitrocellulose was covered with Whatmann 3M paper and a glass plate. The blots were labeled for orientation on the back side of the

nitrocellulose with a black ink pen. The nitrocellulose blots were blocked with 3% BSA-TBS solution for one hour. The blots were incubated at 37°C with absorbed antisera (either a- or b-type) diluted 1:1000 in NETG buffer (150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 50 mM Tris-Cl, pH 7.4). After a two hour incubation, the blots were washed in NETG buffer to remove any unbound antibody.

A peroxidase conjugated to goat anti-rabbit IgG solution diluted 1:10000 was applied to the blots and incubated at 37°C for two h with gentle shaking. After washing, the blots were developed in a TBS solution containing orthophenylenediamine(1mg/ml) and H<sub>2</sub>O<sub>2</sub> (0.03%) as the substrate as described in the colony blot assay. Upon development, any band which possessed the flagellar antigen was considered positive by a yellow color on the nitrocellulose.

## RESULTS

Motility Assays and Flagella Determination. To determine the flagella phenotype of the various *P. aeruginosa* strains listed in Table II-1, cells were examined under a bright field compound microscope. Under 400X magnification, flagellated cells were easily distinguished by their swimming movement patterns from non-motile cells which only possessed

Brownian movement. As shown in Table II-1, all of the Kropenski isolates, the PA103 strains, and nearly all of the CF isolates were Fla<sup>-</sup>, while the MT isolates were nearly equally distributed.

Motility assays based upon a method first described by Adler (Adler, 1966) were performed to confirm the presence or absence of flagella based upon movement. If a bacterium possessed a functional flagella; after a period of time, characteristic growth rings were clearly visible on a motility plate.

Motility assays from representative strains are graphically represented in Figures II-1, II-2, and II-3. The colony diameters were measured over a period of 24 h. Each assay was done in triplicate and the values were averaged. A known positive control (PAO1) was run with each assay. Representative CF isolates are shown in Figures II-1 and II-2. The motile isolates, 86f (Figure II-1) and 402c (Figure II-2) both appeared to show a three-fold increase in colony diameter by seven to eight hours. These strains were more motile than the control strain, PAO1. The non-motile strains showed very little increase in motility over the 24 h period: colony diameters were than 5 mm, 11% of the known positive control PAO1 motility. These strains grew in motility medium, however did not display the growth ring characteristics typical of motile bacteria.

The AK strains and MT strains are represented in Figure II-3. In this figure, all of the isolates were non-motile, hence these strains did not

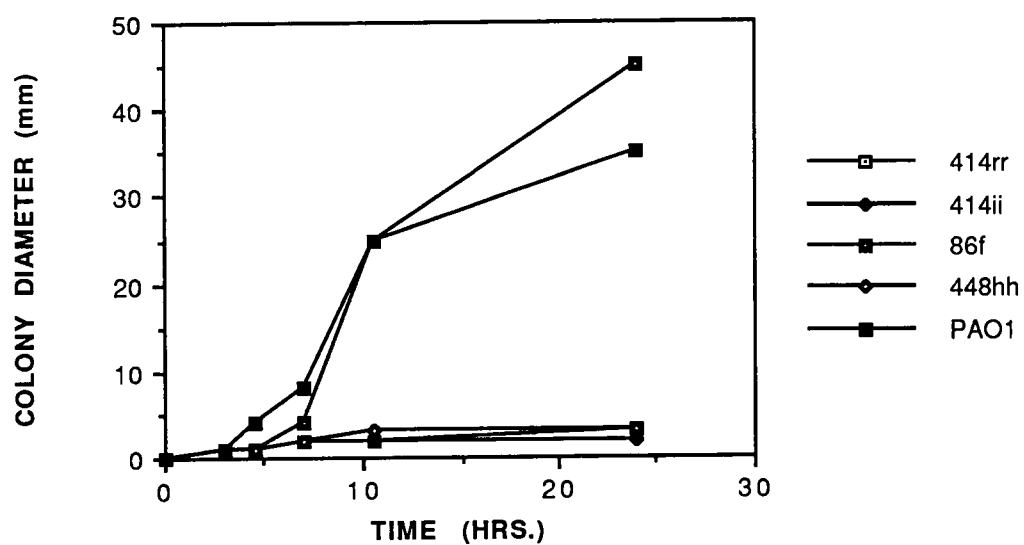


Figure II-1. Migration of specific CF clinical isolates in motility assays. The colony diameter (mm) was measured over a period of 24 hours and compared to known positive and negative controls. Each assay was done in triplicate.

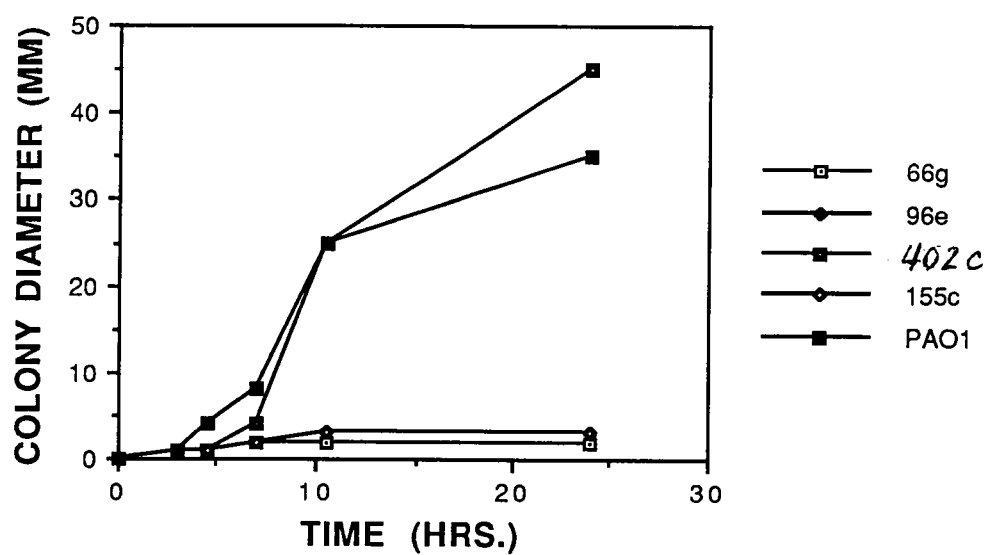


Figure II- 2. Migration of more CF clinical isolates in motility assays. The colony diameter (mm) was measured over a period of 24 hours and compared to known positive and negative controls. Each assay was done in triplicate.

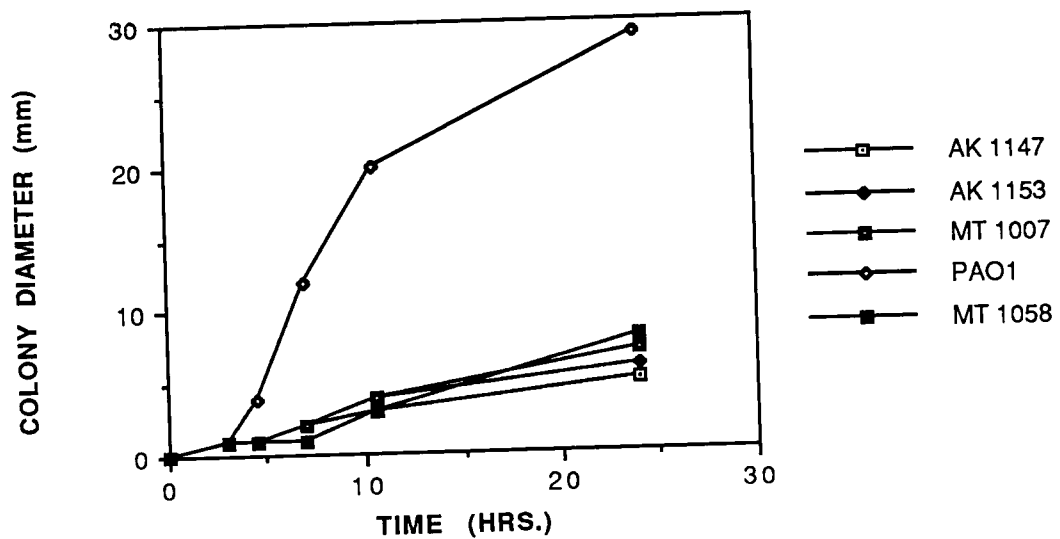


Figure II-3. Migration of specific AK strains and MT strains in motility assays. The colony diameter (mm) was measured over a period of 24 hours and compared to known positive and negative controls. Each assay was done in triplicate.

synthesize a functional flagella. These strains appeared to have larger colony diameters on average than those strains represented in Figures II-1 and II-2. Colony diameters of the AK strains (AK1147, AK1153) were no greater than 6 mm or only 20% of the known positive control motility of PAO1 over the 24 h period. While the MT colonies (MT1058 and MT1007) were not greater than 8 mm in diameter, 27% of the control diameter of PAO1. Similar results were seen with the other MT strains listed in Table II-1. One possible explanation for this difference was the amount of inoculum or different growth rates of the different strains. However, compared to the control strain, it was easy to discern the non-motile strains from the motile ones.

Colony Blot Assays of Fla<sup>+</sup> and Fla<sup>-</sup> Cells. Although the non-motile strains tested produced no detectable flagella, the nature of the mutations were not characterized. Since flagella synthesis and assembly is very complex, mutations which generate a Fla<sup>-</sup> phenotype could involve a number of different genes and not necessarily the structural flagellin filament gene. Upon closer examination, it might be observed that a fraction of these mutants possess partial or defective structures. To determine if the monomeric flagellin protein (antigen) was being synthesized by the Fla<sup>-</sup> mutants, a colony blot was developed initially

using a-type and b-type polyclonal anti-flagella antiserum. In later experiments, anti-b-type flagella monoclonal antibody was also utilized (Anderson et al. , 1989). In all blotting experiments, a positive control, *P. aeruginosa* PAO1 wild-type strain, and a negative control, *Pseudomonas fluorescens* were used.

Colony blot results of the CF isolates are shown in Table II-2. Strains 66g, 423mm, 448bb, although phenotypically Fla<sup>-</sup>, produced detectable or strongly detectable antigen when compared to known Fla<sup>+</sup> and Fla<sup>-</sup> strains. Also, strain 96e produced weakly detectable antigen. This was an unexpected finding since the antigen was detected using whole cells. This suggested that the monomers were being synthesized and secreted outside of the cells. The cells were also lysed with chloroform on nitrocellulose, and upon development the intensity of the reaction was not visibly greater. This suggested that there was not a large concentration of flagellin inside of the Fla<sup>-</sup> Mon<sup>+</sup> cells unable to be secreted out. Fla<sup>-</sup> strains 155c, 414ii, 414nn, 414rr, and 448hh all produced no detectable flagellin antigen in either lysed or whole cells. This finding strongly suggested that at least some of the strains contained a mutation within the structural gene, although the mutation could be elsewhere.

The other strains are listed on Table II-3 (MT strains) and Table II-4 (AK strains and PA103 strains). Although some of the MT strains and all

Table II-2. Colony blots used for the detection of monomeric flagellin antigen in CF clinical isolates.

| <u>Strain</u>         | <u>Flagellin Antisera</u> <sup>a</sup> |                    |                         |
|-----------------------|----------------------------------------|--------------------|-------------------------|
|                       | <u>anti b-type</u>                     | <u>anti a-type</u> | <u>NRS</u> <sup>b</sup> |
| 66g                   | ++                                     | +                  | -                       |
| 86f                   | +++                                    | +                  | -                       |
| 96e                   | +                                      | -                  | -                       |
| 155c                  | -                                      | -                  | -                       |
| 402c                  | ++                                     | +                  | -                       |
| 409c                  | ++                                     | +                  | -                       |
| 414ii                 | -                                      | -                  | -                       |
| 414nn                 | -                                      | -                  | -                       |
| 414rr                 | -                                      | -                  | -                       |
| 423mm                 | +++                                    | +                  | -                       |
| 437e2                 | +++                                    | +                  | -                       |
| 448bb                 | +++                                    | +                  | -                       |
| 448hh                 | -                                      | -                  | -                       |
| PAO1                  | +++                                    | +                  | -                       |
| <i>P. fluorescens</i> | -                                      | -                  | -                       |

<sup>a</sup>Detection of the antigen was determined by the color intensity of each blot compared to known positive and negative control blots.

+++ ,strong detection; ++, detection; +, weak detection; -, no detection.

<sup>b</sup>NRS-Normal rabbit serum.

Table II-3. Colony blots used for the detection of monomeric flagellin antigen in MT strains.

| <u>Strain</u>         | <u>Flagellin Antisera</u> <sup>a</sup> |                    |                         |
|-----------------------|----------------------------------------|--------------------|-------------------------|
|                       | <u>anti b-type</u>                     | <u>anti a-type</u> | <u>NRS</u> <sup>b</sup> |
| M10                   | ++                                     | +                  | -                       |
| MT354                 | ++                                     | +                  | -                       |
| MT519                 | +                                      | -                  | -                       |
| MT1007                | ++                                     | -                  | -                       |
| MT1008                | +++                                    | +                  | -                       |
| MT1032                | ++                                     | +                  | -                       |
| MT1058                | +++                                    | -                  | -                       |
| MR1100                | ++                                     | -                  | -                       |
| MT1129                | ++                                     | +                  | -                       |
| MT1200                | +++                                    | +                  | -                       |
| MT1200-80             | +                                      | -                  | -                       |
| MT1211                | ++                                     | -                  | -                       |
| MT1220                | +++                                    | +                  | -                       |
| PAO1                  | +++                                    | +                  | -                       |
| <i>P. fluorescens</i> | -                                      | -                  | -                       |

<sup>a</sup>Detection of the antigen was determined by the color intensity of each blot compared to known positive and negative control blots.

+++ ,strong detection; ++, detection; +, weak detection; -, no detection.

<sup>b</sup>NRS-Normal rabbit serum

Table II-4. Colony blots used for the detection of monomeric flagellin antigen in AK strains and "other" strains.

| <u>Strain</u>         | <u>Flagellin Antisera</u> <sup>a</sup> |                    |                         |
|-----------------------|----------------------------------------|--------------------|-------------------------|
|                       | <u>anti b-type</u>                     | <u>anti a-type</u> | <u>NRS</u> <sup>b</sup> |
| AK1147                | ++                                     | +                  | -                       |
| AK1152                | ++                                     | +                  | -                       |
| AK1153                | +++                                    | +                  | -                       |
| PA103                 | +++                                    | +                  | -                       |
| PA10329               | +++                                    | +                  | -                       |
| PAO1                  | +++                                    | +                  | -                       |
| <i>P. fluorescens</i> | -                                      | -                  | -                       |

<sup>a</sup>Detection of the antigen was determined by the color intensity of each blot compared to known positive and negative control blots.

+++,strong detection; ++, detection; +, weak detection; -, no detection.

<sup>b</sup>NRS-Normal rabbit serum

of the strains listed in Table II-4 (except the positive control) were Fla<sup>-</sup>, they all produced detectable and strongly detectable antigen when compared to known control strains. As described above for the CF isolates, similar results were seen when the cells were lysed with chloroform. It was interesting that all of these Fla<sup>-</sup> isolates produced detectable amounts of flagellar antigen. The isolates which were Fla<sup>-</sup> but produced detectable levels of monomeric flagellin filament protein were termed Fla<sup>-</sup>, Mon<sup>+</sup>.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Flagellin antigen detected in Fla<sup>-</sup> mutants was examined by comparing the electrophoretic migration of proteins from supernatant samples to the migration patterns of purified monomeric flagellin protein of strain M2. This method is a modified version of one first described by Laemmli (Laemmli, 1970). The results of the silver stained Phast gels of supernatant samples are seen in Figure II-4. In Figure II-4A, supernatant samples from MT1058, AK1153, PA103, MT1007 (Lanes 1, 2, 5, 8) protein bands were present which comigrated with M2 flagellin at 53 kDa. In Lane 4, which contained the 414ii supernatant sample, no band was present. Likewise, in Figure II-4B, Lanes 4, 6, 8; protein bands comigrated with M2 flagellin.

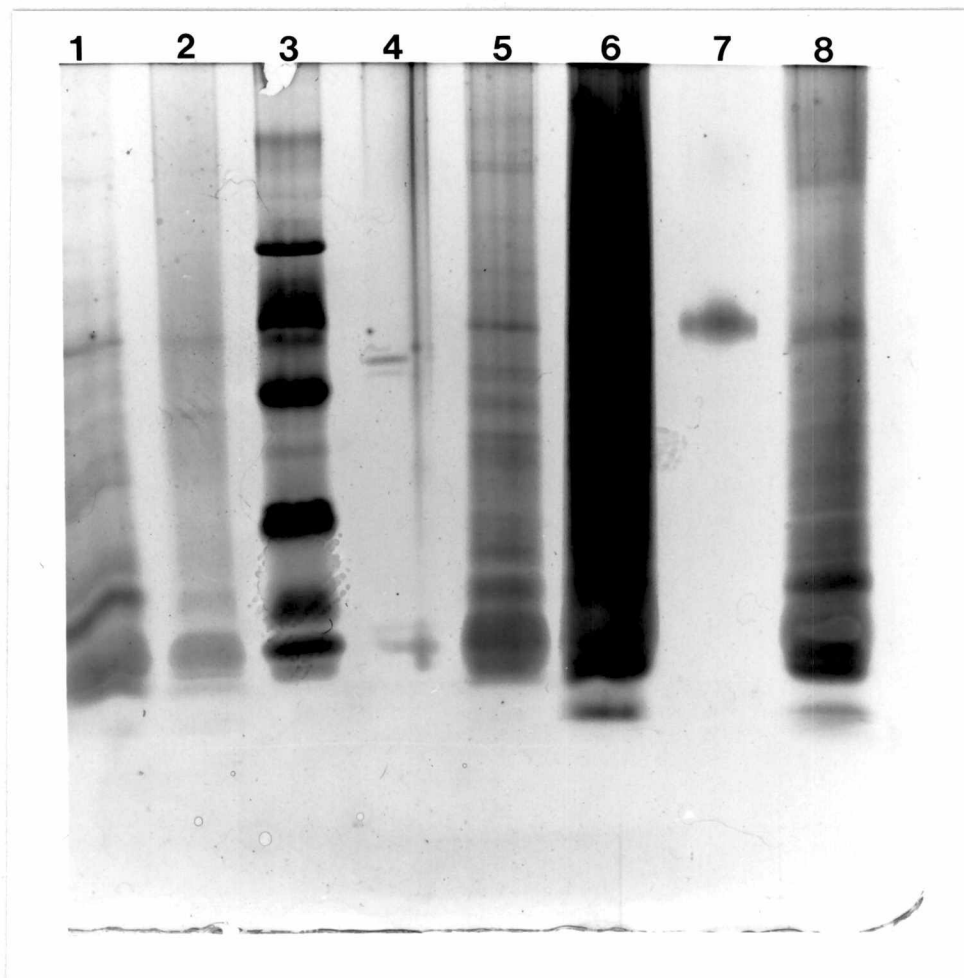


Figure II-4. SDS-polyacrylamide gels of supernatant samples. The migration of the supernatant samples were compared to the migration of purified flagellin from strain M2. The gels were silver stained. A) Lanes: 1, MT1058; 2, AK1153; 3, Marker; 4, 414ii; 5, PA103; 6, M2 Ag; 7, M2 Ag; 8, MT1007.

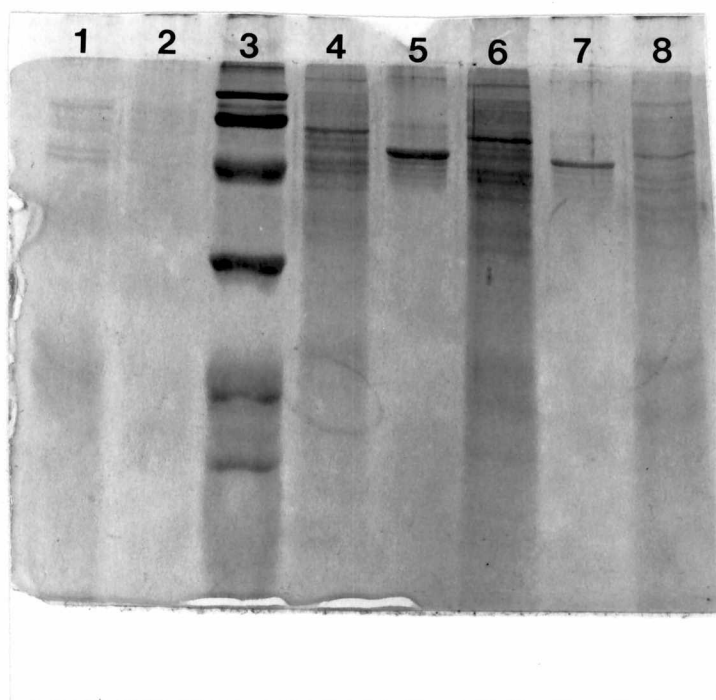


Figure II-4 (continued). B) Lanes: 1, 414ii; 2, 414ii; 3, molecular weight marker; 4, AK1147; 5, M2 Ag; 6, AK1153; 7, M2 Ag; 8, AK1152. AK1147, AK1152, AK1153 (Lanes 4, 6, 8) protein bands also comigrated with M2 purified flagellin.

These data suggested the presence of flagellin antigen in the supernatants of Fla<sup>-</sup> Mot<sup>-</sup> mutant strains grown in MSM, and agreed with the colony blot data. However, the supernatant of *P. aeruginosa* cells contains a large number of extracellular proteins (Woods, 1987). Therefore to confirm the presence of flagella antigen in the Fla<sup>-</sup> mutants, immunoblotting was performed.

Immunoblotting. To confirm the presence of flagellin antigen in the supernatants, immunoblots using anti-flagellin polyclonal antibody was employed as first described by Towbin (Towbin et al., 1979). The immunoblot shown in Figure II-5, a single band of protein from the MT1058, AK1153, MT1007, and AK1147 samples (Lanes 1, 4, 5, 7) was present, supernatant from 414ii (Lane 6) showed no reactivity with the anti-flagellin antibody. These blots showed as in the colony blot assays and SDS-PAGE gels, detectable monomeric flagellin protein in Fla<sup>-</sup> *P.*

*aeruginosa* strains MT1007, MT1058, AK1147, and AK1153. However, no flagellar filament protein was detected in the CF isolate 414ii in colony blot assays, SDS PAGE gels, or immunoblots.

Native Gels of the Supernatant Gels. To determine if the mutants which were Fla<sup>-</sup> Mon<sup>-</sup> excreted flagellin in a monomeric form or in some

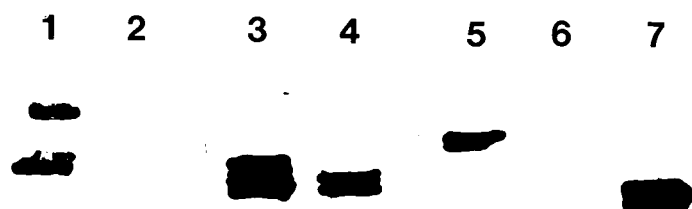


Figure II-5. Immunoblotting of the supernatant samples. Immunoblots were performed as described in Materials and Methods section and the supernatant samples were compared to M2 antigen. Lanes: 1, MT1058; 2, molecular weight marker; 3, M2 Ag; 4, AK1153; 5, MT1007; 6, 414ii; 7, AK1147.

aggregation form, native gels were run on the supernatant samples (Garfin, 1990). As shown in Figure II-6, no monomeric flagellar units seen corresponding to the control purified antigen from M2 (Lane 3). As were detected in supernatants from AK1152, MT1058, PA103, AK1153 (Lanes 1, 2, 3, 7, and 8). Some monomeric flagellin was present in Lane 4 with M2 antigen.

Since no monomeric flagellin was detected in the Fla<sup>-</sup> Mon<sup>+</sup> strains, this suggested that the flagellin was being released in some type of polymerized form, but not as a functional flagellum. It was possible that there was not enough protein to be visible upon staining. Another possibility was that self aggregation of the monomers occurred. This is supported since no strong 53kDa band was seen even in the control M2 lane of the native gels (Lane 4). It has been well documented that under proper conditions, the flagellin monomers will self-aggregate, forming polymers of different sizes (Asakura, 1970).

## DISCUSSION

The mutational loss of flagella in a-type and b-type strains of *P. aeruginosa* has been shown to decrease the virulence in burned-mouse models (Montie et al., 1982). In this study a number of *P. aeruginosa*

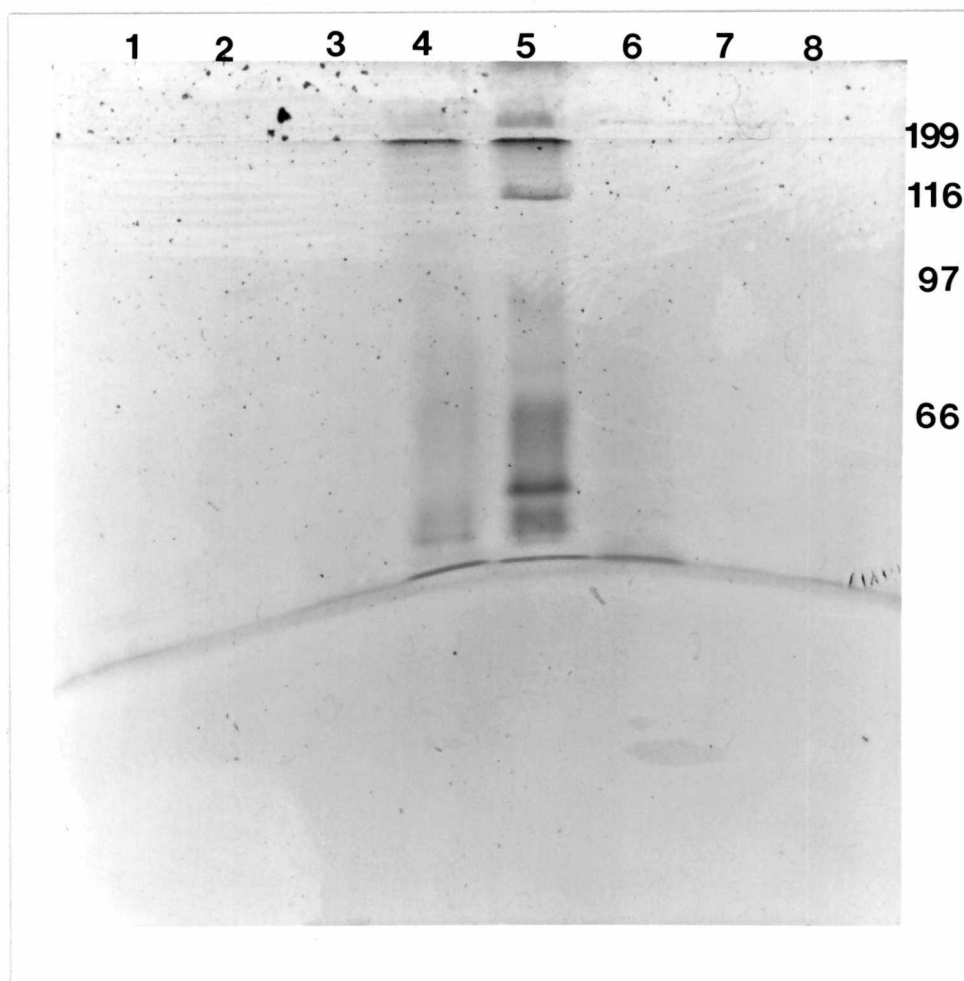


Figure II-6. Presence of aggregated monomers in supernatant samples as determined by native gel electrophoresis. Lanes: 1, AK1152; 2, MT1058; 3, PA103; 4, M2 Ag; 5, molecular weight marker; 6, 414ii; 7, AK1153; 8, MT1007. Numbers on the right are molecular weight standards in kDa.

strains were investigated. It was found that although no functional flagella were assembled, the majority of these Fla<sup>-</sup> strains produced the monomeric protein, flagellin. Therefore, the mutations contained in the flagella region of the genome are present on the DNA in regions other than those encoding the flagellin structural gene.

It is of interest why such a large proportion of these Fla<sup>-</sup> strains are producing flagellin in either a monomeric form or a small polymerized form, and secreting it into the supernatant in detectable amounts. One suggestion is that the location of the mutation is in an area which affects the polymerization or assembly but not synthesis or excretion. Secondly, partial or defective hook or basal structures might be present in at least some of the mutants. This would at least in part, explain the presence of flagellin.

It remains unclear if flagellin proteins detectable in these Fla<sup>-</sup> strains are released as a monomer or in a polymerized form. The native gels should have shown this. Due to the self-aggregating nature of flagellin protein (Asakura, 1970), it is possible the aggregation, occurs after the release of the filament protein. However, the filament is not fully polymerized as determined by the Fla<sup>-</sup> phenotype. Electron micrographs of certain CF strains studied here and previously investigated by Luzar

(Luzar et al., 1985b), and Drake and Montie (Montie et al., 1987), clearly showed no polymerized flagella.

Motility of *P. aeruginosa* may be important in early lung colonization in CF patients, whereas after initial colonization and establishment of chronic infection motility and chemotaxis may be less important. The importance of flagellated cells for the dissemination of *P. aeruginosa* is illustrated by a model proposed by Costerton (Costerton, 1984). He described two types of *Pseudomonas* cells: microcolonies of bacteria (Fla<sup>-</sup>) embedded in alginate and thus less accessible to phage or antibodies; or single motile cells swarming to establish new colonies. This model would explain why many of the CF isolates characterized are Fla<sup>-</sup> (Luzar et al., 1985b).

Interestingly, the only strains in which no monomer was produced were isolates from chronically colonized cystic fibrosis patients. In addition to their Fla<sup>-</sup> and Mot<sup>-</sup> phenotypes, these isolates had deficiencies in a number of virulence factors including: protease and LPS. It has been suggested from experiments using animal models that relatively low invasive virulence may be a common property of strains involved in this type of chronic infection (Luzar et al., 1985b).

From the research presented here, the colony blot assay using anti a-type or b-type antisera appears specific for detecting flagellar filament in

both Fla<sup>-</sup> and Fla<sup>+</sup> strains of *P. aeruginosa*. Testing certain Fla<sup>-</sup> Mon<sup>+</sup> using colony blot technique and gel electrophoresis and immunoblotting, each gave the same results. In addition, results from colony blot from this research and previous ELISA results (Montie, and Anderson, 1988) are in agreement.

The colony blot method provides some advantages for antigen detection over the other methods such as an ELISA for a number of reasons. First, its ability to distinguish a- and b-type strains. Secondly, intact bacteria are used, and therefore isolation and purification of flagella antigen is not required. In addition, no overnight incubation is required for the antigen to bind to an ELISA plate. Thirdly, an important advantage in utilizing this method is its simplicity; positive isolates can be identified visually, eliminating the need for expensive equipment. Finally, unlike immunoblots, no blotting time is required (Nicas et al., 1985).

This method may have diagnostic use for *P. aeruginosa* detection and typing for these reasons: a) The flagellar reactive epitope is well conserved in all isolates. Only two antigen sources are needed, the b-type antigen and a common cross-reacting epitope. b) The flagellum is well exposed, reactive, and present on over 99% of invasive clinical isolates.

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PART III

CLONING AND EXPRESSION OF *PSEUDOMONAS AERUGINOSA*  
FLAGELLIN IN *ESCHERICHIA COLI*.

## INTRODUCTION

*P. aeruginosa* has been recognized for several years as a serious threat to certain compromised individuals (Cross, 1979). Previous data indicated not only that flagella of *P. aeruginosa* contributed to virulence but also flagellar protein serves as a protective antigen. Studies with nonmotile isogenic mutants showed severely reduced virulence (McManus et al., 1980; Montie et al., 1982) and that the flagellar antigen was protective both as an actively administered immunogen (Holder et al., 1982) and in passive studies in compromised animals (Drake, and Montie, 1987; Montie et al., 1987).

Previous work performed by Tsuda and Iino (Tsuda, and Iino, 1983b) has shown by genetic mapping that the flagellar genes of this organism were clustered into two distinct regions. These regions are termed region I and region II and are located in the very late region of the linkage map of the chromosome (Tsuda, and Iino, 1983a). One motility (*mot*) and five flagellum (*fla*) cistrons are identified in region I, and ten flagellum (*fla*) and two chemotaxis (*che*) cistrons were identified in region II. By using multiflagellate (*mfl*) mutants and a sequence coding for mercury resistance in TN501, Tsuda and Iino determined the order of 1 *che* and 14 *fla* cistrons (Tsuda, and Iino, 1983a). The clustering effect of the flagellar genes seen in *P. aeruginosa* is also seen in *E. coli* and *S. typhimurium*

(Komeda et al., 1980; Kutsukake et al., 1980; Parkinson, 1980; Silverman, and Simon, 1977).

In *E. coli* and *S. typhimurium*, more than thirty cistrons are clustered in three distinct regions of the chromosomal genome. In *P. aeruginosa*, genes coding for related functions typically do not show the close linkage characteristic of members of the family *Enterobacteriaceae*. This clustering of the *fla* cistrons suggests that expression of each *fla* cistron is coordinately regulated, constituting operons (Tsuda, and Iino, 1983a). As a first step in studying the composition and function of the Fla gene locus, the structural flagellin gene was cloned into *Escherichia coli*.

Genes encoding exotoxin A (Gray et al., 1984; Lory et al., 1988; Hedstrom et al., 1986), alginate (Flynn, and Ohman, 1988; Goldberg, and Ohman, 1984), pili (Finlay et al., 1986; Strom, and Lory, 1986), and a porin protein of *P. aeruginosa* have been cloned using a triparental mating system and oligonucleotide probes (Goldberg and Ohman, 1984; Ogle et al., 1987).

To isolate and characterize the structural flagellar gene product of *P. aeruginosa*, a clone bank in a suitable vector was constructed. As previously shown, *E. coli* does not usually translate genes efficiently from *P. aeruginosa*; also, narrow host range cloning vectors do not replicate in *Pseudomonas* (Vasil et al., 1982).

To avoid these problems, a cloning system with a broad host range originally developed by Ditta et al. (Ditta et al., 1980) was adopted. The cosmid vector, pLAFR1 is 21.6 kilobases (kb) long, contains a single EcoR1 site, confers tetracycline resistance, and is capable of replicating in a number of Gram - organisms including *P. aeruginosa* and *E. coli*. Large fragments of the *P. aeruginosa* genome DNA were inserted into pLAFR1 to create a clone bank. The flagellar genes in the clone bank were detected by tri-parental mating and complementation analyses. The transformation of large recombinant plasmids into *P. aeruginosa* cells would be difficult unless a conjugative helper plasmid, e.g. pRK2013, was employed. The pRK2013 plasmid contained the conjugal transfer genes (*tra*) of its parental plasmid RK2 and a sequence coding for kanamycin resistance (Figurski, and Helinski, 1979).

The tri-parental mating procedure is derived from Ohman (Ohman, 1986), and the components involved are: one strain of *E. coli* HB101 which carries the pRK2013 helper, another strain of HB101 which carries the pLAFR1-recombinant clones, and the *P. aeruginosa* Fla<sup>-</sup> mutant strain. In solution, the helper plasmid transfers into the *E. coli* strain carrying the recombinant plasmids, pLAFR1-PAO1. Once in these cells, the helper plasmid sets up a conjugal apparatus and attempts to transfer into the *P. aeruginosa* Fla<sup>-</sup> mutant strain, but cannot because the helper plasmid cannot replicated in *Pseudomonas* cells. However, the pLAFR1

recombinant clones take advantage of the conjugal apparatus set up by the helper plasmid. Since the pLAFR1 plasmids retain the *rlx* site, they are mobilized into the *P. aeruginosa* Fla<sup>-</sup> cells and once incorporated, are maintained. Colony blot assays were performed to determine which pLAFR1-PAO1 recombinant plasmid complemented the *P. aeruginosa* Fla<sup>-</sup> mutant.

In the present study, we isolated DNA fragments containing the structural gene of the flagella from a library of *P. aeruginosa* PAO1 by triparental matings. Plasmids with DNA fragments containing the structural gene of the flagella were transformed into *E. coli* HB101 cells. Expression of the *Pseudomonas* flagellin protein in *E. coli* HB101 cells was examined by colony blotting and immunoblotting of electrophoretically isolated protein.

## MATERIALS AND METHODS

Bacteria and Plasmids. *P. aeruginosa* PA103, originally described by Liu (Liu, 1966), was used as the recipient in triparental matings. This organism is deficient with respect to synthesis of a complete flagellar filament, although some flagellin is detectable. Well-characterized flagellin deletion mutants were not available and are not readily isolated.

Since the flagellar operon was clustered in *Pseudomonas aeruginosa*, PA103 was used as a vehicle to clone the cluster containing the structural gene by using a relatively large cosmid fragment (20 to 30 kb), (Hindahl et al., 1987). *E. coli* HB101 provided by Jeffrey Becker was used in transformation experiments. The *P. aeruginosa* PAO1 genomic library pLAFR1-PAO1 harbored in *E. coli* HB101 cells was kindly provided by Dennis Ohman (Goldberg, and Ohman, 1984), as was the helper plasmid, pRK2013, used in the triparental matings.

Growth Conditions. Bacteria were grown in Luria broth as described by Wintenberg-Kelly (Wintenberg-Kelly, and Montie, 1989) and Part II for triparental matings, isolation of plasmids, and transformation experiments. Motility medium for assays and electron micrographs contained Luria broth with 0.3% agar. *P. aeruginosa* PA103 cells containing plasmids were selected in mineral salts medium as described by Allison (Allison et al., 1985) and in Part II and also contained 250 ug of tetracycline per ml. Luria agar was prepared with Luria broth and 1.6% agar. For isolation of flagellin protein from supernatants of transformed *E. coli* cells, bacteria were grown in mineral salts medium, 0.3% Casamino Acids with 0.4% sodium succinate, and 300 ug of tetracycline per ml was used.

Construction of Genomic Library. The pLAFR1-PAO1 genomic library was a gift provided by D. Ohman (Goldberg and Ohman, 1984). The genomic library was constructed by an EcoR1 partial digestion of PAO1 DNA followed by sucrose density centrifugation. DNA fragments of 15 to 30 kilobases were retrieved and ligated into a single EcoR1 site of the broad-host-range vector pLAFR1 originally developed by Ditta et al. (Ditta et al., 1980). The pLAFR1 vector is 21.6 kb long and confers tetracycline resistance.

Plasmid Preparations-Alkaline Lysis and Casse Method. Plasmids harbored in *E. coli* cells were isolated by the alkaline lysis procedure as described by Maniatis (Maniatis et al., 1982). Plasmid molecules were prepared for transformation into *E. coli* from *P. aeruginosa* PA103 cells containing the recombinant clones by the Casse method (Casse et al., 1979). Cells were grown in Luria broth overnight at 37°C and were then harvested by centrifugation and suspended in 10 mM Tris-Cl (pH 8.0) to a concentration of 0.1 g wet pellet per ml. The cells were gently mixed with fresh lysis buffer (10 mM Tris base (pH 12.5), 1 mM EDTA, and 1% SDS). After a short incubation at 37°C, 0.6 ml of 2 M Tris-Cl pH 7.0 was added to the lysate followed by the addition of solid NaCl to a final concentration of 3%. The mixture was incubated for 20 min with gentle rocking, followed by phenol, chloroform, isoamyl alcohol (24:1)

extraction. The upper aqueous phase was removed and mixed with 0.1 volume of 3.0M sodium acetate, followed by 2 volumes of 100% ethanol, and incubated overnight at -20°C. The DNA was pelleted by centrifugation at 8000 x g for 30 min and suspended in Tris-Cl EDTA buffer (T.E.), pH 8.0 (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0). Quantitation and purity of these plasmids were determined by spectroscopy.

DNA Procedures. Restriction endonucleases were purchased from BRL (BRL, Bethesda, MD), and endonuclease digestion of DNA was performed according to manufacturer's directions. Generally, digestion occurred overnight at the specified temperature. In transformation experiments, competent HB101 cells were prepared by the calcium chloride procedure described by Maniatis (Maniatis et al., 1982). Plasmids and restriction fragments were examined by agarose gel electrophoresis, and the size of the DNA fragment was determined by comparison of its migration patterns to known standards.

Agarose Gel Electrophoresis. Most electrophoresis was performed with a sub-cell unit (Biorad, Labs, Richmond, CA). Either 0.5% (1.5 g of agarose, Biorad, Labs) or 0.7% (2.1 g of agarose) gels were prepared by dissolving the agarose in 1X Tris-borate electrophoresis buffer (TBE, 89

mM Tris base, 89 mM boric acid, and 2 mM EDTA) or 1X Tris-acetate electrophoresis buffer (TAE, 40 mM Tris acetate and 2 mM EDTA). The slurry was gently heated with constant stirring, cooled to 42°C, and the dissolved agarose was poured into the gel unit. After the gel was hardened, the comb(s) were removed, and electrophoresis buffer was added to cover the gel by about two mm. The DNA samples were mixed with 5X sample buffer, and loaded into the wells of the submerged gel with a pipetteman. The gels were usually run overnight at 20 V or at 90 V for 5 h. When the marker dye was near the end of the gel, the power was turned off, and the gel was removed and stained in ethidium bromide (30 ul of a 10 mg/ml stock in 300 ml of H<sub>2</sub>O). The stained DNA bands were viewed on a transilluminator and photographed with a Polaroid camera with a red-orange filter.

Triparental matings. The pLAFR1-PAO1 genomic library and pRK2013, both harbored in *E. coli* HB101 cells, were used in triparental matings to transfer by conjugation the PAO1 library into the recipient strain, *P. aeruginosa* PA103. To transfer the pLAFR1-PAO1 plasmids, it was necessary to use a helper plasmid, pRK2013. The pRK2013 plasmids contained the conjugal transfer genes (*tra*) and determinants of kanamycin resistance (Figurski, and Helinski, 1979).

Log-phase *E. coli* HB101 cells containing pRK2013 and the HB101 (pLAFR1-PAO1) clone bank were each grown in Luria broth containing the appropriate antibiotics. The *E. coli* cells were mixed in a 1:1:1 ratio with 0.1 ml of log-phase *P. aeruginosa* recipient cells grown in Luria broth. The suspensions were filtered using 0.45 micron pore size nitrocellulose membranes and incubated on Luria agar plates (cell side up) at 37°C overnight. Following incubation, cells were removed from the filter by being vortexed in a 0.85% NaCl solution. The suspension was diluted with mineral salts medium containing tetracycline (200 ug/ml). The *P. aeruginosa* cells containing pLAFR1-PAO1, but not the auxotrophic *E. coli* HB101 cells, were capable of growth in this medium. Controls of *P. aeruginosa* PA103 and HB101 (pLAFR1-PAO1) were processed separately and identically: growth in the selection medium did not occur for either strain.

Complementation analysis. *P. aeruginosa* PA103 cells containing the recombinant plasmids complementing the Fla<sup>-</sup> mutation were isolated by motility assays. The motility assay consisted of stab inoculation of cells on motility medium (tetracycline 300 ug/ml). For each assay, triplicate plates per strain were incubated at 37°C and examined periodically over six to

twenty-four hours for colony spreading and the distinctive rings characteristic of motile bacteria.

Electron microscopy. Electron micrographs were prepared to compare PA103 Fla<sup>-</sup> and PA103 Fla<sup>+</sup> cells harboring the recombinant clones which complemented the Fla<sup>-</sup> mutation in PA103. Cells were grown in motility medium for 18 to 20 h at 37°C. A loopful of cells were removed from the motility plates at the edges of the colonies and suspended in sterile PBS. Cells were stained with 0.5% phosphotungstic acid, pH 7.0. The specimens were examined using a H-600 Hitachi Transmission Electron Microscope (TEM).

Preparation of supernatants. Transformed HB101 cells were grown to late log phase in mineral salts medium-glucose with 0.3% Casamino Acids at 37°C in a gyratory shaker at 200 rpm. The supernatants were retained following centrifugation and were dialyzed and concentrated by lyophilization.

Colony blots. This assay was modified from the method of Nicas et al. (Nicas et al., 1985) and adapted from a published enzyme-linked immunosorbent assay for flagellar antigen. The method was performed as

described by Wintenberg-Kelly (Wintenberg-Kelly, and Montie, 1989) and in Part II with the exception of the presence of tetracycline. For screening of transformants in *E. coli*, the cells were blotted on plates of Luria agar with 15 ug/ml of tetracycline. For screening of *P. aeruginosa* cells the concentration of tetracycline was 300 ug/ml.

Immunoblots. The proteins were transferred onto nitrocellulose by diffusion for two hours at 37°C. The procedure was carried out as described by Wintenberg-Kelly (Wintenberg-Kelly, and Montie, 1989) and in Part II.

Amino acid composition analyses and Amino (N) terminal analyses.

Flagella protein was hydrolyzed in 6N HCl and 1% phenol for 24 h in sealed vials. Amino acids were derivatized with the PICO-Tag system (Waters operational manual). Derivatization was with phenylisothiocyanate (PITC) to form the amino acid phenthiocarbamyl derivatives (PTC). Analyses were made on a Water's Association amino acid analyzer using a reverse phase HLPC column C VYDAC C18 or BONDAPACTM C 18. N-terminal amino acids were identified by an automated standard Edman degradation using PITC in a Quidral buffer to form the PTA (phenylthiohydantoin) derivatives. These derivatives were

isolated in a “spinning cup” sequencer (Beckman Instrumentation.).

Amino acids were isolated and identified by HPLC analyses.

## RESULTS

Tri-Parental Matings. This assay detected any recombination event involved in the formation of a functional flagellum. The pLAFR1-PAO1 genomic library harbored in *E. coli* HB101 was transformed to PA103 recipient cells by tri-parental matings. Upon screening the entire genomic library, eight clones (designated pKW3, pKW8, pKW23, pKW52, pKW84, pKW123, pKW126, and pKW333) were isolated which complemented the Fla<sup>-</sup> mutation of PA103. Only isolates that showed more than 80% of the motility of PAO1 wild type control strains were retained. The recipient strain PA103 Fla<sup>-</sup>, routinely displayed only 10% of the motility of PAO1. To verify the recombinant plasmid's ability to complement the Fla<sup>-</sup> mutant, the plasmids were transformed into *E. coli* HB101 cells, and again were tri-parentally mated into the same Fla<sup>-</sup> mutant with complementation of motility occurring at a higher frequency.

In later experiments, the eight recombinant clones were tri-parentally mated into *P. aeruginosa* 414ii, a CF isolate which produced no detectable flagellin. Again, the recombinant clones complemented the mutation in 414ii and a functional flagella was produced. The percent motility of the control for the recombinant clones in PA103 and 414ii is shown in Table III-1.

Electron Microscopy. Electron micrographs of complemented PA103 cells are shown in Figure III-1. In the PA103 cells which contained the recombinant clones (pKW333, pKW52, and pKW84), a functional flagella was produced. As illustrated in panel A, no flagellum was present on any PA103 cells examined. The same results were seen for the remainder of the clones. On the basis of the electron micrographs and motility assays, it was evident that these cells produced a functional single-polar flagellum typical of wild-type *P. aeruginosa*. Amino acid analyses (Table III-2) of purified flagella of PA103 (pKW52) showed the content to be essentially identical to those of other *P. aeruginosa* b-type flagella. Notably, there was no histidine, low amounts of aromatic acids, and minute amounts of methionine.

Plasmid preparations from PA103 (pKW3, pKW8, pKW23, pKW52, pKW84, pKW123, pKW126, and pKW333) were examined by electrophoresis in 0.4% agarose gels and stained with ethidium bromide

Table III-1. Migration in motility medium of *Pseudomonas aeruginosa* PA103 cells containing positive recombinant clones.

| <u>Strain</u> | <u>Flagella</u> | <u>Diameter</u><br>(mm) | <u>% control</u> <sup>a</sup> |
|---------------|-----------------|-------------------------|-------------------------------|
| PAO1          | +               | 44                      | 100                           |
| PA103         | -               | 5                       | 11                            |
| PA103-pKW3    | +               | 44                      | 100                           |
| PA103-pKW8    | +               | 45                      | 102                           |
| PA103-pKW23   | +               | 38                      | 86                            |
| PA103-pKW52   | +               | 46                      | 105                           |
| PA103-pKW84   | +               | 47                      | 107                           |
| PA103-pKW333  | +               | 36                      | 82                            |
| PA103-pKW123  | +               | 40                      | 91                            |
| PA103-pKW126  | +               | 39                      | 89                            |

<sup>a</sup>The percent control was given relative to the colony diameter of PA103 Fla<sup>+</sup> cells to wild type PAO1 cells which was 100%.

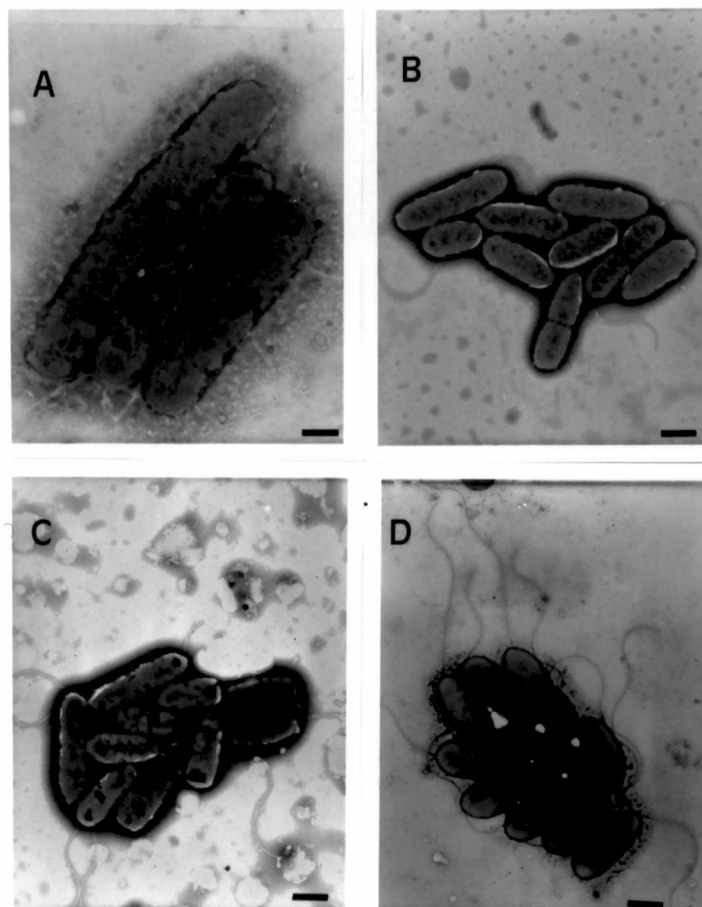


Figure III-1. Electron micrographs of PA103 Fla<sup>+</sup> cells. Cells were stained with 0.5% phosphotungstic acid, pH 7.0. The specimens were examined with an H-600 Hitachi transmission electron microscope.

A) PA103 Fla<sup>-</sup>; magnification, x 20000. B) PA103(pKW333); magnification, x 10000. C) PA103(pKW52); magnification, x 10000. D) PA103(pKW84); magnification, x 10000. The bar represents 1  $\mu$ m except in panel A, where it represents 0.5  $\mu$ m.

Table III-2. Amino acid analyses of *P. aeruginosa* PA103-pKW52 flagellin compared to other b-type flagellin proteins.

|     | M2 <sup>a</sup> | M2  | PJ108 | PA103-<br>pKW52 |
|-----|-----------------|-----|-------|-----------------|
| ASP | 74              | 70  | 75    | 73              |
| GLU | 49              | 47  | 48    | 48              |
| SER | 48              | 53  | 57    | 56              |
| GLY | 51              | 47  | 51    | 54              |
| HIS | 0.6             | 0.6 | 1.0   | 0.8             |
| ARG | 18              | 21  | 20    | 19              |
| THR | 48              | 52  | 47    | 87              |
| PRO | 0               | 8   | 7     | 7               |
| TYR | 4               | 7   | 7     | 7               |
| VAL | 38              | 35  | 32    | 32              |
| MET | 2               | 2   | 2     | 2               |
| ILE | 30              | 27  | 27    | 27              |
| LEU | 43              | 41  | 41    | 40              |
| PHE | 13              | 10  | 12    | 12              |
| LYS | 18              | 20  | 17    | 18              |

<sup>a</sup>Analysis done in a Beckman analyzer.

to visualize the plasmid clones. The size of the clones was 42-47 kb based upon comparisons of known size standards. These plasmids harbored in *Pseudomonas* cells were prepared by the method of Casse et al. (Casse et al., 1979) and were used in  $\text{CaCl}_2$  transformation experiments with competent *E. coli* HB101 cells (Maniatis et al., 1982). Successfully transformed *E. coli* HB101 cells were isolated by their ability to grow in the presence of tetracycline (300 ug/ml) and anaerobically.

Detection of Flagellar Antigen in Transformed *E. coli* Cells. To determine whether *P. aeruginosa* flagellin production occurred and was detectable in transformed *E. coli* cells, colony blots were utilized. The HB101 cells transformed with the positive clones appeared to produce the flagellar protein of *P. aeruginosa* (Table III-3). It also appeared that the *E. coli* cells exported the flagellin protein at levels detectable by colony blotting, even when the colonies were not lysed with chloroform. As shown in Table III-3, the *E. coli* cells containing pKW333 and pKW52 showed a reaction nearly as intense as that obtained with *P. aeruginosa* PA103 cells harboring these plasmids.

It is interesting that there were detectable differences in the intensities of the colony blots of the various transformed HB101 strains (Table III-3). Since the assembly of a flagellum is complex and requires a large DNA-coding region (Tsuda, and Iino, 1983a), it is likely that the difference

Table III-3. Detection of *Pseudomonas aeruginosa* flagellar antigen in transformed *Escherichia coli* HB101 cells by colony blotting<sup>a</sup>.

| Detection of flagellar filament protein <sup>b</sup><br>with the following antisera: |             |          |             |          |
|--------------------------------------------------------------------------------------|-------------|----------|-------------|----------|
| Strain                                                                               | Anti b-type |          | Anti a-type |          |
|                                                                                      | Lysis       | No lysis | Lysis       | No lysis |
| <i>Escherichia coli</i>                                                              |             |          |             |          |
| HB101                                                                                | -           | -        | -           | -        |
| HB101(pLAFR1)                                                                        | -           | -        | -           | -        |
| HB101(pKW8)                                                                          | ++          | ++       | -           | -        |
| HB101(pKW52)                                                                         | +++         | +++      | -           | -        |
| HB101(pKW84)                                                                         | ++          | ++       | -           | -        |
| HB101 (pKW333)                                                                       | +++         | +++      | -           | -        |
| <i>Pseudomonas aeruginosa</i>                                                        |             |          |             |          |
| PAO1                                                                                 | ++++        | ++++     | +           | +        |
| PA103                                                                                | -           | -        | -           | -        |
| <i>Pseudomonas fluroescens</i>                                                       |             |          |             |          |
|                                                                                      | -           | -        | -           | -        |

<sup>a</sup> The presence or absence of flagellar filament protein was determined by a reaction to either anti-M2 (b-type) or anti-1210 (a-type) antisera as described in Material and Methods section. Blots were done in triplicate.

<sup>b</sup> +++++, +++ strong detection; ++, detection; - no detection.

in intensities of the colony blots corresponded to different cloned regions of the flagellin DNA which affected expression.

Secretion of Flagellar Antigen in *E. coli* cells. To investigate the secretion of flagellin protein in *E. coli* HB101 cells, immunoblots (Towbin et al., 1979) of culture supernatant fractions were performed. The prepared supernatant concentrates of the transformed *E. coli* cells were separated (Laemmli, 1970) in polyacrylamide gels by using the Phast System (Figure III-2), and stained with Coomassie brilliant blue. All of the HB101 supernatant samples containing positive clones displayed a protein band with approximately the same Mr (53,000) as the purified b-type PJ108 flagellin protein (Figure III-2, Lanes A, B, C, and E). In other gels, no stained bands were seen in the 53kDa region when supernatant of non-transformed HB101 cells was used. Flagellin purified from PA101 (pKW52) cells (Figure III-2, Lane D) appeared to migrate very closely to the standard b-type flagellin, and immunoblotting analysis demonstrated that the flagellin of PA103 (pKW52) cells was antigenic (Figure III-3, Lane D). In the immunoblot shown in Figure III-3 (Lanes B, C, and E), a single band of protein from each of the transformed *E. coli* supernatants and the control b-type flagellin reacted with the standard b-type polyclonal antiserum. In confirmation, the gel gave blotted bands with an approximate Mr of 53,000.

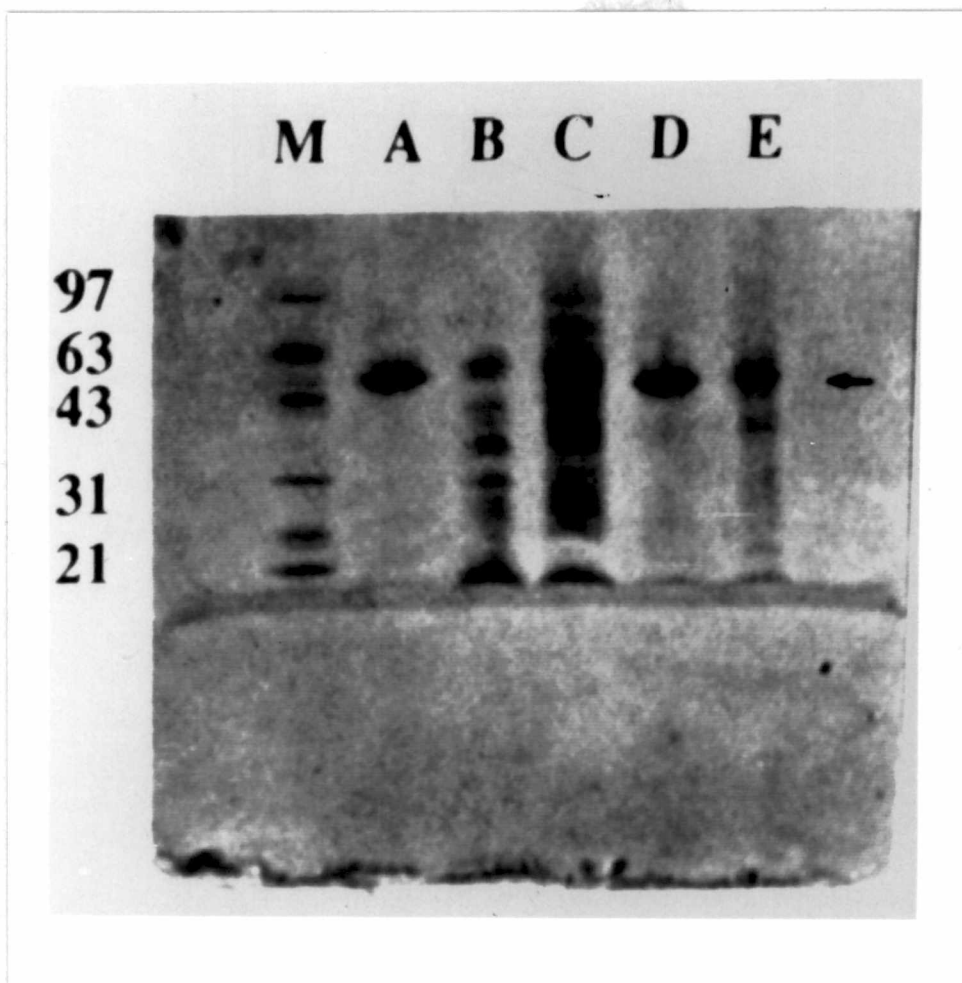


Figure III- 2. SDS-10-15% gradient polyacrylamide gel electrophoresis of transformed *Escherichia coli* HB101 supernatant preparations. Lanes: M, molecular weight markers ( $10^3$ ); A, PJ108 purified b-type flagellin; B, HB101(pKW333); C, HB101(pKW52), D, PA103 (pKW52) purified flagellin; E, HB101(pKW84). The arrow indicates the *Pseudomonas aeruginosa* flagellin protein produced by transformed HB101 cells.

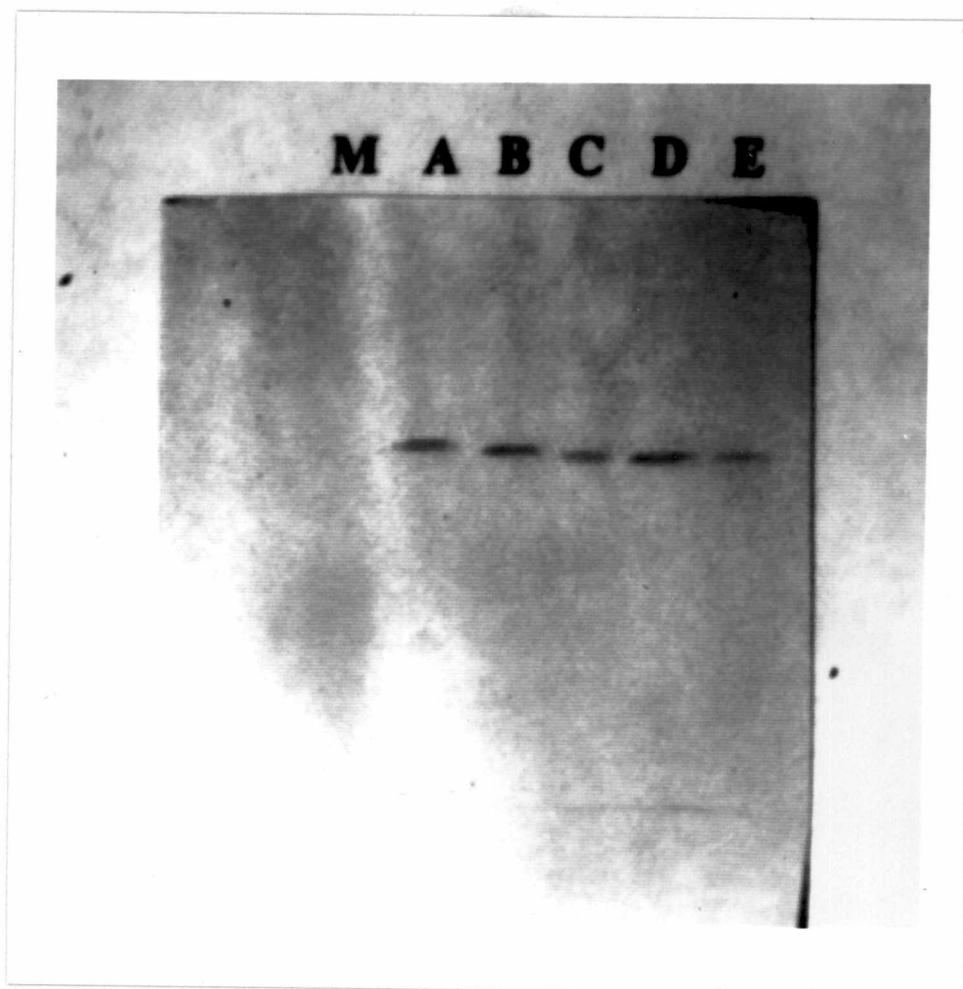


Figure III- 3. Immunoblot analysis of *P. aeruginosa* flagellin protein detected in supernatant concentrates of HB101 cells. Lanes: M, molecular weight markers; A, PJ108 purified b-type flagellin; B, HB101(pKW333); C, HB101(pKW52); D, PA103 (pKW52) purified flagellin; E, HB101(pKW84). The immunoreactive band corresponded to the Coomassie blue-stained *Pseudomonas aeruginosa* flagellin.

Limited Restriction Map of Recombinant Clones. Using the clones in *E. coli*, a limited endonuclease restriction map ( Figure III-4) was completed using the endonucleases EcoR1 and Kpn1. The limited mapping has shown that the plasmids pKW333 and pKW52 probably contain the same *P. aeruginosa* *fla*<sup>+</sup> DNA fragment, whereas plasmids pKW84 and pKW102 appear to contain overlapping but different *fla*<sup>+</sup> DNA.

## DISCUSSION

These experiments showed cloned DNA fragments of *P. aeruginosa* containing the flagellin gene can be cloned into *E. coli* HB101 cells. Use of this tri-parental mating system using the cosmid genomic library has several advantages. First, large fragments have increased the probability of preserving the functional gene clusters. Second, larger inserts were also desirable in that they reduced the absolute number of clones making up the gene bank. A cosmid clone bank in pLAFR1 of a bacterial species with a genomic size equivalent to *E. coli* (4199 kB) would need to contain approximately 820 members to guarantee 99% completeness (Ohman, 1986). Third, the use of pLAFR1 was advantageous because packaging in the phage heads imposed a size selection on the recombinant inserted with a result that all clones in the gene library would be approximately the same



Figure III-4. Partial restriction endonuclease map of *fla*<sup>+</sup> DNA from pKW333 and pKW52. Restriction enzyme sites for EcoR1 (R), Kpn1(K), and the DNA fragments sizes (kb) are represented by the numerals below the map.

size. Fourth, cloning large fragments was necessary to eliminate non-random DNA representation caused by the use of a restriction enzymes to fragment the DNA.

This system proved beneficial because DNA manipulations of the *P. aeruginosa* DNA were done in *E. coli* where such work is easier and more routine, and then the DNA was tri-parentally mated into *P. aeruginosa* cells to score complementation. In this work, the vector system proved to be stable in *E. coli* and *Pseudomonas aeruginosa*.

Upon analysis by colony blots, it appeared that most of the flagellin protein was expressed and transported to the *E. coli* cell surface, since the same results were seen in either the presence or the absence of cell lysis with chloroform. The expression and transport of flagellin out of the cell or to the surface was further indicated by results of immunoblot reactions of *E. coli* extracellular supernatants. However, it cannot be conclusively stated that secretion into the extracellular fluid occurred. It is possible that a small number of cells lysed and released intracellular flagellin protein.

Preliminary experiments with transformed supernatants in nondetergent polyacrylamide gels followed by immunoblots to detect antigen suggested that most of the exported flagellin was in a polymerized form. However, it is possible that self-aggregation of the secreted monomeric protein occurred since the phenomenon of self-aggregation at

or near neutral pHs has been well documented with *Salmonella* flagellin (Macnab, 1987, see Part I).

Results with a number of *P. aeruginosa* mutant strains that synthesize flagellin but cannot assemble the intact filament showed that an apparent polymerized form of the flagellin is found in the extracellular medium. In every strain tested, detection of the putative secreted polymerized flagellin coincided with positive colony blots of the non-lysed *P. aeruginosa*.

There appears to be no leader sequence requirement for flagellin protein export, and the C terminal region of the flagellin protein is thought to be responsible for signaling (Eisenstein, 1987; Hommo et al., 1987), so recognition of export sequences could occur among genera. In this regard, the first nine amino acids of the N-terminal region are the same for both a- and b-type flagellins of *P. aeruginosa*. Also, homology within the region exists between *Pseudomonas* and other G - bacteria as shown in Figure III-5. Similarly, unlike some of the secreted *P. aeruginosa* proteins, cloned pilin genes from *Bacteroides nodarus*, *Neisseria gonorrhoeae*, and *P. aeruginosa* show analogous sequences and are all expressed effectively in *E. coli* independent of a vector promoter (Finlay et al., 1986). Although detected at the cell surface, *P. aeruginosa* pilin protein is not secreted extracellularly (Woods, 1987).

In this research, a tri-parental mating system was used to isolate recombinant clones from a *P. aeruginosa* PAO1 gene bank which

| <u>Comparison of N-terminal Amino Acids in Flagellins</u> |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                                           | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  |
| <i>Pseudomonas aeruginosa</i>                             | *   |     |     |     | *   | *   | *   |     | *   | *   | *   | *   | *   | *   | *   |
|                                                           | Ala | Leu | Thr | Val | Asn | Thr | Asn | Ile | Ala | Ser | Leu | Asn | Thr | Gln | Arg |
| <i>Salmonella typhimurium</i> NH <sub>2</sub> -Met        | *   |     |     |     | *   |     |     |     | *   | *   | *   |     | *   | *   |     |
|                                                           | Ala | Gln | Val | Ile | Asn | Ser | Leu | Ser | Leu | Ser | Leu | Leu | Thr | Gln | Asn |
| <i>Campylobacter coli</i>                                 |     |     |     |     | *   | *   | *   |     | *   |     | *   | *   |     |     |     |
|                                                           | Gly | Phe | Arg | Ile | Asn | Thr | Asn | Val | Ala | Ala | Leu | Asn | Ala | Lys | Ala |
| <i>Bacillus subtilis</i>                                  |     |     |     |     | *   |     | *   |     | *   |     | *   | *   | *   |     | *   |
|                                                           |     |     | Arg | Ile | Asn | His | Asn | Ile | Ala | Ala | Leu | Asn | Thr | Asn | Arg |
| <i>Roseburia cecicola</i>                                 |     |     |     |     | *   |     |     |     |     |     |     | *   |     | *   |     |
|                                                           | Val | Val | Gln | Gln | Asn | Met | Thr | Ala | Met | Asn | Ala | Asn | Arg | Gln | Leu |

Figure III-5. Comparison of amino terminal amino acids in flagellins of different genera. The numbers denote the amino acids from the N-terminal end. The asterisks (\*) denote the exact same amino acid as encoded by other genera.

complemented a Fla<sup>-</sup> mutant and therefore contains the flagellin structural gene within the 22-25 kb PAO1 DNA region. In confirmation, it was discovered that *E. coli* HB101 cells which contain the positive recombinant clones express detectable levels of flagellin in whole and lysed cells. Subcloning of DNA fragments containing the flagellin gene is discussed in Part IV.

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PART IV

TRANSPOSON MUTAGENESIS WITH Tn501 AND SUBCLONING  
THE b-TYPE FLAGELLIN GENE.

## INTRODUCTION

To determine the location of the *fla* gene on the recombinant plasmids, pKW52 and pKW123, two approaches were taken: deletion mapping and transposon mutagenesis. Once the gene was located, a gene replacement method was utilized, whereby it was possible to replace the wild-type chromosomal *fla* gene with a cloned and modified allele (*fla* ::Tn501). Using these methods, the *fla* gene was subcloned in *E. coli*, and mated into the *P. aeruginosa* mutant cells to score complementation.

Transposon mutagenesis can be thought of as biological mutagenesis because its insertion within a gene disrupts the linear continuity of that gene, and the function of that gene is lost (Ohman, 1988). Transposons are segments of double stranded DNA which are able to move from one genetic location to another. They are responsible for a good proportion of DNA movement in the genome. When a transposon inserts into a target site, it moves as a separate piece of DNA, and the transposon itself does not change in size. The complementary sequence to the transposon need not share homology with the transposon for insertion. Transposons usually do not self-replicate, and are considered parasites since they depend upon replication of the host for their own replication (Berg, and Berg, 1987).

Previous work with the transposon Tn5 (Berg, 1977), [kanamycin resistance ( $\text{kan}^{\text{r}}$ )], in this laboratory and the laboratories of others has

shown high frequencies of spontaneous kanamycin resistant *P. aeruginosa* cells (Ohman et al., 1985). Therefore, the transposon Tn501, which encodes mercury resistance, was chosen for the study, because *P. aeruginosa* cells are very sensitive to mercuric chloride. Typically, *P. aeruginosa* cells are resistant to most antibiotics. The transposon, Tn501, is a member of the Tn3 family also known as simple transposons (Weiss, and Falkow, 1983).

Transposon mutagenesis has several advantages as opposed to chemical mutagenesis. The introduction of Tn501 into the genome of *P. aeruginosa* provides a positive selection marker for both strain construction and the analysis of the mutant phenotype. Mutants can also be isolated at a high frequency even though cells are exposed to very low levels of mutagens. Each mutant rarely suffers more than one mutational event. So, the problem of multiple hits is nearly eliminated (Woods, and Sokol, 1985). Also, Tn501 has EcoR1 sites at its extreme termini, and therefore the transposon's DNA does not contribute any measurable amount to the sizes of EcoR1 fragments (Ohman et al., 1985). The mutations can be transferred to different *P. aeruginosa* strains by either transduction or conjugation simply by selecting for mercury resistance. In addition, the region of DNA containing the Tn501 element can be subcloned into *E. coli* by selecting for mercury resistance in the transformants.

This Part contains a presentation of transposon mutagenesis by Tn501 which was utilized to determine the location of the *P. aeruginosa* b-type flagellin structural gene in the pKW recombinant plasmids. The sites of transposon insertions in the pKW plasmids were mapped by analyzing restriction digests. In addition, these mutants were used to introduce site specific mutations in the flagellin of *P. aeruginosa* PAO1. The wild type chromosome *fla* gene was replaced with modified *fla*::Tn501 fragments. By this technique, we have defined the general boundaries of the *fla* gene, and generated several PAO1 flagellin mutants. Based upon the results of deletion mapping and transposon mutagenesis, the smallest fragment containing the *fla* gene was subcloned.

## MATERIALS AND METHODS

Bacterial and Phage Strains. *Pseudomonas aeruginosa* strains used were common a- and b-type laboratory strains including PAO1 (b-type), M2 (b-type), and 170018 (a-type). *E. coli* strains used included HB101, previously described in Part III [(*hsd* S20 *rec* A13 *ara* 14 *pro* A2 *lac* Y1 *gal* K2 *mtl* -1 *xgl* -5 *sup* E44 ) (Boyer and Roullard-Dussoix, 1969) and DH1. Phages used were  $\lambda$ c1857 (*ts* non-lysogenic at 37°C) (Berg, 1977) and F116 L (Holloway et al., 1979).

Plasmids and DNA Procedures. Plasmids used were pLAFR1 (Inc P-1, Tc<sup>r</sup>, rlx, cos) (Friedman et al., 1982) pRK2013 (Col E1, Tra<sup>+</sup>, Km<sup>r</sup>), (Figurski, and Helinski, 1979), pUW942 (ColE1, Tra<sup>+</sup>, Tn501), (Weiss, and Falkow, 1983), and RSF1010 (Inc Q, SM<sup>r</sup>), (Guerry et al., 1974).

Plasmid DNA from *Escherichia coli* strains was isolated using the alkaline lysis procedure of Birnboim and Doly (Birnboim, and Doly, 1979), and purified using PEG as described in the Materials and Methods section in Part III. Plasmid DNA was used in transformation experiments along with calcium chloride treated competent *E. coli* HB101 and DH1 cells as described by Maniatis (Maniatis et al., 1982), and in Part III. T4 ligase and restriction enzymes were purchased from BRL (Bethesda, MD), and used according to the manufacturer's instructions. Restriction fragments were examined by agarose gel electrophoresis as described in Part III. Tn501 insertions into the pKW recombinant DNA were mapped by the change in the known restriction digest pattern.

Transposon Mutagenesis of Cloned Recombinant DNA. To isolate the pKW52 or pKW123 recombinant clones (plasmids) containing Tn501 insertions, a method described by D. Ohman was used (Ohman et al., 1985). This method is very useful because it takes advantage of the ability

of lambda phage to package cosmids such as pKW52 and pKW123, in vivo during its replication.

Initially, purified recombinant plasmids pKW52 and pKW123 were transformed into competent *E. coli* DH1 (RSF1010::Tn501) cells by the calcium chloride procedure described in Part III. *E. coli* DH1 colonies containing both plasmids were selected by isolation on Luria agar containing tetracycline (15 ug/ml) and incubation at 37°C, overnight. A single colony from these plates was used to inoculate five ml of Luria broth and incubated at 37°C, overnight.

These DH1 cells which contained Tn501 and the pKW- recombinant plasmids pKW52 or pKW123 were used to prepare plate lysates of  $\lambda$ c1857 (see Preparations of plate lysates). These lysates were later used to transduce the recombinant clones containing Tn501 insertions into *E. coli* HB101 cells (see Preparation of transductant). The transductants which contained the transposon insertion were selected by mercury resistance. The recombinant plasmids were isolated by repeated isolation of colonies from Luria agar containing mercuric chloride (18 ug/ml) followed by selection on Luria agar containing tetracycline (15 ug/ml). Since a transposon can insert in any region of the genome, colony blots were performed to characterize and isolate *E. coli* clones which no longer produced *P. aeruginosa* flagellin.

Preparation of a Lambda Plate Lysate. Overnight cultures of DH1 cells (RSF1010::Tn501, pKW recombinant plasmid) were used to prepare plate lysates of  $\lambda$ c1857. After incubation, a solution of  $\text{MgSO}_4$  (1.0 M) was added to the culture to a final concentration of 10 mM.  $\text{Mg}^{2+}$  was required for lambda to adsorb to cells. A portion of the overnight culture was removed (0.2 ml), and placed in a sterile tube to which 0.1 ml of lambda stock suspension was added at a concentration to produce a confluent lysis ( $10^7$  PFU/ml). The cells and phage solution were incubated for 10 min at room temperature to allow for adsorption. After adsorption, lambda top agar (Luria broth and 0.7% agar) plus 10 mM  $\text{MgSO}_4$  was added to the suspension, and poured evenly onto a Luria agar plate. The plates were incubated cell side down at  $37^\circ\text{C}$ , overnight, and then three ml of cold Luria broth plus 10 mM  $\text{MgSO}_4$  was added. The overlay solution was removed, and the lysate was sterilized with three to four drops of chloroform, or was filtered sterilized through a 0.45 micron membrane (Gelman Scientific). The titer of the lysate was determined by spot titration.

Preparation of Transductants of Recombinant Clones with Tn501 Inserts.

The cosmid clones::Tn501 were transferred into *E. coli* HB101 cells by transduction (Ohman, 1988). The *E. coli* HB101 recipient cells were

incubated overnight in Tryptone Maltose (TM) broth (10 g Bacto-Tryptone, 5 g NaCl per liter with 0.2% maltose). The cells were harvested by centrifugation and resuspended in 10mM MgSO<sub>4</sub>. The titrated  $\lambda$ c1857 lysate grown with pKW recombinant cosmid::Tn501 strain was added and incubated at room temperature. The mixture was incubated at 37°C for 60 min before plating on selective medium containing mercuric chloride (18 ug/ml).

Gene Replacement in *P. aeruginosa*. Triparental matings described in Part III were used to transfer the pKW::Tn501 recombinant clones to *P. aeruginosa* PAO. Colonies containing the clones were selected by Tc<sup>r</sup>. Plate lysates of the phage F116L were prepared with PAO cells containing the recombinant plasmids. A 0.2 ml sample of lysate (10<sup>9</sup> PFU/ml) was placed upon *P. aeruginosa* strains immobilized on nitrocellulose membrane filters on Luria agar, and was incubated overnight at 30°C. Following incubation, the cells were suspended in five ml of Luria broth, and isolated on Luria agar mercuric chloride (18 ug/ml). To determine if recombination had occurred, isolated colonies were streaked onto Luria agar tetracycline for one to two days at 37°C. If no growth occurred, then the vector was not coinherited. Cells which were Hg<sup>r</sup>, Tc<sup>s</sup> were further

characterized by agarose gel electrophoresis, motility assays and colony blots, to determine if flagella were being synthesized.

Triparental Matings. Triparental matings were utilized to mobilize the pKW::Tn501 recombinant plasmids from *E. coli* HB101 cells to various *P. aeruginosa* flagella containing strains with a helper plasmid, pRK2013 as described in Part III.

Motility Assays. Cells to be tested were stab inoculated as described in Part II in motility medium with Tc (200 ug/ml).

Colony Blot Assay. This assay was used to detect the presence of *P. aeruginosa* flagellin as previously described in Part II.

## RESULTS

Transposon Mutagenesis of pKW Recombinant Clones. Since the fla gene is located on a relatively large DNA fragment (25 kb), to determine its location within this fragment, transposon mutagenesis was used to generate random insertions on the recombinant clones, pKW52 and pKW123. The mutants were screened to isolate cells which no longer

synthesized flagellin. The procedure used to generate these mutations is diagramed in Figure IV-1 and adapted from a procedure first described by White (White, et al., 1983).

Transposition by Tn501 into the pKW clones occurred spontaneously when transformed into an *E. coli* strain, DH1, containing RSF1010::Tn501. Colonies selected were characterized by their Tc<sup>r</sup> phenotype. The pKW recombinant colonies with Tn501 insertions were transduced into *E. coli* HB101 cells. Those HB101 cells which harbored the pKW recombinant clones with Tn501 insertions displayed the Hg<sup>r</sup>, Tc<sup>r</sup> phenotype. These cells were isolated by repeated platings on selective medium.

Characterization of Tn501 Insertions. The recombinant plasmids which contained Tn501 insertions in the flagellin region were selected by colony blot assays using anti-b type flagella polyclonal antiserum. In Table IV-1, representative strains from HB101 cells containing Tn501 insertions in pKW52 are shown. From these results, it appears that Tn501 insertions interrupted the *fla* region in several clones (101, 102, 103, 104), since no positive reactions were seen in either lysed cells or in cells without chloroform lysis. In clone 108, detection of flagellin was visible only after cell lysis suggesting Tn501 had inserted in a region associated with export.

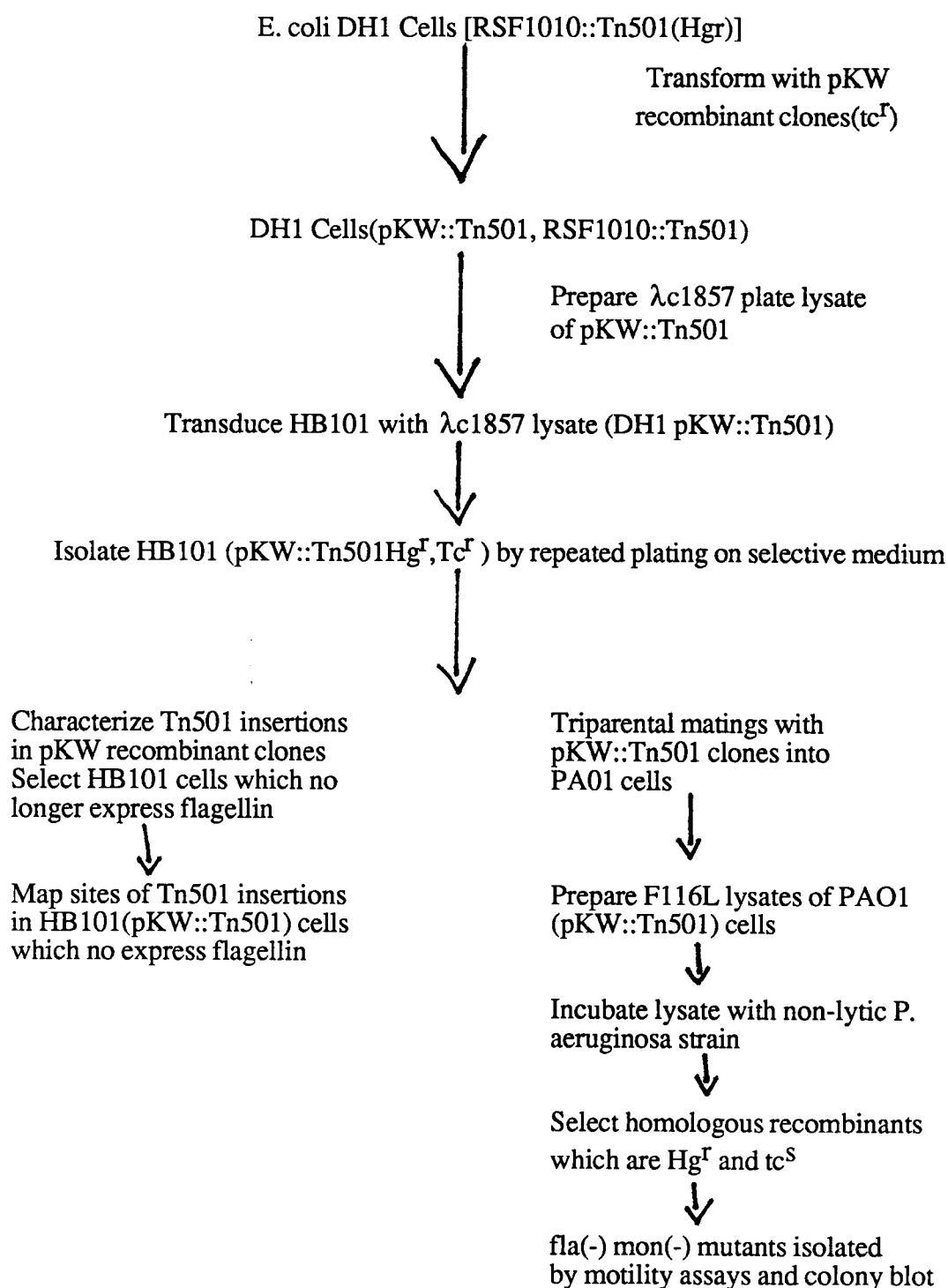


Figure IV-1. Tn501 insertional mutagenesis procedure used to generate mutations in the fla gene.

Table IV-I. Insertions of the *Pseudomonas aeruginosa* fla region with Tn501 in *Escherichia coli* HB101 cells containing pKW52.

| Strain           | anti-b type Polyclonal Antiserum |                 |
|------------------|----------------------------------|-----------------|
|                  | No cell lysis                    | With cell lysis |
| pKW52            | +++                              | +++             |
| pKW52::Tn501-101 | -                                | -               |
| pKW52::Tn501-102 | -                                | -               |
| pKW52::Tn501-103 | -                                | -               |
| pKW52::Tn501-104 | -                                | -               |
| pKW52::Tn501-105 | ++                               | ++              |
| pKW52::Tn501-106 | ++                               | ++              |
| pKW52::Tn501-107 | ++                               | ++              |
| pKW52::Tn501-108 | -                                | ++              |
| HB101            | -                                | -               |
| PAO1             | ++++                             | ++++            |

Similar results with Tn501 insertions in pKW333 are seen in Table IV-2. As described above for pKW52, the Tn501 insertions have interrupted the *fla* region in several clones (301, 302, 305, 307), since no positive reactions were seen in lysed cells or in cells with no lysis. In clone 310, detection of flagellin was visible only after cell lysis suggesting Tn501 inserted in a region associated with export.

To determine the location of the Tn501 insertion sites, restriction digests with EcoR1 and agarose gel electrophoresis were performed on clones in which flagellin was no longer detected. Restriction maps of the Tn501 insertion sites are shown in Figure IV-2. Tn501 has EcoR1 sites at its extreme terminal ends, so the site of its insertion was easily mapped by EcoR1 digestion. In both pKW123::Tn501 and pKW52::Tn501 recombinant clones, the Tn501 insertions were determined to be within a 1.5 kb region.

#### Gene Replacement Technique and Construction of *fla* Mutants in *P.*

*aeruginosa*. Obtaining true gene replacement mutants requires a double crossover event between homologous DNA on each side of the transposon insertion, and subsequent loss of plasmid DNA (Ohman et al., 1985). So, to construct *fla* mutants in *P. aeruginosa*, genomic DNA was replaced with homologous sequences altered by transposon mutagenesis (pKW52::Tn501, pKW123::Tn501). Clones pKW52::Tn501-104 and

Table IV-2. Insertions of the *P. aeruginosa* fla region with Tn501 in *Escherichia coli* HB101 cells containing pKW123.

| Strain            | anti-b type Polyclonal Antiserum |                 |
|-------------------|----------------------------------|-----------------|
|                   | No cell lysis                    | With cell lysis |
| pKW123            | +++                              | +++             |
| pKW123::Tn501-301 | -                                | -               |
| pKW123::Tn501-302 | -                                | -               |
| pKW123::Tn501-303 | +weak                            | +weak           |
| pKW123::Tn501-304 | ++                               | ++              |
| pKW123::Tn501-305 | -                                | -               |
| pKW123::Tn501-306 | +                                | +               |
| pKW123::Tn501-308 | ++                               | ++              |
| pKW123::Tn501-309 | +                                | +               |
| pKW123::Tn501-310 | -                                | ++              |
| pKW123::Tn501-311 | ++                               | ++              |
| pKW123::Tn501-313 | -                                | +weak           |
| HB101             | -                                | -               |
| PAO1              | ++++                             | +               |

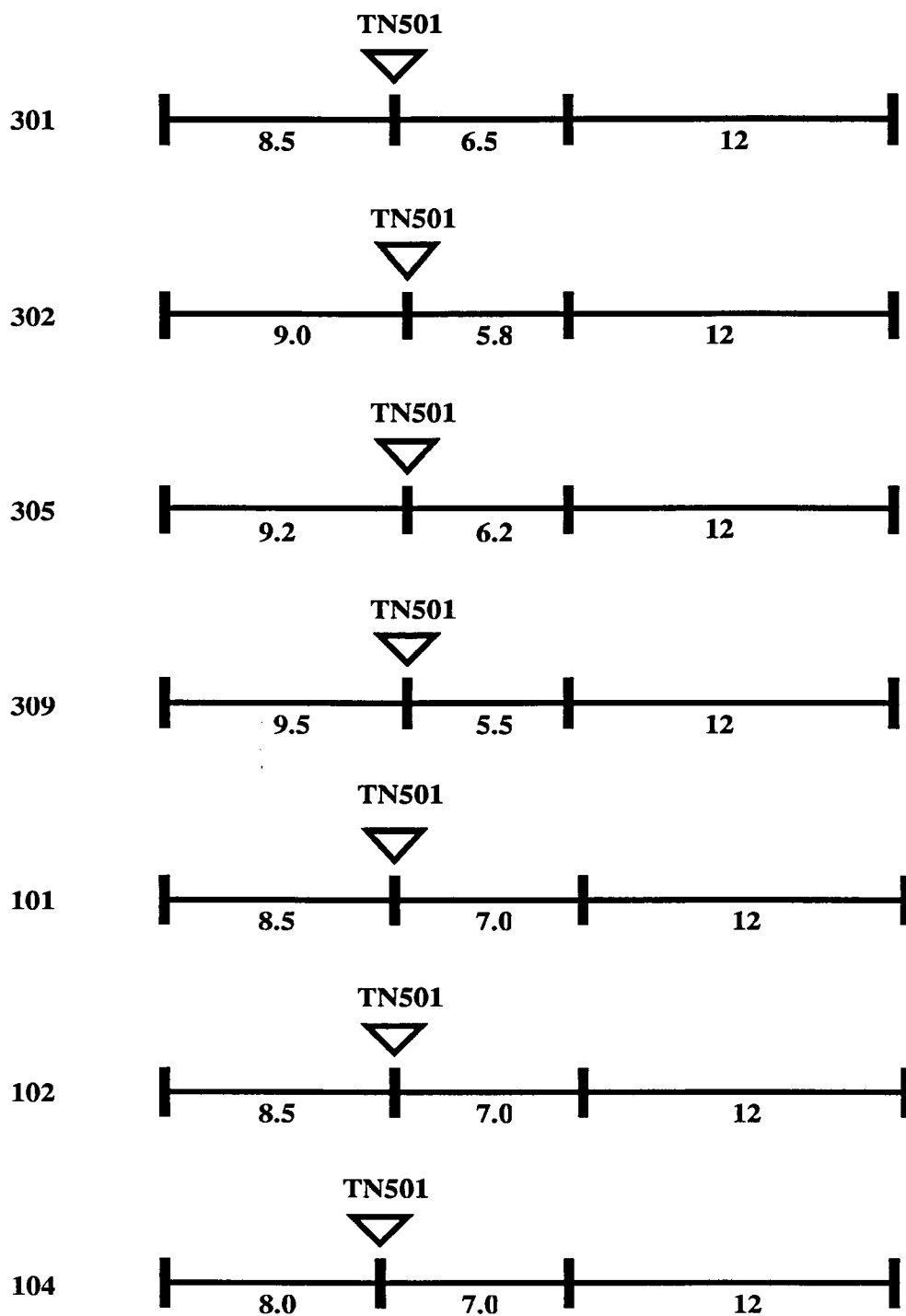


Figure IV-2. Restriction mapping of clones to determine Tn501 insertion sites.

pKW123::Tn501-302 in HB101 cells containing the Tn501 inserts were selected, and triparentally mated in *P. aeruginosa* PAO cells (Goldberg and Ohman, 1984). Plate lysates were prepared with F116L, and the lysates were incubated with non-lytic PAO1 cells. The phage, F116L does not multiply on PAO1, but injects its DNA into this strain (Krishnapillai, 1971). The recombinants were selected on Luria broth agar containing mercuric chloride, and were screened for the Tc<sup>S</sup> phenotype, indicating the cloning vector was not coinherited. Using the gene replacement technique, colonies were obtained at a relatively high frequency (40-200 colonies per plate), and 50-75% were free of the vector as determined by the Tc<sup>S</sup> phenotype.

Cells with Tn501 insertions which were resistant to mercury and were tetracycline sensitive were isolated, and the motility determined by motility assays. The presence or absence of flagella indicated in motility spreading assays, was confirmed by observing the swimming ability of these cells under a microscope. Migrations in motility medium of representative isolates from pKW52::Tn501-104 clones are shown in Table IV-3, while those isolates derived from pKW333::Tn501-302 are shown in Table IV-4. The percent given was relative to the colony diameter of wild type PAO1 cells. Plasmid preparations were prepared of these isolates, and no residual vector (pLAFR1) was detected on agarose gels. The experimental approach was repeated with the same clones and a

Table IV-3. Migration in motility medium of *P. aeruginosa*  
PAO1 cells containing Tn501 inserts derived from pKW52::Tn501-104.

| Strain      | Colony<br>Diameter | % Control | Growth<br>In Tc |
|-------------|--------------------|-----------|-----------------|
| PA01        | 28.0               | 100       | -               |
| PA01-104-1  | 6.35               | 23        | -               |
| PA01-104-2  | 7.95               | 28        | -               |
| PA01-104-3  | 7.05               | 25        | -               |
| PA01-104-4  | 6.60               | 24        | -               |
| PA01-104-5  | 6.9                | 25        | +++             |
| PA01-104-6  | 9.00               | 32        | -               |
| PA01-104-7  | 8.75               | 31        | -               |
| PA01-104-8  | 7.00               | 25        | ++              |
| PA01-104-9  | 6.1                | 22        | -               |
| PA01-104-10 | 8.2                | 29        | -               |
| PA01-104-11 | 6.2                | 22        | +++             |
| AK 1153     | 7.00               | 25        | -               |

Table IV-4. Migration in motility medium of *P. aeruginosa* PAO1 cells containing Tn501 inserts derived from pKW123::Tn501-302.

| Strain      | Colony Diameter | % Control | Growth In Tc |
|-------------|-----------------|-----------|--------------|
| PA01        | 28.0            | 100       | -            |
| PA01-302-1  | 6.40            | 23        | ++           |
| PA01-302-2  | 5.60            | 20        | +            |
| PA01-302-3  | 12.5            | 45        | +++          |
| PA01-302-4  | 7.30            | 26        | +++          |
| PA01-302-5  | 8.00            | 29        | -            |
| PA01-302-6  | 7.50            | 27        | -            |
| PA01-302-7  | 5.80            | 21        | +            |
| PA01-302-8  | 5.00            | 18        | -            |
| PA01-302-9  | 6.5             | 23        | -            |
| PA01-302-10 | 5.6             | 20        | -            |
| AK 1153     | 7.00            | 25        | -            |

different recipient strain, M2. Analogous results were seen when M2 was utilized.

These assays demonstrated a transposon insertion had occurred involving the loss of motility and flagella in wild type cells when the Tn501 inserted into the chromosome. Colony blots were done to confirm the absence of flagellin in PAO1 cells containing the Tn501 inserts from clones 104 and 302. As shown in Table IV-5, when compared with known positive and negative controls, the PAO1 cells which contained the inserts no longer produce detectable levels of flagellin.

*Fla* Gene Replacement of an a-Type Strain with pKW104::Tn501.

Another approach in the gene replacement experiments was to replace the *fla* region of a wild-type *P. aeruginosa* a-type strain (170018) with b-type DNA from pKW104::Tn501 by insertional mutagenesis. As shown in Figure IV-3, the same procedure described was used for gene replacement of the *fla* gene in PAO1 (b-type) cells. The a-type cells which displayed the Hg<sup>r</sup>, Tc<sup>s</sup> phenotype were selected. Upon analyses by colony blotting with polyclonal and monoclonal anti-170018 flagella antiserum and motility assays, shown in Table IV-6, the Tn501 region inserted into the *fla* region of 170018 and flagellin was no longer being synthesized or synthesized at detectable levels. These results showed that the a-type and b-type flagellin regions share similar DNA sequences, and that

Table IV-5. Colony blots demonstrating the absence of *Pseudomonas* flagellin antigen (gene replacement) in PAO1 cells containing Tn501 genomic insertions.

| Strain                | anti-b type Polyclonal Antiserum |                 |
|-----------------------|----------------------------------|-----------------|
|                       | No cell lysis                    | With cell lysis |
| PA01-104-1            | -                                | -               |
| PA01-104-2            | -                                | -               |
| PA01-104-7            | -                                | -               |
| PA01-104-9            | -                                | -               |
| PA01-302-6            | -                                | -               |
| PA01-302-9            | -                                | -               |
| PA01                  | ++++                             | ++++            |
| <i>P. fluorescens</i> | -                                | -               |
| HB101                 | -                                | -               |

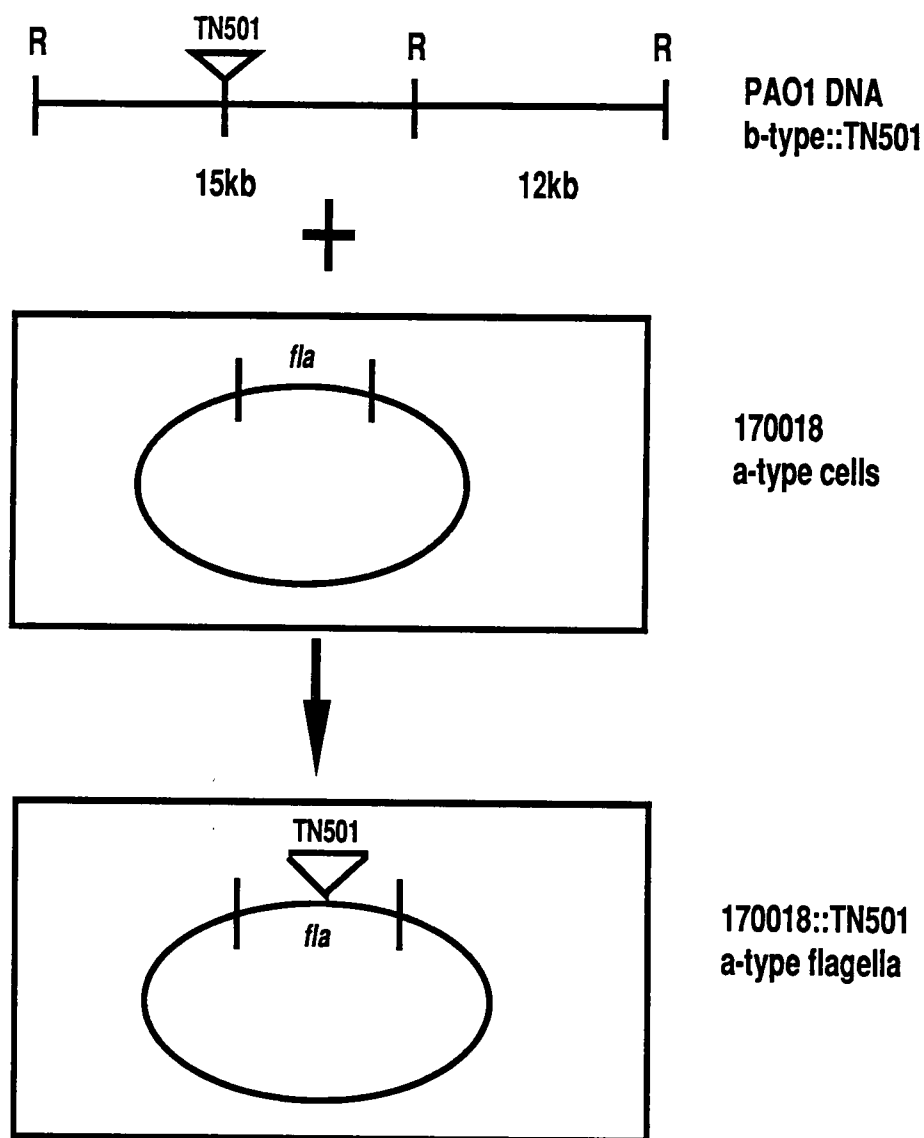


Figure IV-3. Fla gene replacement of an a-type strain with pKW104::Tn501 (b-type DNA).

Table 6. Analyses of phenotype of the *fla* gene replacement experiment of an a-type strain with pKW104::Tn501.

| Strain       | Analyses of phenotype           |                  |
|--------------|---------------------------------|------------------|
|              | Colony blot<br>anti-a antiserum | Motility<br>(mm) |
| 170018-104-1 | -                               | 6.8              |
| 170018-104-2 | -                               | 7.2              |
| 170018       | ++++                            | 33.0             |
| PA01         | -                               | 29.5             |

homologous recombination between 170018 and recombinant plasmids had occurred.

#### Deletion Mapping and Subcloning of the *P.aeruginosa* PAO1 *fla* Region.

Deletion mapping was used to locate the *fla* gene within the PAO1 DNA fragment of pKW52 and pKW123, as shown in Figure IV-4. Either plasmid was digested with EcoR1, to produce a 15 kb and 12 kb piece of PAO1 recombinant DNA as visualized on stained agarose gels. The fragments were excised and electroeluted or eluted using the by *Gene*

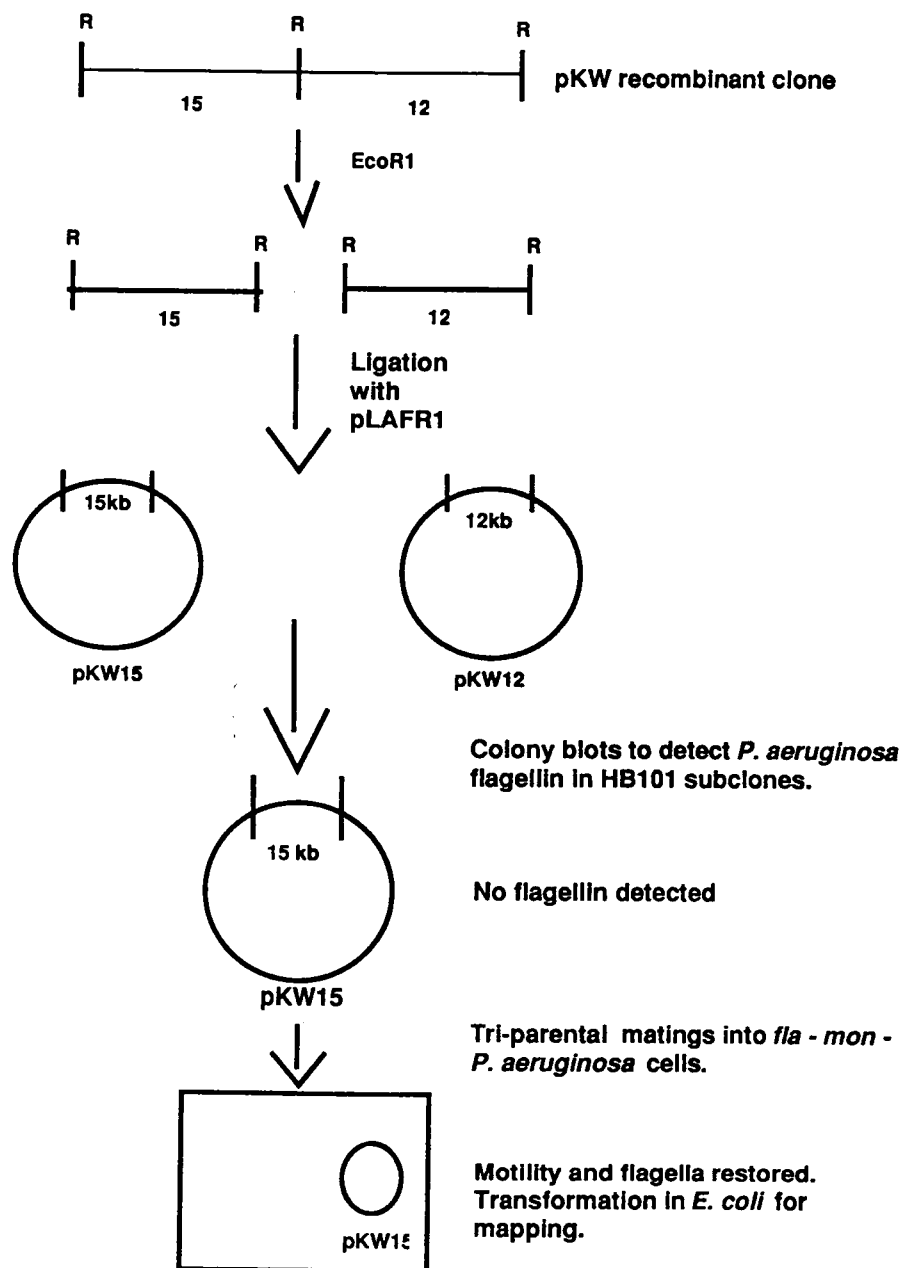
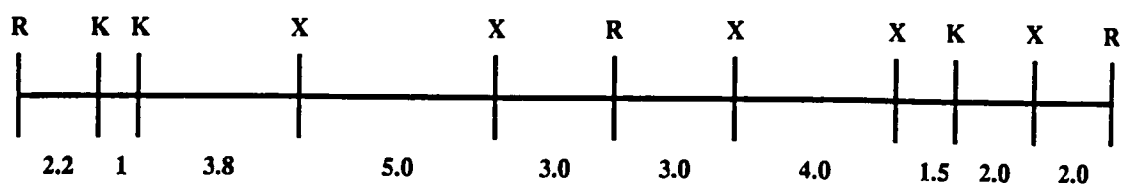
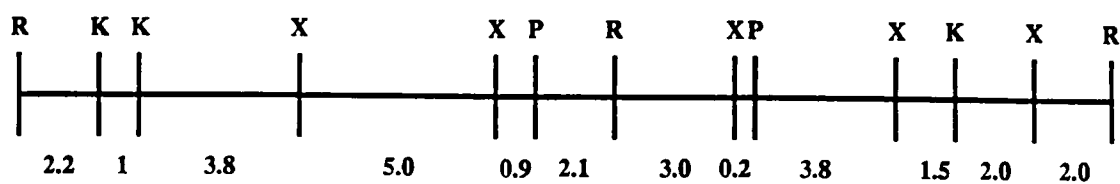


Figure IV-4. Subcloning of the *P. aeruginosa* PAO1 *fla* region in the recombinant clones pKW52 AND pKW123.

### pKW123



### pKW52



R - EcoR1  
 K - Kpn I  
 X - Xho I  
 P - Pst I

Figure IV-5. Restriction mapping of the *Pseudomonas aeruginosa* Fla region in pKW52 AND pKW123 recombinant clones.

*Clean* procedure from agarose gels and each was ligated to the broad host range vector, pLAFR1. The new subclones, pKW12 and pKW15 were tested for flagellin expression in *E. coli* HB101 cells, and it was found that only pKW15 plasmid produced detectable levels of flagellin, as determined by colony blotting. The subclones were triparentally mated into *P. aeruginosa* PAO1 *fla*<sup>-</sup> *mon*<sup>-</sup> cells generated by transposon mutagenesis, and again the pKW15 fragment, and not the pKW12, produced flagellin and restored motility in the *P. aeruginosa* mutant.

A more detailed endonuclease restriction map generated from single and double restriction digests was constructed from clones pKW52 and pKW123 as shown in Figure IV-5. There were slight differences in the restriction digest pattern of these clones. As shown in Figure IV-5, PstI sites were found only in pKW52.

Upon comparison of the restriction maps shown in Figure IV-5, and the transposon insertion sites represented in Figure IV-2, it was concluded that the Tn501 transposons lie within a 5.0 kb Xho I fragment. This fragment was located within the 15kb fragment, and therefore these results agreed with those from deletion mapping experiments. Experiments were performed to subclone the 5.0kb fragment, and to look for synthesis of flagellin. The 5.0kb fragment was excised and electroeluted or eluted by the *Gene Clean* method from agarose gels and blunt end ligated into the SmaI site of pLAFR1. Detectable levels of

flagellin were produced in *E. coli* HB101 and *P. aeruginosa* PAO1 *fla* ::Tn501 cells as judged by motility assays.

## DISCUSSION

The use of molecular genetic techniques for the analysis of *P. aeruginosa* has not progressed as rapidly as in other genera such as *E. coli*. Several reasons have contributed to this slow progress. A reliable system for transposon mutagenesis in *P. aeruginosa* has been slow compared to the enterics because of the small number of suitable delivery vectors available for the transposons (Stapleton et al., 1984). *P. aeruginosa* is naturally resistant to even high concentrations of most antibiotics. Therefore, distinguishing between the transposon antibiotic marker and spontaneous resistance has proven to be difficult, as seen in the Tn5 system. Also, regional specificity of transposon insertion has been reported for Tn1 in *P. aeruginosa* (Krishnapillai et al., 1981).

In spite of problems inherent in working with *P. aeruginosa* cells, an adaptation of a method described by Ohman using Tn501 was successful (Ohman, et al., 1985). The location of the *fla* gene in the recombinant clones was determined and *P. aeruginosa* isogenic mutants deficient in the *fla* gene were generated. As discussed in Part II, chemical mutagenesis

has provided no mutants deficient in the flagellin structural gene. Such mutations were found in CF isolates. However, of the cells that were screened, the site specific mutagenesis procedure produced a large percent of isogenic flagellin mutants.

Since the gene coding for the flagellin protein is about 1.5kb in length, there were over 20 kb of *P. aeruginosa* DNA on the recombinant plasmids pKW52 and pKW123 that were not in the *fla* gene. To locate the gene on a fragment of DNA, deletion mapping was performed. Fragments were cut with EcoR1 and the plasmid was recircularized with ligase. The effect was determined by colony blots in *E. coli* and the by ability of the subclone to complement the *P. aeruginosa fla mon* mutant. Initially, it was determined that the *fla* gene was located on a 15kb fragment. Transposon mutagenesis with Tn501 confirmed this, and pin-pointed the gene within a 5.0 kb Xho1 fragment.

Transposon mutagenesis of cloned DNA is a useful tool to locate specific genes on a recombinant plasmid. This system was based upon a technique originally described by White (White et al., 1983). Tn501 has been the transposon of choice because spontaneous Hg<sup>r</sup> in *P. aeruginosa* is rare. The use of Tn501 (Hg<sup>r</sup>) resulted in generating a number of mutations in the PAO1 DNA of the recombinant plasmids, pKW52 and pKW123. Selection by mercury resistance and colony immunoblots identified several clones with mutation in the flagellin structural gene.

The location of the gene was determined by mapping the Tn501 insertion sites. In all of the clones, the sites were within a 1.5 kb region.

To generate isogenic, site specific mutants in *P. aeruginosa*, a gene replacement technique was used in which fragments of cloned *P. aeruginosa* DNA previously mutated with the Tn501, were transduced from one *P. aeruginosa* strain to another with the F116L phage. Transductants were positive for gene replacement when their phenotypes were Hg<sup>r</sup>, Tc<sup>s</sup>. The sensitivity to tetracycline indicated the pLAFR1 vector was not coinherited. Future work will involve sequencing the b-type flagellin gene. Once the entire sequence of the b-flagellin monomer has been determined, the inferred amino acid sequence will be determined. One objective in sequencing the gene is to predict the hydrophilic peptides which might be antigenic. These peptides could be synthesized and tested for immunogenicity.

An interesting research direction derived from the sequence work will involve comparison of the inferred amino acid sequence of *P. aeruginosa* flagellin to known amino acid sequences of other bacterial flagellins. It will be of interest to determine if the N-terminal and C-terminal residues of flagella contain common sequences required for export in several bacteria. Since signal sequences are required for export of flagellin (Eisenstein, B. 1987). Also, the sequence of the b-type flagellin will be compared with the published a-type flagellin sequence (Totten, and Lory,

1991). With this information, the structure and function relationships between the a- and b-type flagellins could be determined. This comparison could be important with regard to the location of phosphorylated tyrosines of both flagellins.

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PART V

PHOSPHORYLATED TYROSINE IN THE FLAGELLUM FILAMENT  
PROTEIN OF *PSEUDOMONAS AERUGINOSA*.

## INTRODUCTION

This research effort was built around a central theme of developing a better understanding of the flagellin filament protein of *P. aeruginosa*. Therefore the second half of this research, discussed in the next two chapters, has focused upon a biochemical approach to isolate and characterize a posttranslational modification occurring in the flagella filament protein. Currently little is known or understood about the biosynthesis, secretion, and assembly of the flagella of *P. aeruginosa*; this work was begun in hopes of investigating these aspects.

Posttranslational modifications are common in eukaryotes and prokaryotes. One type of post translational modification which proteins undergo is phosphorylation. Certain observations prompted the investigation to determine whether flagella of *P. aeruginosa* are phosphorylated. First, phosphorylation is a common modification in eukaryotic systems from fungi to mammals, and it is involved in a large number of cellular reactions. Secondly, several different modes exist for posttranslational modification of bacterial flagella and pili (Macnab, 1987). In *P. aeruginosa*, N-methyl phenylalanine is an N-terminal component of a major class (type IV) pilin, and is found in pili protein related to this group and in other widely divergent genera (Strom, and Lory, 1991). In *S. typhimurium* flagellin, N-methyl lysine has been

isolated (Joys, 1988). Thirdly, in *Campylobacter* flagellum filaments, a posttranslational modification has been reported (Logan et al., 1989). *Campylobacter coli* flagellin contains modified serine residues generated by phosphorylation, most of which occurs within a region containing two nine amino acid repeating peptides separated by 34 unique amino acids. The later report was of particular interest because of the polar arrangement of true flagellum in *Campylobacter*, is analogous to *P. aeruginosa*.

Phosphorylation was first demonstrated in 1956 by Krebs and Fischer (Krebs, and Fischer, 1956). It was shown that the activity of rabbit skeletal muscle glycogen phosphorylase was regulated by reversible phosphorylation. The idea of phosphorylation in prokaryotes remained a controversy for years because of the inability to reproduce results demonstrating the existence of a protein kinase (Cozzzone, 1988). In the last decade, however, this uncertainty has been diminished by conclusive evidence of the existence of protein kinases in prokaryotes.

A number of different protein kinases have been characterized in enteric bacteria. These include isocitrate dehydrogenase kinase (Garnak, and Reeves, 1978), the Che A protein kinase, and the NtrB protein kinase (Stock, et al., 1989). However, these kinases phosphorylate their protein substrates at either serine or threonine residues (Wang, and

Koshland, 1981). By contrast, the presence of tyrosine kinase in enteric bacteria has not demonstrated (Foster et al., 1989).

Evidence for phosphorylation of proteins by protein kinase was first obtained simultaneously, and independently, in both *E. coli* and *S. typhimurium*. The NADP-dependent isocitrate dehydrogenase (IDH) of *E. coli* was the first bacterial phosphoprotein identified (Garnak, and Reeves, 1978). The enzyme catalyzes the metabolism of isocitrate at a branch between the Krebs Cycle and the glyoxylate bypass. Depending on the nature of the carbon source, IDH may or may not be phosphorylated. The phosphorylation reaction leads to inactivation of the enzyme (Nimmo, 1984). The inactivation is mediated by the negative charge of the phosphate (Thorsness, and Koshland, 1987).

Another phosphoprotein found in *E. coli* is dnaK protein. It modulates the heat-shock response in bacteria, and is required for the replication of DNA (Paek, and Walker, 1987). This protein is phosphorylated in exponentially growing bacteria.

Phosphorylation also plays a critical role in signal transduction systems. It is now recognized that several global regulatory networks in bacteria are controlled by transcriptional regulators of signal transduction systems. (Dieteric et al., 1989). These systems are responsible for the ability of the bacteria to respond to changes in nitrogen, phosphate, oxygen, osmolarity, and chemical gradients (Ninfa et al., 1988). The model for this system is

that one component is a sensor which reacts to environmental stimuli while the second component, the effector, receives the signal from the sensor and is activated. The sensors have conserved C-terminal domains while the effectors share similar N-terminal domains (Stock et al., 1989).

It appears that sensory transduction in the nitrogen (Ntr) and chemotaxis (Che) systems involves a common protein phosphotransfer mechanism. It is also suggested that the homologies between modulator proteins reflect conserved protein kinase function, and the homologies between effector proteins reflect conserved phosphoacceptor activities. Therefore, bacterial cells are capable of using common biochemical reactions and signal transduction modules to relay a variety of unrelated messages from the environment to specific components of the genetic apparatus. Thus, in phosphate regulation, Pho R is a phospho-regulated kinase and Pho B is a phospho-transcriptional factor (Wanner, 1987); in osmoregulation of porin expression, Env Z is the kinase that phosphorylates Omp R. The question of cross-talk occurring in vivo and its effect in global regulation is a question that will need to be answered.

It has been estimated that about 5% of the total proteins in *E. coli* undergo phosphorylation, suggesting that the modification is specific (Cozzzone, 1988). Phosphorylation has been found in a number of other bacterial species. In most cases, phosphoproteins have been detected through their ability to incorporate radiolabeled [ $^{32}\text{P}$ ] phosphorous, and

the nature of the phosphorylated moiety has been characterized chemically and enzymatically. The most common modifications in all cells occur on the amino acids tyrosine, threonine, and serine (Cooper et al., 1983).

## MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Chemicals. *P. aeruginosa* wild-type strain PAO1 was graciously supplied by A. Kropinski, Queen's University, Kingston, Ontario, Canada. Strain M-2, originally isolated from the small intestine of a CF-1 mouse, was kindly provided by I. A. Holder, Shriner's Burn Institute, Cincinnati, Ohio (Drake, and Montie, 1987).

Cells were grown in two liters of modified mineral salts medium [ $4 \times 10^{-4}$  M  $K_2HPO_4$ ,  $2.2 \times 10^{-4}$  M  $KH_2PO_4$ ,  $7.5 \times 10^{-3}$  M  $(NH_4)_2SO_4$ ,  $2.0 \times 10^{-4}$  M  $MgSO_4$ ,  $6 \times 10^{-6}$  M  $FeCl_3$ , and 0.4% sodium succinate] and incubated at 30°C for 20 h on a gyratory shaker. The total phosphate concentration was decreased to approximately twofold over the limiting levels (0.62 mM). [ $^{32}P$ ]Phosphoric acid (ICN Biomedical, Inc., or Dupont Biotechnical Systems) was added at a concentration of five micro Curies (uCi) per ml, and the cells were grown at

30°C for 22 hours. Phosphoamino acid standards (Sigma Chemical Co., St. Louis, MO.) were prepared at a concentration of 20 mM in 0.3 N HCl.

Isolation and Purification of Flagellum Filaments. The cells were concentrated by repeated centrifugation at 5,000 x g at 4°C. After concentration, the cells were suspended in 10 mM phosphate buffer (pH 7.0) (35 ml/liter of cells) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and the flagella were removed from the cells by shearing in a Waring blender for 30 sec on low speed. The sheared cell suspension was centrifuged at 5,000 x g for 30 min at 4°C. The supernatant containing flagellum filaments was again centrifuged at 16,000 x g to remove any contaminating cellular debris. The supernatant was retained, and the flagellum filaments were pelleted by ultracentrifugation (24,000 rpm for 3 h at 7 °C), the pellets were suspended in 30 mM Tris chloride (pH 8.0), dialyzed overnight, lyophilized.

Isolated flagellum filaments were purified primarily to remove lipopolysaccharide by gel filtration. The partially purified sample (approximately 2 mg) was dissolved in one ml of sample buffer (15 mM Tris [pH 8.0], 0.1 M EDTA [pH 8.0], 0.1 mM phenylmethylsulfonyl fluoride, 1% sodium azide, 0.1% sodium deoxycholate), and loaded onto a

Sephacryl S-300 superfine column containing 30 mM Tris chloride (pH 8.0) and 2 mg of deoxycholate buffer per ml. Flagellum filaments emerged as undissociated protein at the void volume. After lyophilization, the combined flagellar fractions were passed through a Sephadex G-25 column equilibrated with a 10 mM Tris chloride (pH 8.0) buffer to remove excess deoxycholate and EDTA. Again, the flagellar filaments emerged at the void volume. The flagellar sample was passed through a second Sephadex G-25 column with 0.1%  $\text{NH}_4\text{OH}$  as the running buffer to desalt the preparation.

Electrophoresis and Immunoblots of Labeled Flagella. Flagellum samples were dissolved in SDS-EDTA dissociating buffer as described in Part II. Samples containing one to four ug of flagellum protein were applied to SDS-10 to 15% polyacrylamide Phast gels and separated by the Phast system (Pharmacia Diagnostics, Piscataway, N. J.) as described in in Part II. After electrophoresis, the gels were stained with Coomassie brilliant blue. Immunoblotting was done as previously described. (Wintenberg-Kelly and Montie, 1989) and Part II. A yellow band color was positive. Location of  $^{32}\text{P}$ -labeled proteins was determined by autoradiography with Kodak S-Omat R film (Eastman Kodak Co., Rochester, N. Y. ) in darkness at  $-70^\circ\text{C}$  for 48 to 72 hr.

#### Preparation of Acid Hydrolysates and Trypsin Digests of Flagellin.

The labeled flagellin protein (250 ug) was hydrolyzed in 200 ul of 6 N HCl at 110°C for 45 min. in a sealed hydrolysis vial. The samples were lyophilized and then suspended in 200 ul of 0.3 N HCl. For trypsin digests, purified lyophilized flagellin was suspended in 0.1 M Tris-Cl (pH 8.0) containing 0.1% deoxycholate at a ratio of 5:1 (wt/vol) minus the volume of trypsin used. After a 30 min incubation period at 70°C to denature the protein, trypsin was added at a ratio of 1:50 (trypsin:prep). The suspension was incubated for 30 min at 37°C. The trypsin was inactivated by incubation at 100°C for 5 min, and the trypsinized flagella peptides were lyophilized.

Thin Layer Electrophoresis (TLE) of Acid Hydrolysates. The labeled flagellin protein (0.5 mg) was hydrolyzed in 200 ul of 6 N HCl at 110°C for 45 min in a sealed tube. The sample was then lyophilized and suspended in 400 ul of 0.3 N HCl. Approximately 15 ul of the flagellar hydrolysate samples and 60 ug of phosphoamino acid standards were spotted one ul at a time, and dried with a stream of air onto thin-layer chromatography cellulose paper (Eastman Kodak) as described by Cooper et al. (Cooper et al., 1983), and separated in either pH 3.5 buffer

(10:100:1890 pyridine-glacial acetic acid-distilled H<sub>2</sub>O) or pH 1.9 buffer (50:156:1794 88% formic acid-glacial acetic acid-distilled H<sub>2</sub>O) at 18 mA and 350 V for one and one half to three hours, respectively. After drying, the chromatograms were sprayed with a ninhydrin solution (0.5% ninhydrin in acetone-4.5% collidine), and spots were developed by exposure to a stream of hot air. Locations of unlabeled phosphoamino acid standards and unknown peptides were determined by ninhydrin staining, and labeled amino acids and peptides were detected by autoradiography and scintillation counting.

High Pressure Liquid Chromatography (HPLC) Analysis. For HPLC analysis, 100  $\mu$ l (100 to 125  $\mu$ g) of hydrolyzed flagella in 0.3 N HCl was mixed with 400  $\mu$ g each of phosphotyrosine, phosphothreonine, and phosphoserine in 300  $\mu$ l of 0.1 M Tris chloride (pH 8.0). This sample was injected into a VYDAC C<sub>18</sub> column connected to a Waters HPLC system with a solvent system consisting of 0.1% trifluoroacetic acid-acetonitrile (Lottspeich and Henschen, 1985). One minute fractions were collected, and 20  $\mu$ l samples were spotted on glass fiber filter discs and dried. The radioactivity was assayed by scintillation counting.

Quantitation of Phosphorylated Compounds. The relative amount of each sample containing phosphate radioactivity was determined by scraping

each lane, which had been divided into one centimeter squares, and quantitating the radioactivity present by scintillation counting. The relative location of labeled phosphoamino acids was determined by comparing them with comigration of ninhydrin-stained phosphoamino acid standards. Results were expressed as a percentage of radioactivity recovered in the phosphoamino acids and phosphopeptides.

## RESULTS AND DISCUSSION

In separate experiments, two b-type strains, PAO1 and M2, were grown in the presence of  $^{32}\text{P}_i$  overnight (5 uci/ml). Flagellum filaments were sheared from intact cells, and flagellin protein was purified as previously noted (Montie et al., 1987) and modified as previously described by Wintenberg, et al. (Wintenberg-Kelly et al., 1990) and in the Materials and Methods of this Part. Isolation of b-type 53 kDa subunits from purified preparations is shown on stained gels in Figure V-1A. Autoradiograms obtained from stained gels showed that two to four ug (dry weight) of protein applied to Phast gels readily gave positive banding as shown in Figure V-1B. The different concentration of labeled protein corresponded to the different intensities seen in the autoradiogram. A more intense band was seen in Lane 4, which contained four ug of flagellin

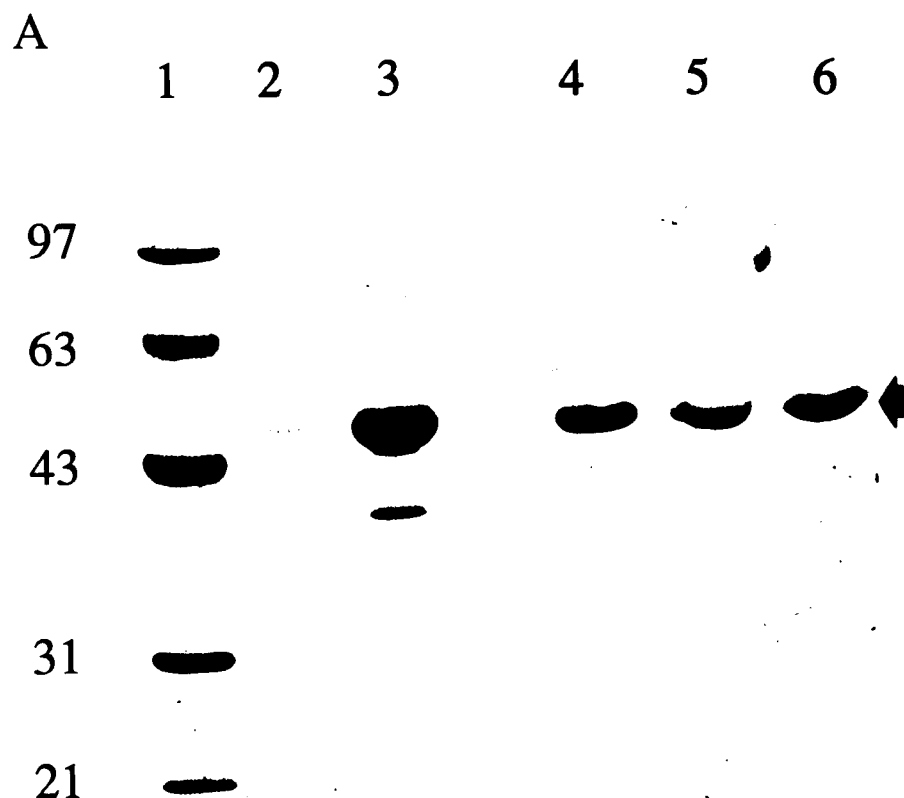


Figure V-1. Phosphorylation of *Pseudomonas aeruginosa* flagellum filament protein. (A) Coomassie blue-stained polyacrylamide gel. Lanes: 1, molecular mass markers (kilodaltons); 2 and 3, standards of 53-kDa flagellin from PJ108 and PAO1; 4, 5, and 6, isolated labeled PAO1 flagellin (4, 2, 3 ug, respectively). Arrow denotes 53 kDa band.

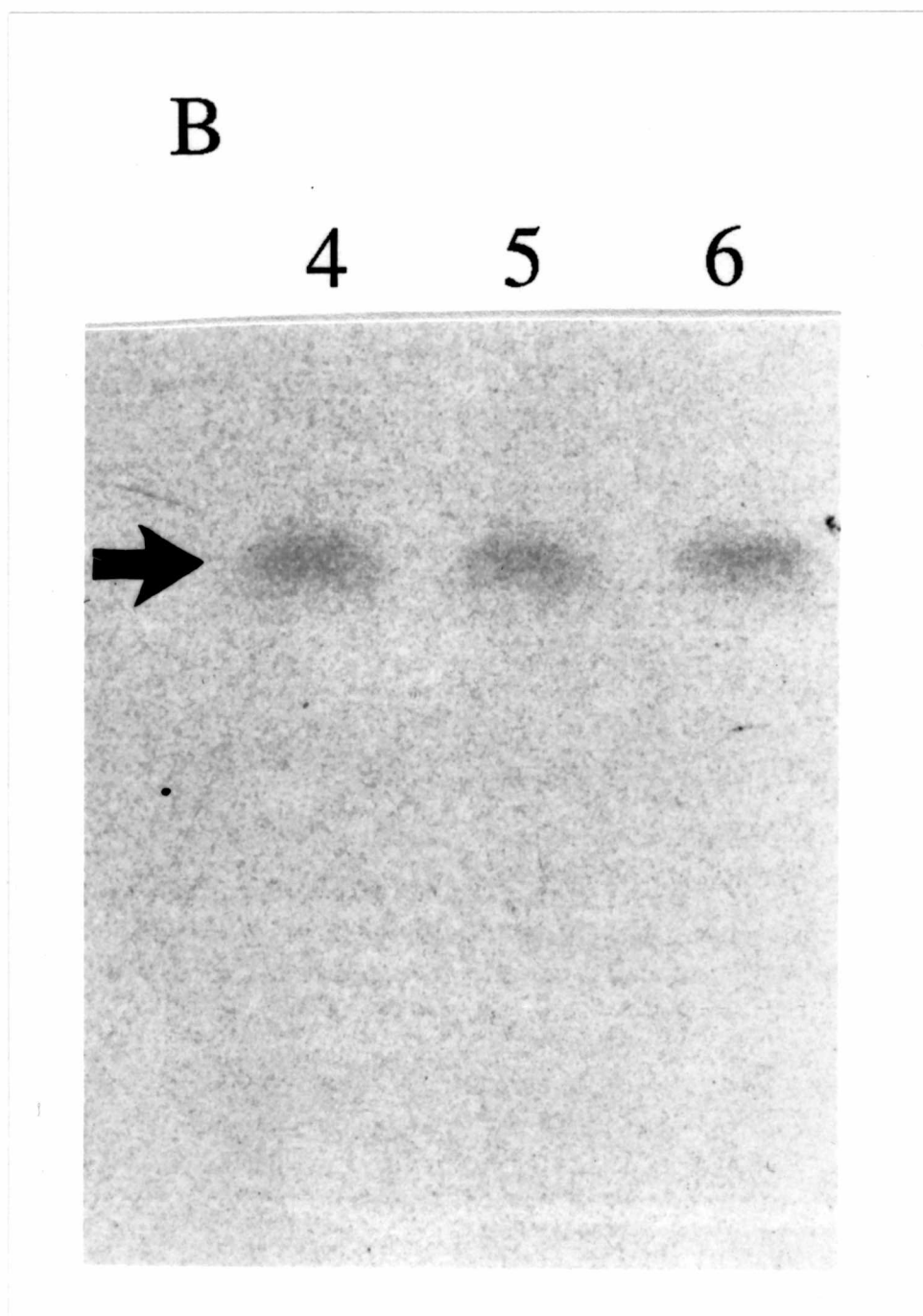


Figure V-1 (continued). (B) Autoradiogram of panel A, lanes 4, 5, and 6, as describe above. Arrow denotes 53 kDa band.

than Lane 5, which contained only two ug of protein. This indicated that the labeling seen in Figure 1B was directly related to the concentration of flagellin protein present. Results from immunoblotting experiments also gave radioactive bands corresponding to 53 kDa flagellin when subjected to autoradiography as shown in Figure V-2.

Since purified  $^{32}\text{P}$ -labeled PAO1 flagellin was readily detectable, these experiments were repeated with another b-type strain, M-2, to eliminate the possibility of any unusual properties associated with a single strain. Analogous results were obtained with M-2 flagellin at all levels of purification. It was concluded from the data that phosphate was covalently bound to flagellin.

The phosphoryl linkage hypothesized was most likely an O-ester linkage to serine (p-ser), threonine (p-thr), or tyrosine (p-tyr) since there is no histidine in the flagellin, and acyl residues are very unstable (Cozzone, 1988). To identify the O-phosphates, samples were separated by TLE. Labeled phosphoproteins and  $^{32}\text{P}_i$  migrate towards the anode.

The rate of migration in pH 3.5 buffer was  $\text{P}_i > \text{P-ser} > \text{p-thr} > \text{p-tyr} > \text{phosphopeptides}$  (Martensen, 1984). Phosphoserine or phosphothreonine were suspected as likely candidates to be isolated because these amino acids were the ones most commonly phosphorylated in *E. coli* (Cooper et al., 1983; Cozzone, 1988). However, this was not case. Examination of partial acid hydrolysis (two to four h) of flagellin by TLE in pH 1.9 buffer

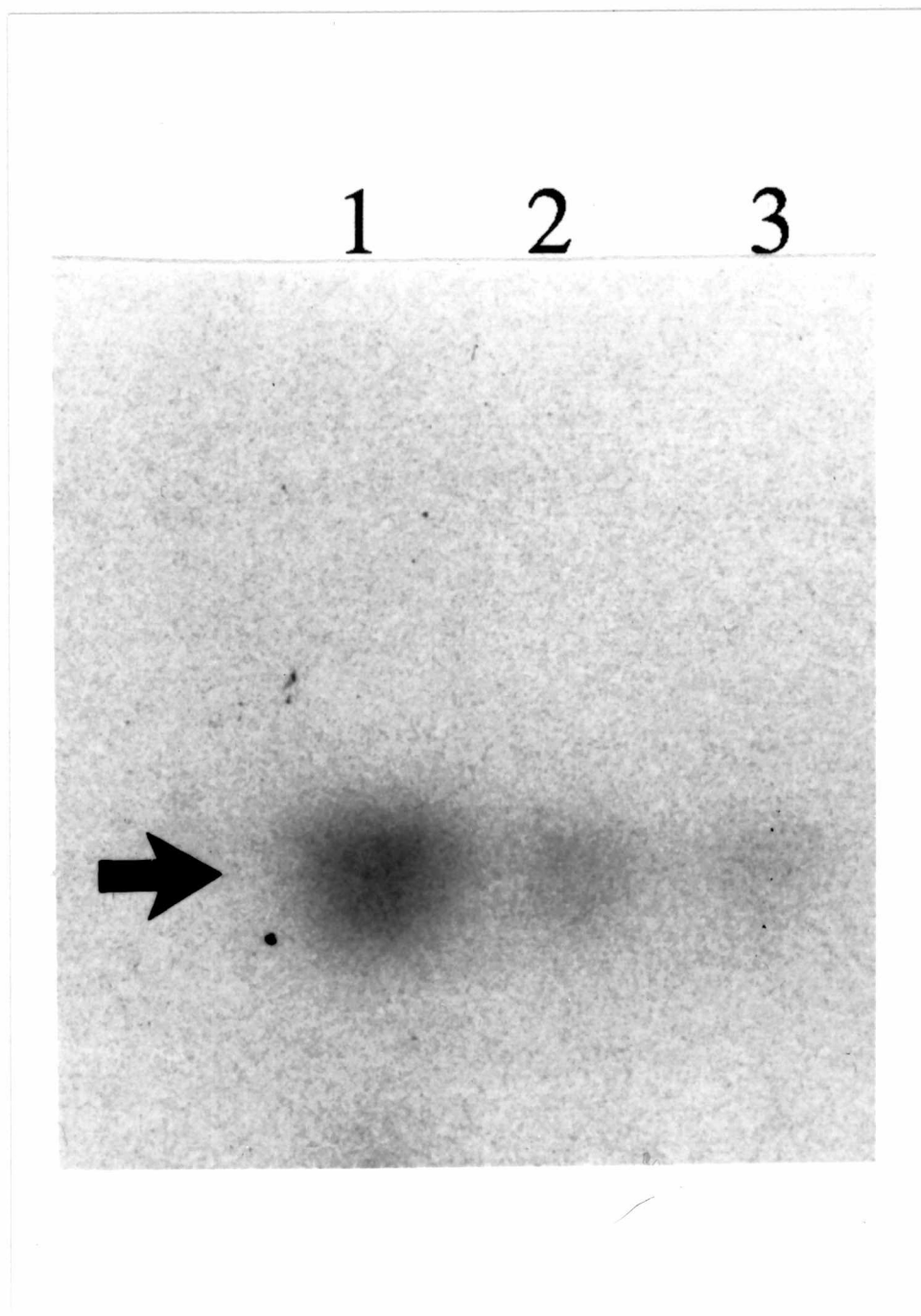


Figure V-2. Autoradiogram of immunoblot of  $^{32}\text{P}$  labeled flagellin. The immunoblots were obtained as described in Materials and Methods. Lanes 1, 2, and 3, labeled PAO1 flagellin. Arrow denotes 53 kDa band.

(2.5% formic acid, 7.8% glacial acetic acid in H<sub>2</sub>O) showed all of the activity in phosphotyrosine with very little to no activity in p-ser or p-thr (Table V-1). To avoid degradation of labeled phosphoamino acids, the hydrolysis time was reduced to 45 min and the partial acid hydrolysates were examined by TLE in a pH 3.5 buffer (0.5% pyridine, 5% glacial acetic acid in H<sub>2</sub>O). Hydrolysate samples gave approximately 250 cpm/ug of protein. Results from autoradiography of chromatograms separated in pH 3.5 buffer, unexpectedly showed that most of the radioactivity appeared in phosphotyrosine (Figure V-3).

For quantitative estimations, TLE chromatograms were sectioned and radioactivity in all fractions was compared. Results showed 34% radiolabel in phosphotyrosine and negligible activity in phosphoserine and phosphothreonine, with most of the remaining radiolabel associated with putative ninhydrin-positive peptides (Figure V-3 and Table V-1). The phosphotyrosine was clearly resolved in the single-dimension electrophoresis in pH 3.5 buffer (Cooper et al., 1983). Three slowly migrating peptides, designated P-2, P-3, and P-4 (Table V-1), retained over 50% of the total amino acid radiolabel. Also a peptide, P-8 was present. Two similarly migrating components designated P-3 and P-4 were observed in TLE separations at pH 1.9 (Table V-1). In some experiments, small amounts of phosphotyrosine were seen in pH 1.9 separations, but overall, the combination of partial acid hydrolysis and pH

Table V-1. Analysis by TLE of radiolabeled compounds in partial acid hydrolysates (45 min) of M2 flagellum filaments<sup>a</sup>.

| Component          | Separation buffer   |           |
|--------------------|---------------------|-----------|
|                    | pH 1.9 <sup>c</sup> | pH 3.5    |
| Phosphotyrosine    | 7 (57)              | 34(2,870) |
| Phosphoserine      | _c                  | _c        |
| Phosphothreonine   | _c                  | _c        |
| P-2 <sup>d</sup>   | 3(30)               | 13(1,100) |
| P-3 <sup>d</sup>   | 41(335)             | 37(3,148) |
| P-4 <sup>d</sup>   | 38(314)             | 4(400)    |
| P-8 <sup>c,d</sup> | 11(87)              | 12(1,000) |

<sup>a</sup>Analogous results were seen with flagella of strain PAO1. Results are shown as percent total radioactivity excluding free Pi; Numbers in parentheses are counts per minute.

<sup>b</sup>2-h hydrolysates showed similar results.

<sup>c</sup>In some experiments, P-8 overlapped with p-ser and p-thr, obscuring accurate determinations of the compounds.

<sup>d</sup>P denotes peptide and number denotes migration from origin in centimeters.

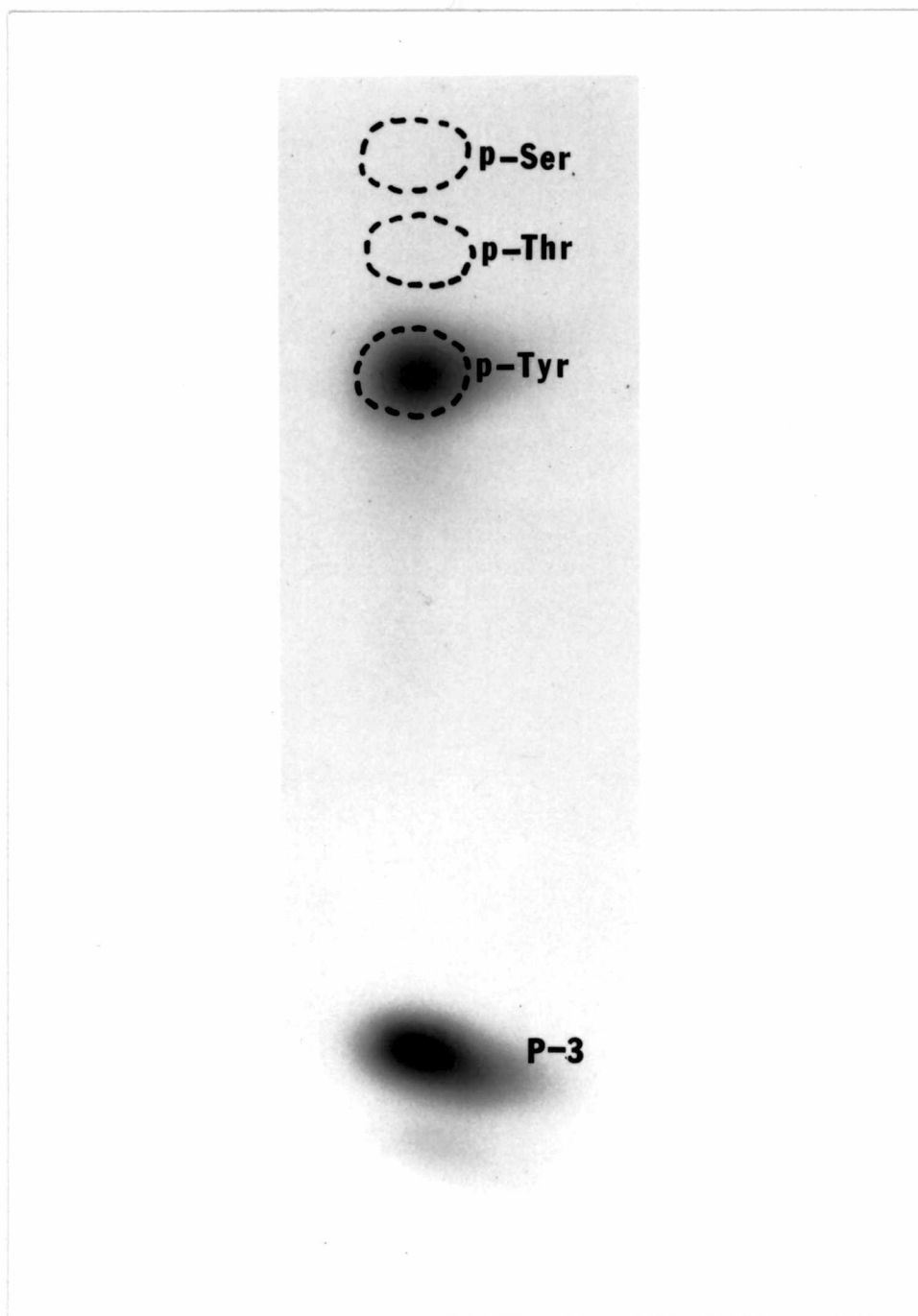


Figure V-3. Autoradiogram of TLE of hydrolyzed filament protein. Migration of unlabeled standards was determined by ninhydrin staining, and labeled amino acids were detected by autoradiography. p-ser, phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine.

1.9 buffer apparently released labeled Pi from tyrosine, and reduced increased in the pH 1.9 buffer compared with the pH 3.5 buffer, indicating hydrolysis of the phosphoryl linkage.

Confirmation of these results was obtained by HPLC separations of 45-min hydrolysates on C<sub>18</sub> columns (Figure V-4). A significant amount of activity was associated with an early appearing 5-min peak corresponding to phosphotyrosine and a peptide-like component. The presence of phosphotyrosine in fraction five was confirmed by its adsorption spectrum with a peak at 265 nm and by TLE assay (Table V-2 and Figure V-5). No evidence was obtained for the presence of any other labeled amino acid. However, fraction 12 contained a single component that migrated on TLE assays as a ninhydrin-positive peptide and was responsible for all the radioactivity in that fraction.

To further confirm that the ninhydrin positive spots represented peptides (P-2, P-3, P-4, P-8), the individual peptides from b-type flagella were eluted from a TLE chromatogram and subjected to pronase digestion for 20 h at 37°C. The treated samples were separated by TLE along with non-treated samples. The chromatogram was cut, and counts were determined (Table V-3). Peptides P-3, P-4, P-8 were substantially reduced in radioactivity (by 40 to 87%) compared to untreated controls. In conclusion, evidence indicates that *P. aeruginosa* flagellin filament protein is phosphorylated in vivo, and that a substantial portion of the

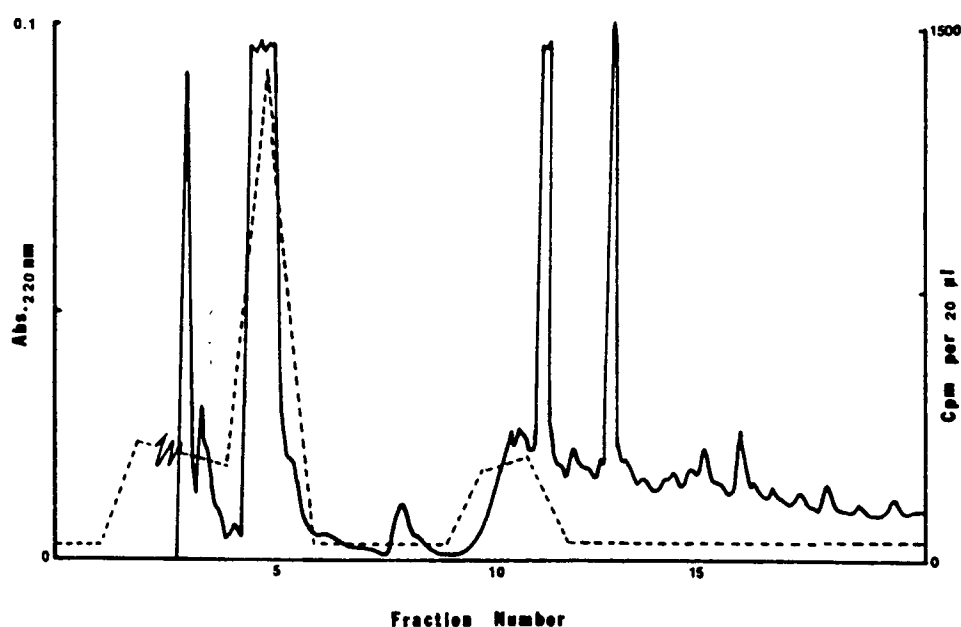


Figure V-4. HPLC profile and fraction radioactivity of hydrolyzed filament protein. One-minute fractions were collected, and 20 µl samples were spotted and the radioactivity assayed by scintillation counting as described in Materials and Methods. Solid line represents  $A_{220}$  of fractions; dashed line represents counts per fraction.

Table V-2. Analysis by TLE of radiolabeled compounds in partial acid hydrolysates (45 min) of M2 flagellum filaments<sup>a</sup>.

| Compound           | HPLC fraction in pH 3.5 buffer <sup>b</sup> |             |
|--------------------|---------------------------------------------|-------------|
|                    | Fraction 5                                  | Fraction 12 |
| Phosphotyrosine    | 60(1850)                                    | <1(4)       |
| Phosphothreonine   | <1(2)                                       | 0(0)        |
| P-2 <sup>d</sup>   | 13(400)                                     | <1(5)       |
| P-3 <sup>d</sup>   | 23(724)                                     | 99(4,096)   |
| P-4 <sup>d</sup>   | 2(86)                                       | <1(8)       |
| P-8 <sup>c,d</sup> | <1(20)                                      | 0(0)        |

<sup>a</sup> Analogous results were seen with flagella of strain PAO1. Results are shown as percent total radioactivity excluding free Pi; Numbers in parentheses are counts per minute.

<sup>b</sup> No radioactivity was detected in HPLC fractions 13 and 14, which seemed to correspond to phosphothreonine and phosphoserine fractions.

<sup>c</sup> In some experiments, P-8 overlapped with p-ser and p-thr, obscuring accurate determinations of the compounds.

<sup>d</sup> P denotes peptide and number denotes migration from origin in centimeters.

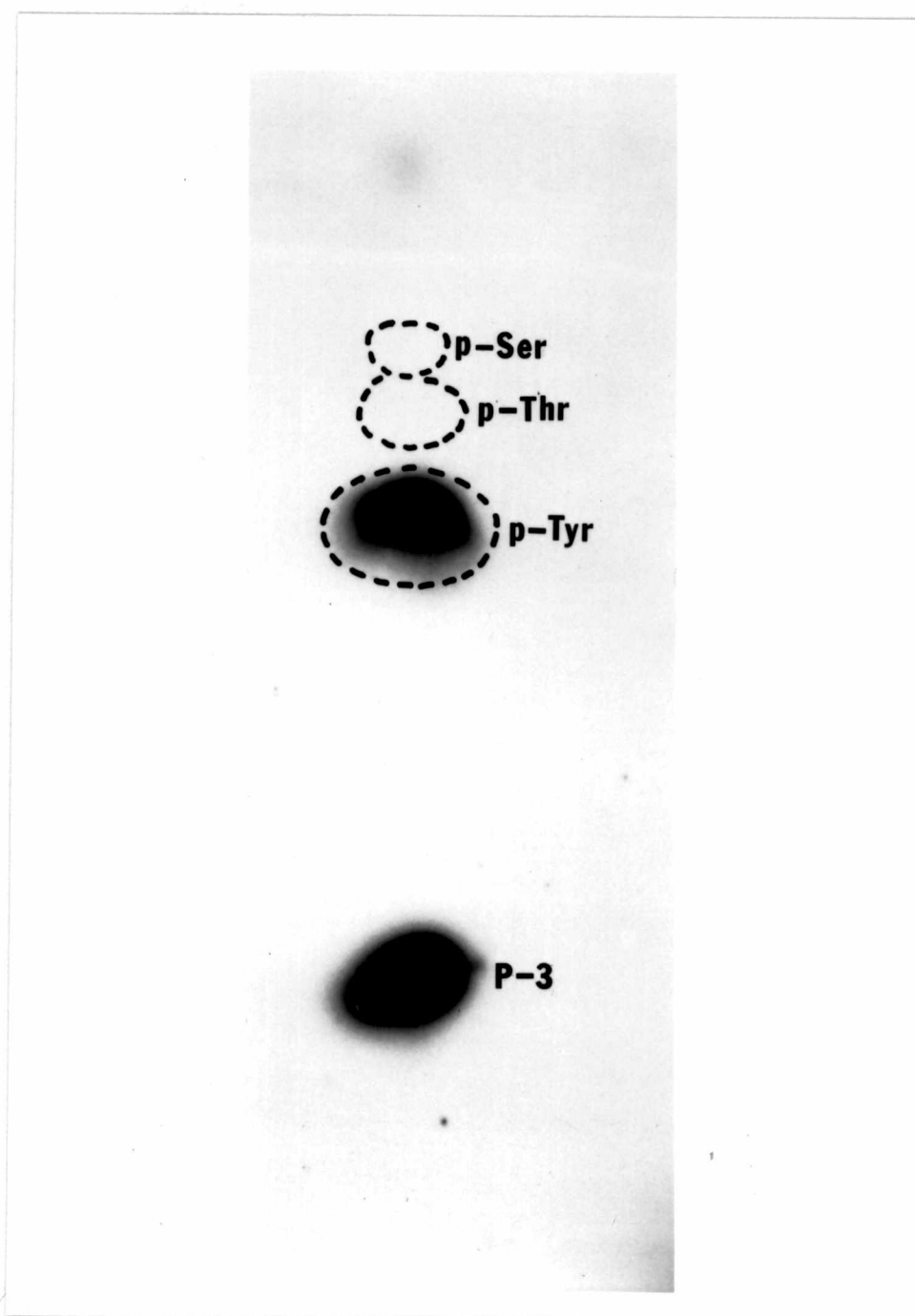


Figure V-5. Autoradiogram of TLE of the peak fraction (number five) of HPLC-purified hydrolyzed filament protein. Migration of unlabeled standards was determined by ninhydrin staining, and labeled amino acid components were detected by autoradiography. p-ser, phosphoserine; p-thr, phosphothreonine; p-tyr, phosphotyrosine.

Table V-3. Reduction in specific activity by pronase digestion of peptides generated from acid hydrolysates.

| Peptides <sup>a</sup> | <u>Counts per minute</u> |                   |
|-----------------------|--------------------------|-------------------|
|                       | No pronase treatment     | Pronase treatment |
| P-3                   | 95                       | 56                |
| P-4                   | 431                      | 57                |
| P-8                   | 177                      | 85                |

<sup>a</sup>The peptide numeral denotes the distance migrated from the origin in centimeters.

phosphate is bound to tyrosine. Previous amino acid analyses have consistently shown the presence of seven to eight tyrosine residues per 53-kDa subunit. It will be of interest to determine whether all the tyrosines present per subunit are phosphorylated and whether every 53-kDa subunit is phosphorylated. Based on preliminary data we estimated that there is approximately 1.0 mole of  $^{32}\text{P}$  per mole of flagellin protein.

The presence of p-ser or p-thr has not been completely excluded, but these compounds were not readily detectable in acid hydrolysates. Experiments employing alternative hydrolysis procedures are to be carried out, to examine the components of the incompletely digested peptides. These results will determine whether phosphotyrosine is the phosphorylated amino acid present in these peptides. At present, the function(s) of posttranslational modifications of flagellum filament protein is unknown. Based upon preliminary evidence (see Part VI), the current hypothesis suggests a role for phosphorylation affecting the filament structure, thereby altering motility. It cannot be ruled out that this posttranslation modification is associated with export. These current ideas are discussed in Part VI.

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CHAPTER VI

COMPARATIVE ANALYSIS OF TYROSINE PHOSPHATE IN BOTH a-  
AND b-TYPE FLAGELLINS OF *PSEUDOMONAS AERUGINOSA*.

## INTRODUCTION

The purpose of the research discussed in this chapter was to further characterize the structure of the flagellin filament protein in *P. aeruginosa*, particularly the protein's relationship to the site and location of phosphorylation. This posttranslational modification might influence flagella function, or assembly and export of the flagellin subunits.

It has been known for some time that certain flagellins are posttranslationally modified, e.g., N-methyl lysine is found in *Salmonella* flagellin (Joys, 1988). Also, in *Campylobacter* flagella, phosphorylation of serine residues has been indicated (Logan et al., 1989). In *P. aeruginosa*, N-methyl phenylalanine is an N-terminal component of a major class (type IV) pilin, and is also found in pili protein in widely-divergent genera (Paranchych, and Frost, 1988; Strom, and Lory, 1991). The biological significance of these modifications remains unknown (Foster et al., 1989).

Tyrosine phosphorylation, although occurring less frequently than modifications of serine and threonine, contributes to very specific and important regulatory events in animal cells. These include several hormone receptors (e.g., insulin and epidermal growth factors), a variety of oncogene proteins, regulation of retroviral replications, and tubulin function and motility (Hayashi et al., 1987; Hunter, and Cooper, 1985;

Matten et al., 1990). Flagella protein phosphorylation is an important feature of motility in unicellular eukaryotes such as *Chlamydomonas* (Tash, 1989).

Although thought to be of little significance until recently, posttranslational phosphorylation in bacteria is now recognized as playing a vital role in individual enzyme regulation (Dannelly et al., 1989; Hurley et al., 1990; Robertson, and Reeves, 1989), as well as in global control mechanisms (Stock et al., 1990). The presence of tyrosine phosphorylation in prokaryotes remains controversial. The presence of p-tyr in prokaryotes has been well documented (Cozzzone, 1988). In fact, wild type *E. coli* and *S. typhimurium* extracts have been found to yield significant proportions of p-tyr. Upon partial acid hydrolysis, p-tyr constituted about 9% of the total recovered phosphoamino acids (Foster et al., 1989). However, the majority of p-tyr found in prokaryotes is thought to be generated by partial acid hydrolysis of proteins to which a nucleotide or nucleic acid is linked via a phosphodiester bond to the phenolic hydroxyl of a tyrosine residue (Foster et al., 1989). In enteric bacteria, protein kinases phosphorylating serine and threonine have been well characterized, however; no tyrosine protein kinase activity has been isolated in these bacteria (Foster et al., 1989).

In contrast, Vallejos and associates (Vallejos et al., 1985), however, have documented the presence of phosphotyrosine in several membrane-

associated proteins from the photosynthetic bacterium, *Rhodospirillum rubrum*. More importantly they have also identified a tyrosine kinase in bacterial extracts that phosphorylates certain synthetic substrates containing a single tyrosine residue as the only apparent phosphoacceptor site. Phosphatase activity has not been reported associated with the reaction. In addition, no one has ever reported repeating this work (Kaplan, S., personal communication).

If a phosphoprotein containing p-tyr or p-ser is isolated from cells grown in the presence of labeled Pi, it is important to characterize the type of linkage to serine or tyrosine. To demonstrate the presence of an O-phosphomonoester linkage as opposed to a phosphodiester linkage, the labeled samples are treated with phosphatase, and characterized by the release of labeled Pi. Failure to distinguish released Pi indicates the presence of a phosphodiester linkage. This type of linkage is typically confirmed with snake venom nuclease, which removes labeled nucleotide ligated through a 5'-phosphodiester to tyrosine (Martenson, 1984).

In a preliminary report, the presence of phosphotyrosine in b-type flagellin has been demonstrated (Wintenberg-Kelly et al., 1990) and in Part V. In this research, these findings have been extended to show phosphotyrosine present in several a- and b- type flagellins. Evidence is presented that phosphotyrosine is associated with the flagellin by a monophosphoester linkage.

## MATERIALS AND METHODS

Materials. Phosphoric acid [ $^{32}\text{P}$ ] was purchased from ICN Biomedical Inc. or Dupont Biotechnical Systems. Sephacryl S-300 and Sephadex G-25 and the Phast Gel PAGE System were obtained from Pharmacia Fine Chemicals. Nitrocellulose for immunoblots, molecular weight markers, and horse radish peroxidase were purchased from BioRad Labs. Film (X-ray) for autoradiography and thin layer cellulose paper were from Eastman Kodak Company. Anti-phosphotyrosine monoclonal antibody was obtained from Upstate Biotechnology Inc. Diaminobenzidine, collodine, ninhydrin, trypsin, and the phosphoamino acids were purchased from Sigma Chemical Co. All other chemicals were from Baxter Scientific.

Bacterial Strains and Growth Conditions. *P. aeruginosa* strains used in this study were common laboratory strains previously typed as a- or b-type antigenic strains (Allison et al. , 1985). Bacteria were grown in 2 liters of modified mineral salts medium (MSM) as previously described (Wintenberg-Kelly et al., 1990) and in Part V. [ $^{32}\text{P}$ ] Phosphoric acid was added at a concentration of 5 uCi/ml for all strains. Late log cells were obtained by growth at 30°C for 22 h. To obtain mid-log cells, *P.*

*aeruginosa* grown overnight was adjusted to an O.D. of 0.04, and then were grown at 30°C for about five h until the O.D. 595 equaled 0.25 units (Craven, and Montie, 1985).

Isolation and Purification of Flagella Filaments. Flagella from the a- and b-type cells were isolated and purified as previously described (Wintenberg-Kelly et al., 1990) and in Part V. Briefly, after concentrating the cells, the flagella were removed by shearing, and isolated by differential centrifugation. The flagella were purified by gel filtration. In all cases, the intact flagella filaments eluted at the void volume.

Amino Acid Compositions and N-terminal Amino Acid Analyses. Amino acid and N-terminal analyses were carried out as described in Part II.

SDS Gel Electrophoresis. Purified flagellin samples were separated as previously described in Part II. All gels were stained with Coomassie brilliant blue. Location of <sup>32</sup>P-labeled proteins was determined by autoradiography in darkness at -70°C for 48-96 h as described in Part V.

Immunoblotting with anti-Phosphotyrosine Monoclonal Antibody or With anti  $\alpha$ -type Flagellin Polyclonal Antibody. Immunoblots were prepared as previously described in Part II. The blots were incubated with the anti-phosphotyrosine monoclonal antibody (diluted 1:500) for three hours. After incubation, peroxidase conjugated to goat anti-mouse immunoglobulin G diluted 1:5000 was applied to the blots. Development of the blots was with 0.5 mg/ml of diaminobenzidine in TBS for 10 to 15 min, in the dark. Immunoblots with labeled flagellin polyclonal antibody were performed as described in Part V.

Preparation of Acid Hydrolysates, Trypsin Digests, and Pronase Digestion of Flagellin. The labeled flagellin protein (250 ug) was hydrolyzed as described in Part V. For trypsin digests, purified lyophilized flagellin was suspended in 0.1 M Tris-Cl (pH 8.0) containing 0.1% deoxycholate at a ratio of 5:1 (w/v) minus the volume of trypsin used. After 30 min at 70°C to denature the protein, trypsin was added at a ratio of 1:50 (trypsin:prep). The suspension was incubated for 30 min at 37°C. The trypsin was inactivated by incubation 100°C for five min, and the trypsinized flagella peptides were lyophilized. Labeled flagellin samples (100 ug), previously treated with trypsin or acid hydrolyzed, were incubated with four ug of pronase in 10 mM Tris-Cl pH 7.4, overnight at 37°C. The samples were

heated at 100°C for five min and loaded on cellulose (see Part V) and separated by TLE.

#### Phosphatase Treatment of Tryptic Digest and Whole Labeled Flagellin.

Tryptic digest of labeled flagellin or whole label flagella were treated with 0.06 units to 0.1 units of calf intestinal alkaline phosphatase (Worthington Enzymes, Freehold, N. J.), and incubated for 30 to 60 min at 37°C. The samples (four to five ug) were spotted on cellulose paper for TLE separation as previously described. Treatment with bacterial alkaline phosphatase (Worthington Enzymes, Freehold, N. J.) was essentially the same except the incubation times were extended to one h.

#### Thin Layer Electrophoresis of Acid Hydrolysates or Trypsin-treated

Flagellin. Approximately 15 ul (20 ug) of either the flagellin hydrolysates or trypsin-treated flagellin samples and six ul of phosphoamino acid standards (20 mM) were spotted and separated in pH 3.5 buffer on thin layered chromatography cellulose paper as previously described (Wintenberg-Kelly et al., 1990; Cooper et al. , 1983), and Part V. Locations of unlabeled phosphoamino acid standards and unknown peptides were determined by ninhydrin staining, and labeled amino acids and peptides were detected by autoradiography or scintillation counting of sectioned and scraped plastic backed cellulose.

High Pressure Liquid Chromatography (HPLC) Analyses. For HPLC analyses, 174  $\mu$ l (174  $\mu$ g) of strain PAO1 and 80  $\mu$ l (80  $\mu$ g) of strain 2993 flagella digested with trypsin were used. The samples were injected into a VYDAC C<sub>18</sub> column connected to a Waters HPLC system using a solvent system consisting of 0.1% trifluoroacetic acid-acetonitrile as previously described (Wintenberg-Kelly et al., 1990). One minute (1 ml) fractions were collected. Samples were spotted on Whatman filter paper and assayed by autoradiography.

Gel Elution of Labeled Flagellin. Homogeneous 10% SDS-polyacrylamide gels containing labeled purified flagellin were run as previously described in Part II. All lanes of each gel were loaded with the same amount of the same protein. After separation, the two end lanes were removed and stained with Coomassie Blue. During staining the remaining gels were stored in minimal amount of water at 4°C.

Following staining, the gel strips were aligned with the unstained gels, and the area corresponding to the flagellin stained gel in the unstained gel was removed. The gel was macerated through a small syringe into a conical glass tube. To the conical tube, enough sterile water was added to cover the macerated polyacrylamide, and the tubes were stored at 4°C overnight. Afterwards, the tubes were placed in a gyratory shaker at room temperature for several hours. The samples were centrifuged, the

eluent removed, lyophilized and resuspended in 100 ul of trypsin buffer. Trypsinization of the gel eluent was performed for one h.

Organic Extraction of Labeled Flagellin. Purified "cold" flagella was mixed at a ratio of 3:1 (w/w) to the labeled flagella to act as a carrier. To the flagella, sterile water was added at a ratio of 1:1 (w/v) followed by 10% cold tri-chloroacetic acid (TCA) at the volume, so the final concentration of TCA was 5%. After mixing and incubation on ice for two h, the samples were centrifuged at 10,000 x g for 15 min. The protein pellets were washed in absolute EtOH and centrifuged again. The protein pellets were suspended in the same volume of a chloroform-methanol solution, and mixed at a ratio of 3:1 (v/v). Following incubation period of two h on ice, and centrifugation, the protein pellets were again washed in absolute EtOH and centrifuged. The pellets were then sequentially extracted in the same volumes of acetone, followed by EtOH and ether. The final protein samples were centrifuged and resuspended in 300 ul of trypsin buffer. Denaturation of the flagellin at 70°C and trypsinization was performed.

Phosphodiesterase Assays of Supernatant Samples. Supernatants of log cells and late log cells grown in either MSM or modified MSM, were prepared as described in Paer II. The phosphodiesterase assay was based

upon an increase in absorbancy at 400 nm resulting from the hydrolysis of p-nitrophenyl thymidine-5'-phosphate (NTP). For the standard curve, 0.75 ml of Tris-salts buffer (0.11 M Tris-Cl, pH 8.9, 0.11 M NaCl, and 15 mM MgCl<sub>2</sub>) was mixed with 0.25 ml of substrate. To this mixture 10 ug of phosphodiesterase was added, and the change in absorbance was measured at regular intervals over a five min period. For the supernatant samples the amount of phosphodiesterase was compared by mixing 20X Tris-salts buffer, NTP substrate, and the supernatant sample at a ratio of one:one:two. The change in absorbance was measured over a five min period at regular intervals, and compared to the other supernatant samples.

## RESULTS

Isolation and Purification of [<sup>32</sup>P] Labeled a- and b- Antigenic Types of Flagellin. Labeled flagella preparations isolated by differential centrifugation were purified by several steps of molecular sieving. In Figure VI-1A, partially purified a-type flagella protein from strain 170018 (two mg) was passed through a Sephacryl S-300 column. The protein resolved was monitored by absorption at 265 nm, and assayed for <sup>32</sup>P activity via scintillation counting. Flagella fragments emerged at the

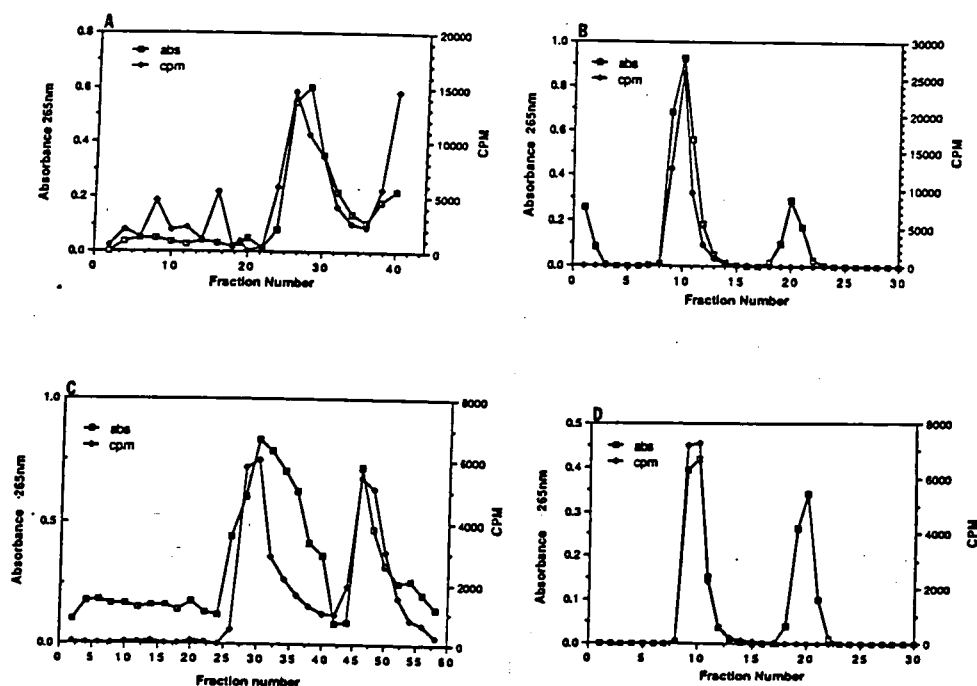


Figure VI-1. Purification of *Pseudomonas aeruginosa* flagellin and incorporation of  $^{32}\text{P}$  into the flagella. Panel A and B, strain 170018, Panel C and D, strain 5940. Open squares ( $\square$ ) represent absorbance values at 265 nm, and closed squares ( $\blacksquare$ ) represent specific activity (cpm). In panels A and C, fractions from a Sephacryl S-300 column were assayed for radioactivity. In panels B and D, column fractions from a Sephadex G-25 column were also assayed.

void volume (fractions 22-34), and coincided with a radioactive peak. This initial peak was followed closely by a second radioactive peak which has previously been shown to contain LPS associated with a non-flagellin protein (Montie et al., 1987). In Figure VI-1C, similar results were seen in the purification of a-type flagella from strain 5940. Flagella were further purified and isolated over two G-25 columns. The final eluent from the second Sephadex G-25 column of 170018 flagella (fractions 8-13) is shown in Figure VI-1B. This peak coincided with the only radioactive peak eluted. Nearly identical results were seen for 5940 flagella as shown in Figure VI-1D, with elution in fractions eight through twelve. It was important to show that  $^{32}\text{P}$  was associated with the flagella at all stages of purification of the intact flagella filament. Similar elution profiles and radioactive peaks were seen for other a-type and b-type flagella tested.

Purified labeled flagella protein was also characterized by SDS-PAGE. The presence of radiolabeled b-type flagellins had previously been demonstrated in gels (Wintenberg-Kelly et al., 1990) and Part V. Purified preparations of a-type flagellins were separated by electrophoresis in 10 to 15% gradient gels, and stained with Coomassie blue (Figure VI-2A). As expected, the  $M_r$  of strain 2993 was 49 kDa (lane 1), and the  $M_r$  of strain 170018 was 45 kDa (lane 2). Autoradiographic patterns showed radioactivity directly associated with a-type flagellins as shown in Figure

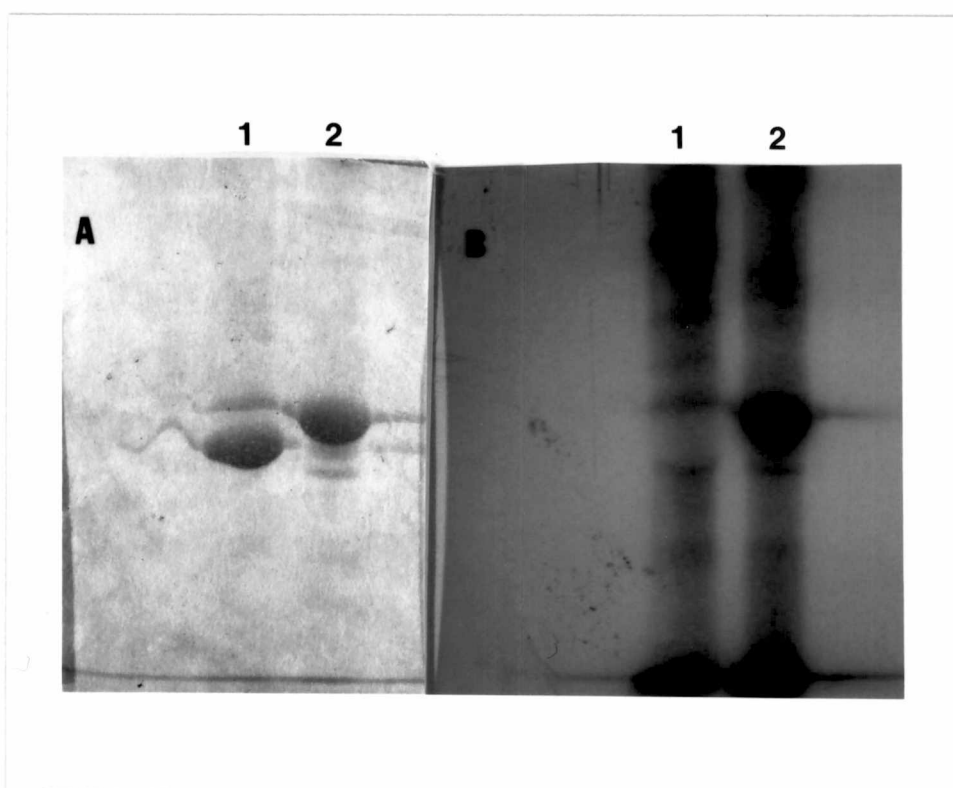


Figure VI-2. The presence of radiolabeled a-type flagellins. A) Coomassie blue stained gel with a-type purified flagellin (3 ug) B) Autoradiogram of the Coomassie blue stained gel. Lane 1, 2993 purified flagellin; Lane 2, 170018, purified flagellin. The arrows denote respective flagellin protein.

VI-2B. Autoradiograms of immunoblots also showed radioactivity directly associated with a-type flagellins. Therefore the phosphorylated flagellin is a characteristic of both flagellin types.

Immunoblotting With Anti-Phosphorylation Monoclonal Antibody. To confirm the presence of unmodified phosphotyrosine in both the a- and b-type flagellin, immunoblots using an anti-phosphotyrosine monoclonal antibody (MAb) were employed. The immunoblot in Figure VI-3 (lanes 1, 2, 4, and 5), showed a single reactive band corresponding to the Mr of the a- and b-type flagellins.

It was difficult to use this MAb in routine blotting assays, since it did not always given reproducible results possibly because of the lack of sensitivity. Therefore, a more sensitive assay using  $^{125}\text{I}$  was developed by S. South (South et al., unpublished results). Instead of the secondary antibody conjugate, goat-anti mouse IgG conjugated to peroxidase; this assay utilized goat-anti mouse IgG conjugated to  $^{125}\text{I}$ . The blots were developed on X-ray film, and have confirmed the presence of p-tyr. This lack of a strong reaction is not surprising since experimental evidence indicates that only one of the tyrosines is phosphorylated per subunit. Therefore the  $^{125}\text{I}$ -secondary antibody is now used routinely because it is a superior method.

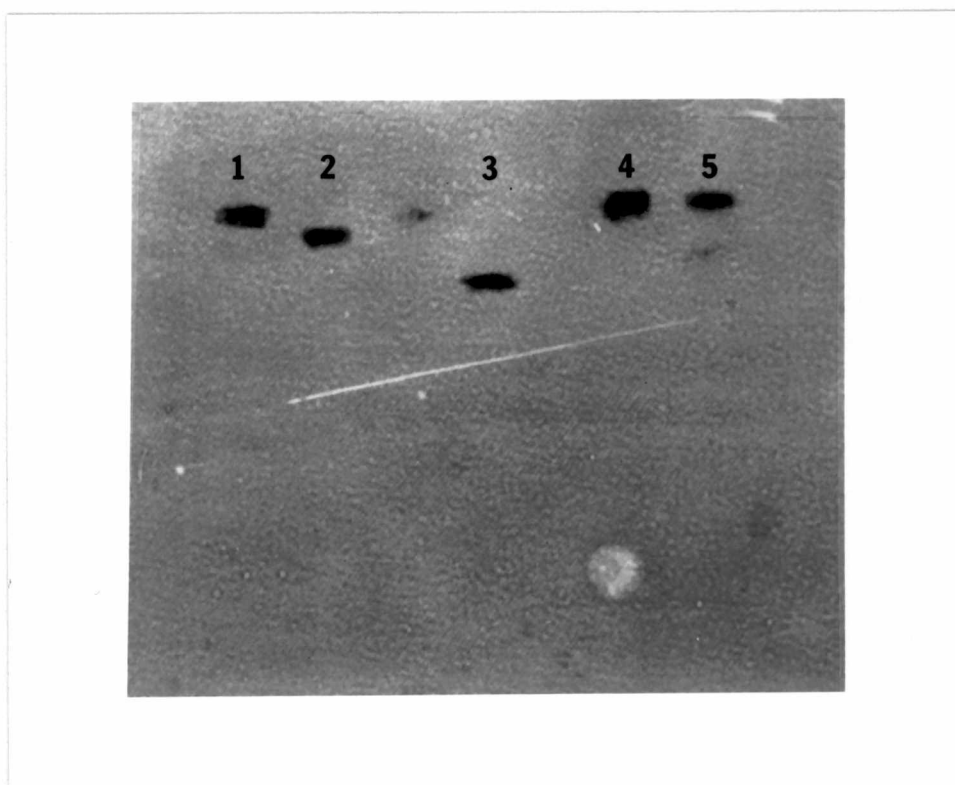


Figure VI-3. Determination of phosphotyrosine in the flagella filament. (A) Coomassie blue stained gel with a- and b-type flagellin (3 ug) were separated by electrophoresis in a 10-15% polyacrylamide gel. (B) Immunoblot of phosphotyrosine with specific anti-phosphotyrosine MAb. Lanes: 1, 5940 (a-type); 2, 170018 (a-type); 3, molecular weight marker; 4, PAO1 (b-type); 5, M2 (b-type).

### Comparative Phosphopeptide Analysis of Flagellins in Partial Acid

Hydrolysates. Recently, the presence of phosphotyrosine in acid hydrolysates (45 min to 120 min) of b-type flagellin was determined by TLE analysis (Wintenberg-Kelly et al., 1990). Using the same methodology, the presence of p-tyr has been determined in a-type flagellins. This approach allowed for comparison of phosphotyrosine containing peptides which were incompletely hydrolyzed in all of the flagellin types. The partial acid hydrolysates were examined by TLE in pH 3.5 buffer. As shown by the autoradiograms of the electropherograms in Figure VI-4, (A and B), a substantial portion of the amino acid label comigrated with phosphotyrosine in both a- and b-type flagellin. No other phosphoamino acids were observed. Peptides were numbered relative to the distance (cm) migrated from the origin. The remainder of label in both types was associated phosphopeptides: P-2, P-3, P-4. An interesting finding was that in all of the b-type hydrolysates tested, the peptide P-8 observed on the autoradiogram was not present in any of the a-type strains tested (Figure VI-4, A and B). These phospho-components were subjected to pronase digestion, and were degraded as were the b-type phosphopeptides described in Part V.

For quantitative estimations of radiolabeled distribution in various peptides, chromatograms were sectioned, and the radioactivity in all fractions was compared (Table VI-1). In the b-type hydrolysates, 24-34%

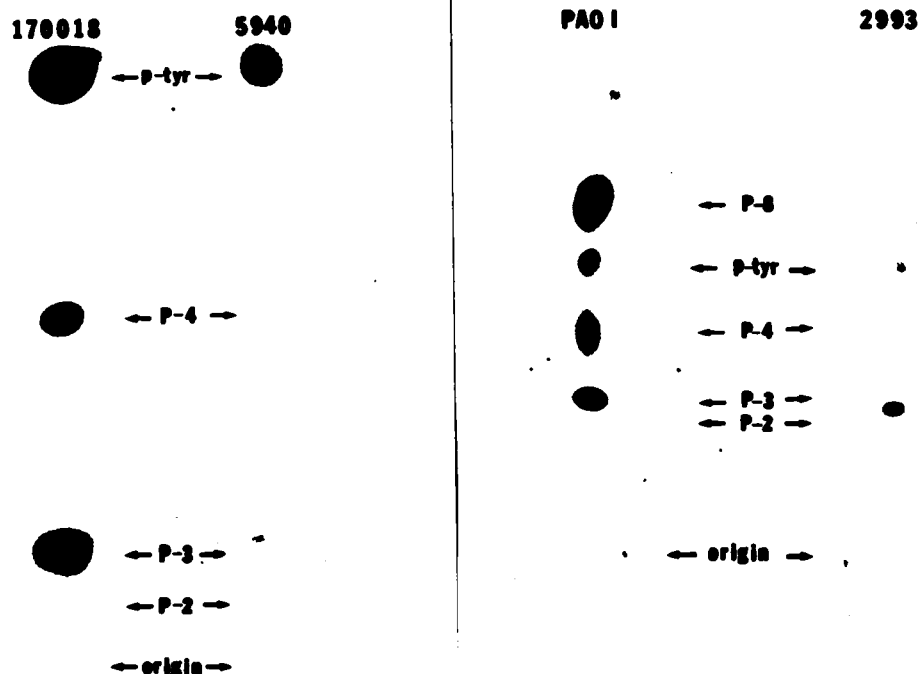


Figure VI-4. Autoradiogram of TLE of partial acid hydrolyzed flagellum filament protein (a- and b-type). Approximately 10 ug of treated protein was loaded per lane. Panel A) PAO1 (b-type), 2993 (a-type); Panel B) 170018 (a-type), 5940 (a-type). p-tyr is phosphotyrosine. P denotes partial acid hydrolysis resistant phosphopeptides, and the number represents distance migrated (cm) from the origin.

Table VI-1. Analysis by TLE of radiolabeled compounds in partial acid hydrolysates (45 minutes) of a-type and b-type flagellum filaments.

| Compound           | Percent total radioactivity |             |               |                |           |
|--------------------|-----------------------------|-------------|---------------|----------------|-----------|
|                    | <u>a-types</u>              |             |               | <u>b-types</u> |           |
|                    | <u>5940</u>                 | <u>2993</u> | <u>170018</u> | <u>PAO1</u>    | <u>M2</u> |
| p-tyr <sup>b</sup> | 49(677)                     | 37(105)     | 43(651)       | 34(2870)       | 24(582)   |
| P-2                | 6(95)                       | ND          | 16(240)       | 13(1000)       | 2(40)     |
| P-3                | 18(255)                     | 20(60)      | 19(287)       | 37(3148)       | 14(335)   |
| P-4                | 12(158)                     | 35(101)     | 11(159)       | 4(400)         | 13(314)   |
| P-8                | 0                           | 0           | 0             | 12(1000)       | 48(210)   |

<sup>a</sup>Results are shown as percent radioactivity excluding free Pi; numbers in parentheses are counts per minute. Counts in phosphoserine and phosphothreonine were 6% or below and were not considered significant.

<sup>b</sup>p-tyr is phosphotyrosine. Almost 50% of the total amino acid label was associated with P-2, P-3, and P-4 .

of the label was associated with phosphotyrosine while 37-49% was associated with phosphotyrosine in the a-type flagellin strains tested.

Variation in Tyrosine Content of Flagellins. Since the molecular weights of flagellin varied among a-types and between a-types to b-types, the amino acid content of each flagellin was examined, specifically looking for differences in the number of tyrosines (Table VI-2). Amino acid analyses of purified flagellin showed the lack of histidine, but the presence of methionine and proline. The latter amino acid was not reported previously probably because of the insensitivity of ninhydrin reaction. Other amino acids, such as phenylalanine, remained relatively constant in number while methionine increased. Since the  $M_r$  was decreased in a-types by 1,000 to 7,000 daltons, the decrease in amino acids was expected. The number of tyrosines of each strain investigated is seen in Table VI-3, along with the relative specific activity. It proved difficult to calculate the relative specific activity based upon moles of  $P_i$  and moles of flagellin. Therefore differences seen in Table VI-3, probably reflect errors in determining the moles of  $P_i$  in the MSM medium, and the relative specific activity is approximately one for all a- and b-type strains tested. In the future it will prove important to determine the precise number of tyrosines phosphorylated per subunit.

Table VI-2. Amino acid analyses of *P. aeruginosa* flagellins.

|                  | STRAINS    |           |              |               |             |             |
|------------------|------------|-----------|--------------|---------------|-------------|-------------|
|                  | M2a<br>(b) | M2<br>(b) | PJ108<br>(b) | 170018<br>(a) | 5940<br>(a) | 2993<br>(a) |
| ASP              | 74         | 70        | 75           | 63            | 67          | 80          |
| GLU              | 49         | 47        | 48           | 45            | 47          | 50          |
| SER              | 48         | 53        | 57           | 41            | 47          | 40          |
| GLY              | 51         | 47        | 51           | 47            | 50          | 40          |
| HIS              | 0          | 0.6       | 1.2          | 3.6           | 1.6         | 0           |
| ARG              | 18         | 21        | 20           | 19            | 16          | 19          |
| THR              | 48         | 52        | 47           | 35            | 40          | 40          |
| ALA              | 91         | 87        | 87           | 64            | 69          | 80          |
| PRO              | 0          | 8         | 7            | 10            | 7.8         | 0           |
| TYR              | 4          | 7         | 7            | 6.7           | 4.9         | 3           |
| VAL              | 38         | 35        | 32           | 26            | 28          | 30          |
| MET              | 0          | 1.5       | 1.5          | 4.2           | 3.5         | 3           |
| ILE              | 30         | 27        | 27           | 20            | 23          | 30          |
| LEU              | 43         | 41        | 41           | 35            | 35          | 55          |
| PHE              | 13         | 10        | 12           | 11            | 11          | 10          |
| LYS              | 18         | 20        | 17           | 16            | 15          | 20          |
| kDa <sup>b</sup> | 53         | 53        | 53           | 45            | 46.5        | 50          |

<sup>a</sup>Analysis in a Beckman analyzer with ninhydrin detection reagents.

<sup>b</sup>The molecular weight is represent in kilodaltons (kDa).

Table VI-3 Comparative  $^{32}\text{P}$ i labeling of flagella preparations.

| Flagella type   | Relative specific activity <sup>a</sup> | Number tyrosines |
|-----------------|-----------------------------------------|------------------|
| M2 (b-type)     | 1.4                                     | 7                |
| PAO1 (b-type)   | 1.6                                     | 7                |
| 170018 (a-type) | 1.3                                     | 7                |
| 5940 (a-type)   | 0.6                                     | 5                |

<sup>a</sup>Moles of Pi/mole of flagellin, based on b-type weight of 53 kDa and a-type flagellin weights, 170018 of 45 kDa, and 5940 of 47 kDa.

#### Phosphatase Treatment of Labeled Tryptic Digests and Whole Flagellin.

Phosphotyrosine is not a common O-phosphate modification in prokaryotic systems except when attached by a phosphodiester bond with a nucleotide (Foster et al., 1989). Thus, it was important to establish if the p-tyr modification on the flagellin protein of *Pseudomonas aeruginosa* was generated by a phosphorylation reaction or a nucleotidylation reaction.

Since the  $^{32}\text{P}$  component in flagellin comigrated with p-tyr after partial acid hydrolysis, and was stable in alkali, it was considered proof of an O-phosphate linkage, and phosphotyrosine. However, a  $^{32}\text{P}$  labeled

phosphoprotein isolated from cells grown in the presence of  $^{32}\text{Pi}$  could contain phosphodiester linkages (e.g., nucleotidylation) to serine or tyrosine, and partial acid hydrolysis of these phosphodiester linkages produce O-phosphates (Martensen, 1984).

To demonstrate the presence of an O-phosphomonoester linkages, the labeled flagellins were treated with two types of phosphatase enzymes (Martensen, 1984). Calf Intestinal Alkaline Phosphatase (CIP) or Bacterial Alkaline Phosphatase (BAP) were used to treat labeled whole flagella and trypsinized samples of labeled flagellin. After treatment, the treated samples were compared to untreated samples by TLE analyses, and a greater release of  $^{32}\text{Pi}$  which migrates further on the chromatogram was observed.

Representative autoradiograms of CIP-treated flagella (South et al. , unpublished results) or BAP-treated flagella is shown in Figure VI-5 and Figure VI-6, respectively. As shown in Figures VI-5 and VI-6, labeled tryptic digests of flagellin samples treated with phosphatase, released considerably more  $^{32}\text{Pi}$  than untreated samples. A more quantitative approach of the data was utilized to determine Pi release after phosphatase treatment as shown in Tables VI-4 and VI-5. Treatment with CIP showed

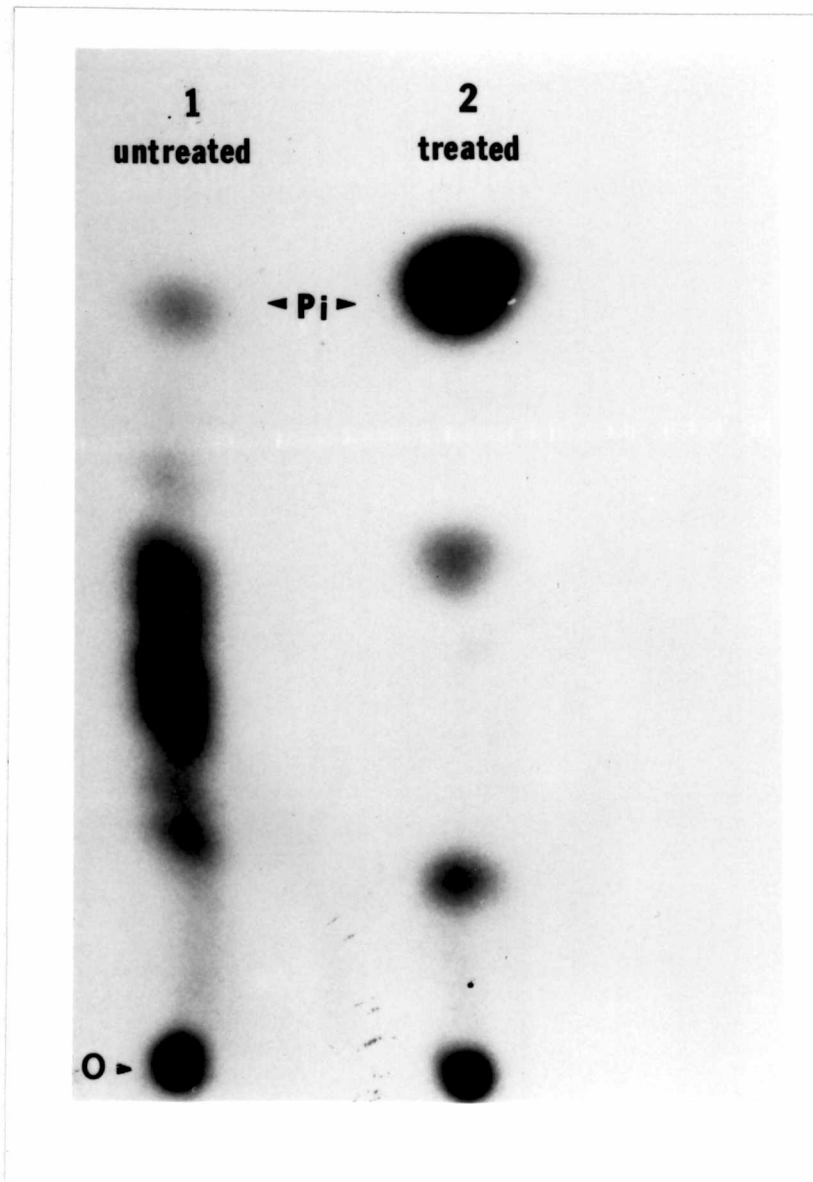


Figure VI-5. Release of  $^{32}\text{P}$  from flagellin phosphopeptides following treatment with CIP. U; untreated tryptic digest; T, CIP treated tryptic digests of labeled flagellin. "Pi" represents  $^{32}\text{P}$  and "O" represents the origin.

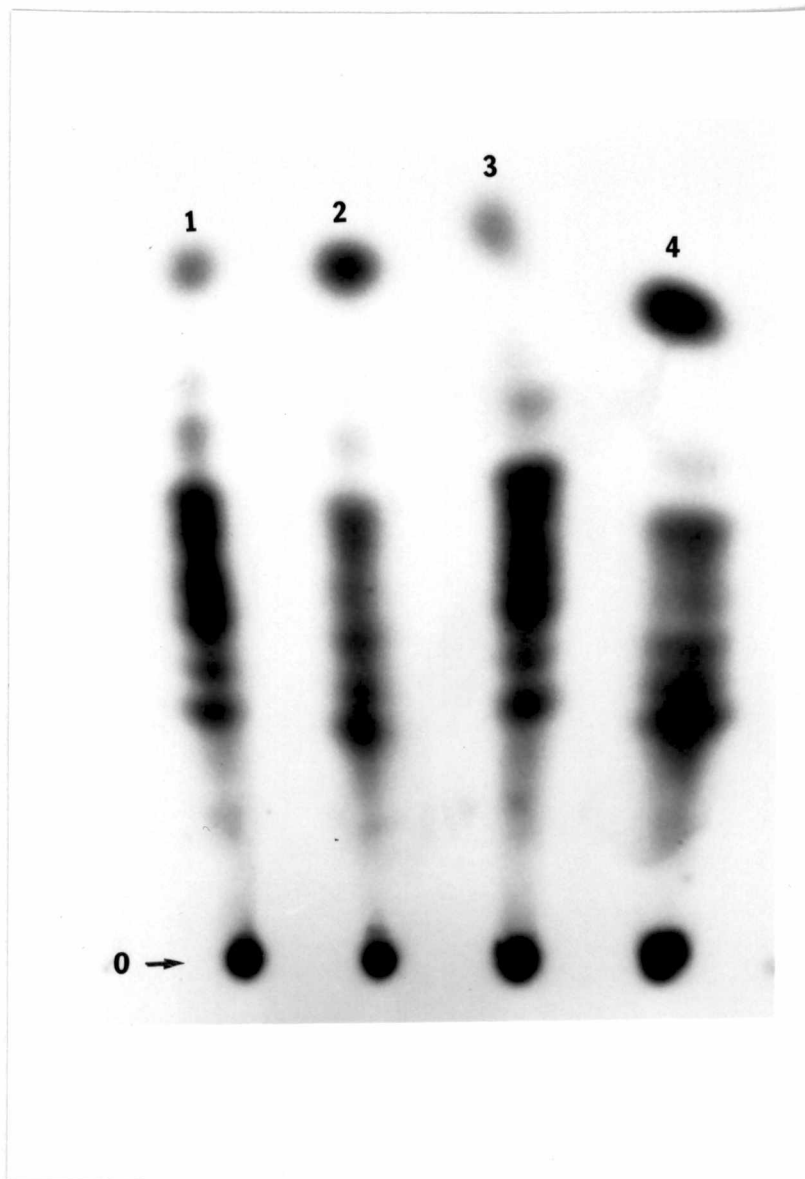


Figure VI-6. Release of  $^{32}\text{P}$  from flagellin phosphopeptides from strain M2 following treatment with BAP. Approximately 15 ug of protein was loaded for the TLE. Lanes: 1, untreated tryptic digest (30 min); 2, tryptic digest (30 min) treated with BAP; 3, untreated tryptic digest (5 h); 4, tryptic digest (5h) treated with BAP. The spot migrating the furthest from the origin is  $^{32}\text{P}$  and "O" represents the origin.

Table VI-4. Release of  $^{32}\text{Pi}$  from flagellin peptides by calf intestinal alkaline phosphatase.

| <u>Sample</u>      | <u>cpm</u> | <u>% Difference</u> |
|--------------------|------------|---------------------|
| <u>Peptides</u>    |            |                     |
| Control            | 855        |                     |
| with enzyme        | 349        | 59% decrease        |
| <u>Released Pi</u> |            |                     |
| Control            | 139        |                     |
| with enzyme        | 488        | 251% increase       |

<sup>a</sup>Treatment of 20 ug of tryptic digest for 30 min at 37°C, pH 8.0, with 0.06 units of calf intestinal alkaline phosphatase. The sample (4 ug) was spotted for TLE separation.

Table VI-5. Release of  $^{32}\text{Pi}$  from flagellin peptides by bacterial alkaline phosphatase.

| <u>Sample</u>      | <u>cpm</u> | <u>% Difference</u> |
|--------------------|------------|---------------------|
| <u>Peptides</u>    |            |                     |
| Control            | 655        |                     |
| with enzyme        | 291        | 56% decrease        |
| <u>Released Pi</u> |            |                     |
| Control            | 41         |                     |
| with enzyme        | 259        | 500% increase       |

<sup>a</sup>Treatment of 20 ug of tryptic digest for 30 min at 37°C, pH 8.0, with 0.10 units of bacterial alkaline phosphatase. The sample (40 ug) was spotted for TLE separation.

a drastic increase in  $^{32}\text{Pi}$  released (Table VI-4), while differences with BAP treatment were less intense (Table VI-5).

Examination of Radiolabeled Tryptic Phosphopeptides. To determine the location of specific sites in the flagellin that were phosphorylated, tryptic digests of labeled flagellins were prepared. Flagellin samples were digested from 30 min to five h. It was determined that total digestion occurred in 30 min as observed by the disappearance of gel bands and an increase in intensity of labeled peptides. In addition, the same tryptic pattern was seen in a five h digestion of flagellin as in the 30 min digestion (Figure VI-6).

The labeled tryptic digests of b-type peptides and a-type tryptic digests resolved by TLE are represented in Figure VI-7 and Figure VI-8, respectively. TLE analyses of a-type and b-type tryptic peptides showed seven to eight labeled peptides present. These results were somewhat surprising since the number of tyrosines in the a-type flagellin is much less than eight (Table VI-3), and experimental evidence mentioned previously indicated that only one tyrosine is phosphorylated per subunit. In an attempt to substantiate the true number of tryptic phosphopeptides present, several approaches were taken including: a) HPLC analyses of tryptic peptides, b) gel elution of the labeled flagellin protein band, and c) organic extraction of purified labeled flagellin.

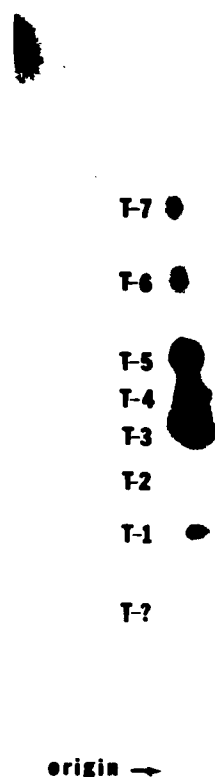


Figure VI-7. Autoradiogram of b-type flagellin tryptic phosphopeptides separated by TLE. Approximately 15 ug of the trypsinized peptides were separated by TLE. Eight phosphopeptides were distinguished and indicated as T-1 through T-8.

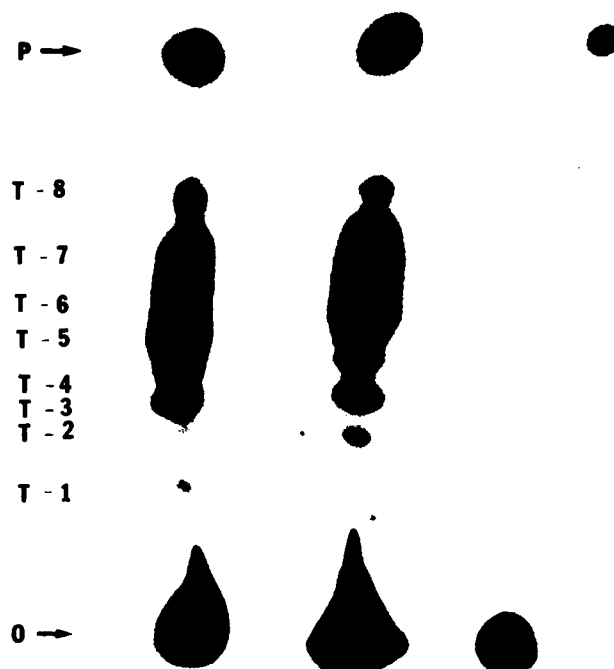


Figure VI-8. Autoradiogram of a-type flagellin tryptic phosphopeptides separated by TLE. Approximately, 15 ug of the trypsinized peptides were separated by TLE. Lane 1, 170018 (a-type) and Lane 2, 2993 (b-type). Eight phosphopeptides were distinguished and indicated as T-1 through T-8. The arrow represents  $^{32}\text{P}_i$  and "O" represents the origin.

HPLC Analyses of Phosphorylated Peptides. Although phosphorylated peptides were not resolved well in the HPLC VYDAC C<sub>18</sub> columns used, the results obtained confirmed that a number of peptides were phosphorylated (Figure VI-9). Approximately four labeled peptides emerged in fraction five. Several other phosphopeptides emerged in fraction eight and thirteen (Figure VI-9). Similar HPLC elution profiles, including the presence of early emerging fractions, were seen in the tryptic digests of a-type flagellins as shown in Figure VI-9. Preliminary data indicated that two peptides common to b- and a-type flagellins emerged in fraction 13.

TLE Analyses of Tryptic Digests of Gel Eluted Labeled a-Type and b-Type Flagellin. The specific activity of the labeled gel eluted protein was weak, and it proved difficult to recover enough protein to develop patterns on autoradiograms. In Figure VI-10, two distinct bands are visible in lane one (170018) and lane two (2993) of labeled trypsinized eluted flagellin. These spots corresponded to putative peptides T-1 and T-3 of the tryptic digest pattern in Figure VI-8. Also faintly visible, especially in lane one, was an unresolved pattern corresponding to T-4 through T-7 or T-8. Peptide T-2 was not visible in any gel eluted autoradiograms. A similar experiment with labeled eluted M2 trypsinized flagellin showed only one band corresponding to T-3.

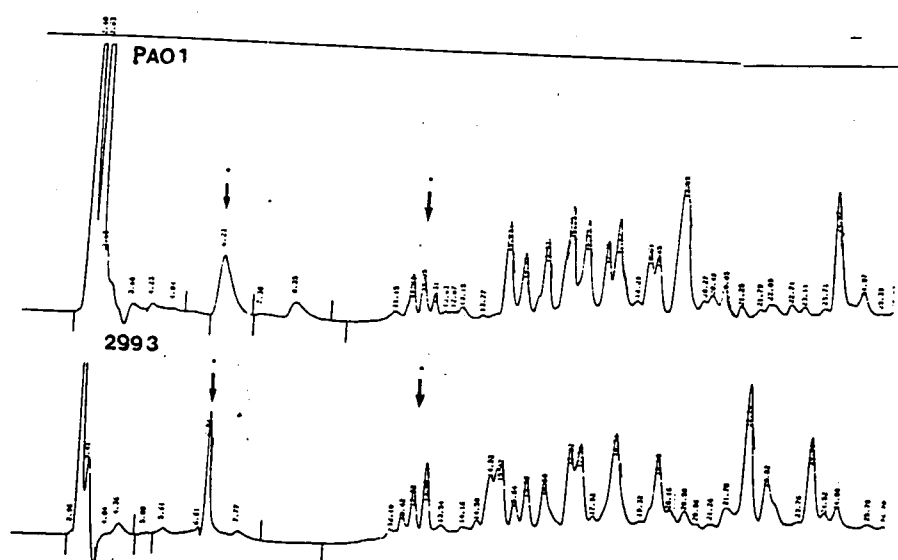


Figure VI-9. Examination of tryptic digests by HPLC. Labeled purified flagellin was subjected to trypsin digestion for 30 minutes at 37°C. The labeled samples (175 ug PAO1 and 80 ug of 2993) were each injected into a VYDAC C<sub>18</sub> column and separated by reverse phase HPLC.

Radioactive peaks were detected in fraction five (retention time of 6 min) and fraction 13 (retention time of 14 min). Peptide profile peaks represent relative absorption at 220 nm.



Figure VI-10. TLE analysis of labeled a-type flagellin eluted from polyacrylamide gels and treated with trypsin. Lane 1, 170018; Lane 2, 2993. The "O" represents the origin and the arrow represents T-4 through T-8. Similar results were seen with b-type flagellin from strain M2.

It is possible that this elution procedure did not remove all of the SDS associated with the gel bands, and residual SDS interfered with the trypsin digestion. This hypothesis is supported by the presence of an unusually large radioactive spot at the origin of the M2 sample. This non-migrating spot was most unlikely undigested protein, which would not migrate in this system.

TLE Analyses of Organic Extracted Labeled Flagellin. The inconsistency in the appearance of multiple peptide and with the previous calculation of only one tyrosine phosphorylated per subunit remained unsolved. Also, the presence of seven to eight tryptic peptides in both the a- and b-type flagellins remained puzzling since the number of tyrosines varies from three to seven. It was possible that contaminating phospholipid or carbohydrate moieties were associated with the tryptic phosphopeptides from purified flagellin filament protein because, for example, it is difficult to remove all of the lipopolysaccharide from flagella and LPS contains high amounts of phosphoesters.

To determine if any contaminating phospho-components were present, the flagellin was extracted with organic solvents before trypsinization and TLE analysis, to remove extraneous contaminants. As shown in Figure VI-11, there were fewer spots present on the extracted sample of flagellin from strain 170018. If compared to a tryptic digest of 170018 flagellin

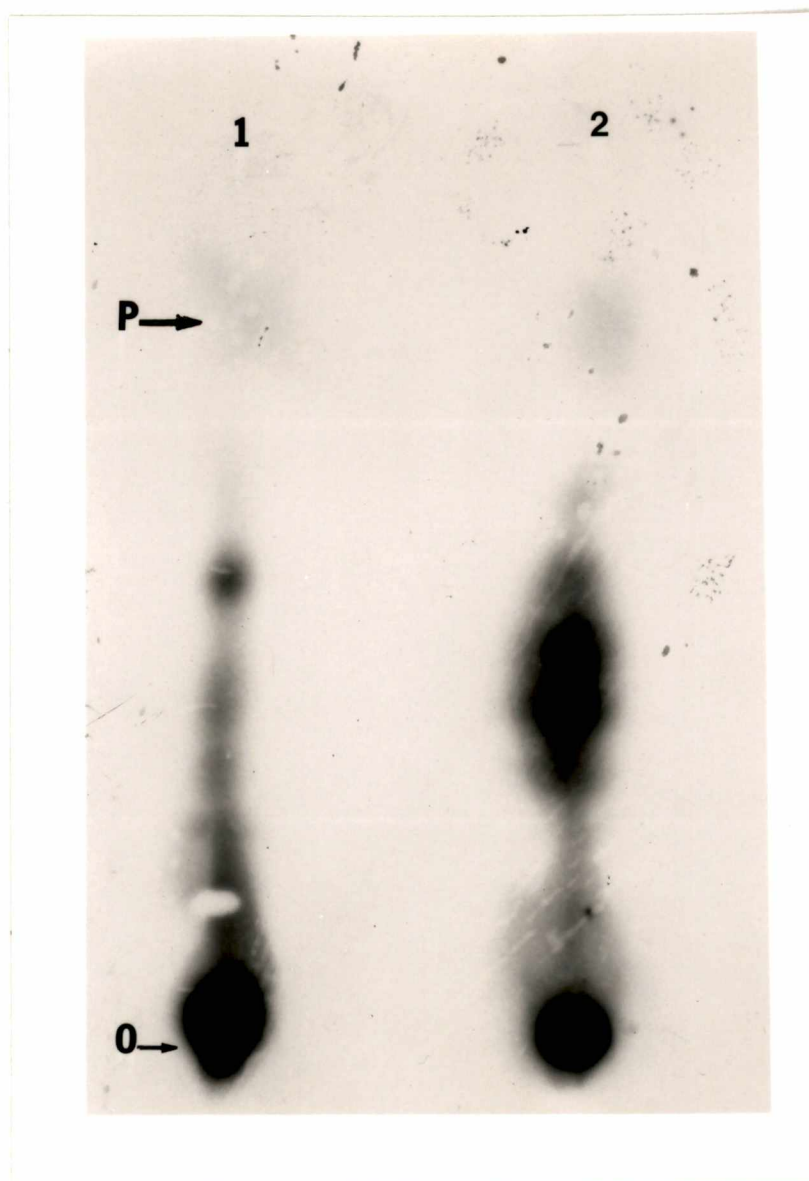


Figure VI-11. TLE analysis of tryptic digest of flagellin from 170018, following organic extraction. Approximately 12 ug of protein were loaded in each lane. Lane 1, tryptic digest of organic extracted flagella; Lane 2, organic extracted flagella. The arrow represents <sup>32</sup>Pi and "O" represents the origin.

(Figure VI-8), it appeared that the two lower spots, T-1 and T-2 were absent in the organic extracted flagellin. The upper spots which corresponded to T-3 through T-7 or T-8 were present. This experiment was repeated, and the same results were obtained.

These tryptic peptides from 170018 were digested with pronase at 37°C, overnight, and characterized by TLE. The autoradiogram showed (Figure VI-12) that only one spot which corresponded to T-1 remained after pronase digestion. Similar results were determined for 2993 labeled flagellin. Peptide T-2 was not visible in Figure VI-12, therefore it was difficult to tell if it was susceptible to pronase.

Phosphodiesterase Assays and Characterization of Labeled Flagellin Isolated From Log Cells. It has been shown by (Meyer, and Stadtman, 1981) that *P. aeruginosa* late log cells grown under low phosphate are under stress and produce and export several enzymes. These include alkaline phosphatase, phospholipase, and more recently a phosphodiesterase has been reported. Therefore, if a nucleotide is linked via a phosphodiester bond to the phenolic hydroxyl residue of tyrosine in the flagella of growing *P. aeruginosa*, then phosphodiesterase could cleave the bond and generate p-tyr in the supernatant. As a result, unmodified p-tyr would be detected in partial acid hydrolysates, and would be susceptible to phosphatase. To probe this question, phosphodiesterase



Figure VI-12. TLE analysis of tryptic digested flagellin from strain 170018, treated with pronase. Approximately 10 ug of treated and untreated flagellin. Lane 1, tryptic digest of flagellin; Lane 2, tryptic digest and treatment with pronase. The arrow represents  $^{32}\text{Pi}$  and the "O" represents the origin.

enzyme assays were done to determine the difference in concentration of the enzyme produced by log cells versus late log cells grown in MSM or MSM with low phosphate (0.62 mM). Tyrosine phosphate is detected in equivalent amount at both growth phases.

Examination of the supernatants isolated from cells in different growth phases and different phosphate concentrations; showed a four fold difference in the amount of phosphodiesterase found in cells grown in different concentrations of phosphate. More importantly, while there were detectable levels of phosphodiesterase in the supernatant of late log cells grown in MSM with low phosphate, no enzyme was detectable in log cell supernatants from the same medium.

Consequently, in log cells, where phosphodiesterase was not detectable, if the p-tyr was linked to a nucleotide then there should be a very little amount of free p-tyr present.

To test this hypothesis, flagella were isolated from  $^{32}\text{P}$ -labeled log *P. aeruginosa* M2 cells, grown to mid-log phase and purified as previously described. The purified labeled flagella were examined by the same techniques as flagella from late log cells. In every instance there appeared to be no difference between the amounts of p-tyr found in flagellin from log cells or late log cells. Autoradiographs from SDS-polyacrylamide gels and immunoblots of labeled flagellin isolated from log cells demonstrated a band corresponding to the  $M_r$  of 53,000. Partial acid hydrolysates and

tryptic phosphopeptides showed the same patterns on electropherograms. In addition, the tryptic peptides from log-grown flagellins were susceptible to phosphatase to the same degree as flagellin from late log cells. In confirmation, p-tyr Mab reactions of log flagellins were positive. This evidence strongly suggests the presence of tyrosine phosphate in the flagellin filament of *P. aeruginosa* cells.

## DISCUSSION

The presence of phosphotyrosine in both antigenic types of flagellin has been confirmed. In agreement with an earlier preliminary report investigating b-type flagellin (Wintenberg-Kelly et al., 1990), a-type flagellin also exhibits phosphotyrosine and not phosphothreonine nor phosphoserine. Since the two straight chain phosphoesters of phosphoserine and phosphothreonine are more stable in acid compared to phosphotyrosine, they should have been observed if present under the partial acid hydrolysis conditions employed for characterization of phosphotyrosine. The evidence that two anti-phosphotyrosine MAbs from different sources reacted with purified flagellin strongly indicates the presence of free p-tyr. To eliminate any difficulties with reproducing results, a more sensitive assay using  $^{125}\text{I}$  is routinely being used in the

laboratory. This assay also has the advantage that the blots are recorded on x-ray film and are permanent.

Upon examination of partial acid hydrolysates of a-type flagellin, the results are consistent with previous findings (Wintenberg et al., 1990) that p-tyr is the only detectable phosphoamino acid in the filament protein. The remainder of the label is associated with phosphopeptides referred to as P-2, P-3, and P-4. These findings are also consistent with the findings that one major phosphopeptide (P-8) (Figure VI-4) is found only in acid hydrolysates of b-type flagellins, and not in the three a-types examined (Wintenberg-Kelly et al., 1990). This would reflect the sequence differences among the b-type and a-type variable regions as evidenced by molecular weight and amino acid difference (Montie, et al., 1987). It is very likely that certain peptides and/or phosphoesters are more vulnerable to acid exposure and hydrolysis. In fact, most of the phosphopeptides (except P-4) are completely hydrolyzed upon four hours of acid hydrolysis but only p-tyr is seen even though it is present in decreased amounts (Wintenberg-Kelly et al., 1990). Release of inorganic phosphate from tyrosine is continually detected under the electrophoretic conditions used at pH 3.5. This observation demonstrates the lability of this ester bond.

The origin of the phosphotyrosine has been important to establish because it has been implied that no unmodified tyrosine phosphate exists in

bacteria since only one known tyrosine kinases have been reported (Vallejos et al., 1985). Phosphotyrosine isolated in acidic or alkaline hydrolysates of cytoplasmic proteins were shown to originate primarily from nucleotidylated glutamine synthetase in *E. coli* (Foster et al., 1989).

To demonstrate the presence of an O-phosphomonoester linkage, the labeled flagellins were treated with two types of phosphatase enzymes. Characterization of the linkage was facilitated using flagellin samples digested with trypsin, since proteins are poor substrates for either of these enzymes (Martenson, 1984). This is especially true for intact flagella where the molecular weight is one million or greater. Trypsinized flagellins were readily susceptible to phosphatase as seen by the large increase in  $^{32}\text{P}$  released and with the concomitant decrease in radioactivity of tryptic spots. An interesting observation was that some of the tryptic spots (T-1 and T-2) were not as susceptible to phosphatase treatment as the other spots (T-3 through T-7 or T-8).

A comparison of two different phosphatases showed that BAP was not as active as the CIP enzyme. However this was not surprising, since this observation has been substantiated with several proteins and peptides. Also as previously observed, intact protein is a poorer substrate for phosphatase. As proposed, failure to have released  $\text{P}_i$  in this reaction would have indicated the presence of a phosphodiester linkage, however this does not seem to be the case.

An alternative argument could contend that a phosphodiesterase is cleaving p-tyr in the medium of stressed cells. Under stress conditions, *P. aeruginosa* produces and secretes a phosphodiesterase (Meyer, and Stadtman, 1981) which if flagellin linkage to a nucleotide is present, would be cleaved to generate free p-tyr. However, evaluation of supernatants of *P. aeruginosa* grown in different phosphate concentrations, and cells in different growth phases showed no detectable levels of phosphodiesterase present in the supernatant of low phosphate log cells. However, free p-tyr was readily identified in flagellin of log cells upon partial acid hydrolysis. Upon characterization, no difference was found between p-tyr of flagellin from log cells and late log cells. Therefore, the evidence strongly indicates the phosphate is associated with tyrosine via a monophosphoester linkage in the flagellin filament protein.

The amount of phosphate labeling in the various flagellins does not necessarily appear to be associated with the total number of tyrosines in the flagellin filament protein. Based upon amino acid analyses, generally, there are fewer numbers of tyrosines in the a-type flagellins than the b-type flagellins. However, upon examination of tryptic digest patterns of a- and b-type flagellins, they were identical producing seven to eight spots. It would be of interest to determine if one or all of the tyrosine residues in each flagellin type were phosphorylated and to locate these sites in the sequence. The inferred sequence of an a-type flagellin obtained by

nucleotide analysis (Totten, and Lory, 1990) placed the tyrosines residues in the central, variable region. N-terminal amino acid analyses of several a- and b-type flagellins through residue 15 gave the following sequence: A-L-T-V-N-T-N-I-A-S-L-N-T-Q-R. This identified amino acid sequence, which was in exact agreement with the inferred sequence (Totten, and Lory, 1990), confirmed both the absence of aromatic amino acids near the N-terminal flagellin, and further indicated that both flagellin types contained significant regions of common sequences (Rotering, and Dorner, 1989).

To explain the presence of seven to eight tryptic peptides several different procedures were attempted to determine if any contaminating phospho-components were associated with the flagellin protein. It is well known that LPS is in close association with many surface components of G - bacteria including flagella and components of *P. aeruginosa*, as discussed in Part I. Organic extractions were performed to remove any contaminating phosphorylated compounds. TLE analyses of the organic extracted flagellins showed reduced numbers of tryptic spots, more specifically, T-1 and T-2 were absent, while T-3 through T-8 were present. These results were in agreement with the phosphatase experiments shown in Figures V and VI. The phosphopeptides (T-3 through T-8) were more susceptible to phosphatase than T-1 and T-2. This indirectly suggested that T-1 and T-2 may not be purely peptide in nature, possibly contaminating

phospho-components. However, the lack of susceptibility to those enzymes could merely reflect the presence of a large more hydrophilic peptide.

Tryptic digests of gel eluted a- and b-type flagellins showed similar results. The difference between gel elution and organic extraction digests was the presence of T-1 in the gel eluted tryptic digests of flagellin. One explanation for this difference is a phospho-component could be associated with the gel band. Analysis of labeled tryptic digests by HPLC has confirmed the presence of a number of phosphopeptides.

It is difficult to explain the number of spots seen on autoradiograms of tryptic digested flagellins when the number of tyrosines vary from a few to eight. One consideration is the large molecular weight of flagella and the ability of the flagellin subunits to self-aggregate. Even after denaturation, it is possible that a portion of the flagellin subunits are self-aggregating, folding up and thereby preventing trypsin from acting efficiently. If this occurs, there could be a mixed population of different sized polypeptides generated. Even though the number of tyrosines differ in the a- and b-type flagellins, the fact that the same tryptic digest pattern of phosphoproteins is generated; indirectly supports the idea that probably the same number of tyrosines are phosphorylated in both flagellin types.

One explanation of the low specific radioactivity observed in Table VI-3, is the possibility that a phosphate-rich subunit region exists in the

proximal filament, and this region is necessary for efficient and proper distal subunit assembly. This arrangement would explain the calculations of one, or less than one, tyrosine phosphorylated per subunit. This situation might be analogous to those bacterial flagella filaments containing two different subunits in *Helicobacter pylori* (Kostrzynska et al., 1991), or three in *Caulobacter ssp.* (Minnich, and Newton, 1987) in the same filament. In these cases, one or two subunits are contiguous with the proximal end, and the other type more prevalent in the remainder of the filament. In *Rhizobium meliloti*, one subunit predominates in the proximal portion and is believed to direct assembly of the second subunit aggregate in the distal portion (Pleier, and Schmitt, 1991).

Recent data from this laboratory has suggested that filament phosphorylation may be controlled by the pho regulon. Flagella from *P. aeruginosa* grown in limiting phosphate (<1 mM) contained much higher amounts of phosphotyrosine when compared to flagella filaments isolated from bacteria grown in sufficient phosphate (>1 mM). These differences were detected using an anti-p-tyr MAb. In the case of the flagella from the high phosphate cells, the presence of phosphotyrosine was barely detectable. Additional evidence supporting this general idea has been found by S. South (South et al., unpublished data). Wild-type *P. aeruginosa* grown in high phosphate were less motile than bacteria grown in low phosphate. These results have been shown in both motility

spreading assays and by capillary tube assay. In contrast, motility assays of a *P. aeruginosa* *phoB* mutant grown in high and low phosphate showed no difference in motility. It is possible phosphorylation was deregulated in the mutant as might be predicted. If these regulatory features are common to flagellin phosphorylation in bacteria, this might explain why phosphorylation might have been overlooked in some previous experimental situations (high phosphate) using other bacterial genera. Nitrogen limiting conditions increase chemotaxis to amino acids (Craven, and Montie, 1985). By analogy to the enteric model, increased flagellin phosphorylation in limiting nitrogen might show that phosphorylation of flagellin is generally regulated by nutritional stress rather than more narrowly related to aspects of "pho" regulation (Dieteric et al., 1989, Stock et al., 1990).

At the present time, it is difficult to provide an explanation for the physiological purpose of the p-tyr occurrence in flagellin. The role of phosphorylation associated with filament assembly can not be completely ruled out. It would be important understand how flagellin protein, once translated, can be effectively sorted by the cell and designated as flagellin. The flagellin subunits are unusual in that unlike pilin (Sastry et al., 1983), they probably contain no signal sequence; but contain some internal export signal, probably at the C-terminal conserved region (Lavallie, and Stahl, 1989). Little is known about the pathway for export. It is believed that

once entering the membrane, the flagellin subunits can travel through the basal body, hook and finally channel through the filament to the tip of the growing flagellum for assembly (Vogel et al., 1991). Questions remain about flagella phosphate turnover rates and flagella synthesis in *P.*

*aeruginosa* dividing cells where only one daughter cell receives the ancestral flagellum and a new polar flagellum must then be newly synthesized following septation (Suzuki, and Iino, 1980). A recently published derived amino acid sequence for an a-type flagellin showed that it contained two tyrosines located centrally at positions 155 and 219 in the so-called variable region of the PAK a-type flagellin (Totten, and Lory, 1990). Other bacterial flagellins show similar dispositions of tyrosines in the central variable, hydrophilic region. Phosphotyrosines found in the variable region of the flagellins suggest that this modification is not be involved in export because sequences common to all flagellins at the N-terminal and C-terminal end have been primarily implicated in these processes (Kuwajima et al., 1989).

Preliminary data from assays of supernatants of labeled Fla<sup>-</sup> Mon<sup>+</sup> cells show the presence of labeled flagellin in autoradiograms of polyacrylamide gels. This suggests that the cell is capable of phosphorylation but not assembly of the filament. However, the precise nature of these mutations are unknown, therefore more proof is necessary to exclude phosphorylation in the role of assembly. It would be interesting

to generate a mutant incapable of phosphorylating the flagellin, and observe if assembly occurs.

In preliminary experiments it was observed that *E. coli* HB101-pKW52 cells labeled with  $^{32}\text{P}$ -phosphoric acid, do not produce any detectable phosphorylated *P. aeruginosa* flagellin. However, *P. aeruginosa* flagellin monomers were detected in polyacrylamide gels and immunoblots.

Recent evidence obtained by S. South, indicates that growth on limiting phosphate (0.62 mM) increases motility but not chemotaxis, as has been reported previously with growth on limiting nitrogen (Craven, and Montie, 1985). Since the response increases motility but not directly chemotaxis, it is possible that the response is not a result of "cross talk". In the latter situation a second "stress" condition (limiting nitrogen) leads to increased phosphorylation of an equivalent *P. aeruginosa* Che Y protein, from a nitrogen controlled kinase (see Part V, Introduction) which in turn controls the switch for mechanical regulation of flagellum rotation (Stock et al., 1989).

Another reasonable interpretation of these observations is that increased phosphorylation of flagellin in some way directly changes the filament rotational efficiency. For example, it has been well documented that a change in the environment such as pH or viscosity, changes the shape of the flagella thereby changing its swimming patterns (Manab, 1987). The tyrosines of the flagellin are located close to the exposed, hydrophilic

variable region of the flagellin. Therefore, it is possible that an increase in phosphorylation of these tyrosine or tyrosines changes the shape of the flagella and the motility. Evidence from a Fla<sup>+</sup> Mot<sup>-</sup> strain, 1200-80, is consistent with this hypothesis. Labeled flagella from this paralyzed strain showed very weak spots corresponding to the seven to eight tryptic peptide patterns for the a- and b-type flagellins.

Another interpretation of these observations is that increased phosphorylation of the flagellin changes the motility by the introduction of a negative charge. This modification would result in increased motility by interaction with water molecules.

In conclusion, it has been determined that phosphotyrosine is present in a- and b-type flagellins. The distribution patterns of acid hydrolyzed and tryptic peptides, separated by TLE, are the same for a- and b-type flagellins. The susceptibility of p-tyr flagellins to two phosphatase enzymes, and reactivity to p-tyr MAbs, indicate that unmodified tyrosine phosphates occur. Although the exact biological role of this modification of flagellin is unknown, it is believed that phosphorylation plays an important role in some flagellar function.

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## VITA

Kimberly Kelly-Wintenberg was born to Norma and Charles A. Kelly on the tenth of May, 1961, in Kingsport, Tennessee. She attended Kingsport public schools, and graduated from Dobyns-Bennett High School in June 1979.

The following fall she entered East Tennessee State University in Johnson City, Tennessee, where she majored in Microbiology. During this time she was employed as an assistant manager of a multiplex theatre. She graduated with honors in December 1983 with a Bachelor of Science degree.

In the fall of 1986 she entered the Department of Biochemistry, and in the spring of 1987 she began graduate studies in the Department of Microbiology at the University of Tennessee, Knoxville. In the fall of 1988 she was married to Alan L. Wintenberg. She was awarded the Doctor of Philosophy degree in December, 1991. She is a member of the American Society for Microbiology and the Protein Society.