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EVIDENCE FOR PROTEIN TYROSINE KINASE ACTIVITY IN
PSEUDOMONAS AERUGINOSA

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

Suzanne Lawson South
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DEDICATION

This work is dedicated to my husband, who has given me
immeasurable love and support.

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ABSTRACT

Pseudomonas aeruginosa flagellin exhibits a single repeating subunit protein designated either as b-type (53 kDa) or a-type (45 to 52 kDa). Previous data demonstrated that b-type flagellins are phosphorylated at tyrosine *in vivo*. Data presented extends that finding to include a-type flagellins, as well. Thin layer electrophoresis of flagellar protein treated with alkaline phosphatase demonstrated a release of ^{32}Pi , indicative of a phosphomonoester linkage. Immunoblots employing anti-phosphotyrosine monoclonal antibody showed that unmodified phosphotyrosine existed in both flagellar types. Experiments were designed to demonstrate phosphorylation of flagellar protein *in vitro*. Evidence presented showed flagellin was labeled by $\gamma^{32}\text{P}$ ATP, but not by $\alpha^{32}\text{P}$ ATP, when incubated with cell envelope fractions. Results suggested that autophosphorylation of a 42 kDa membrane protein occurred. No kinase activity was detected in cytoplasmic fractions. Confirmation of tyrosine kinase activity was shown by labeling of synthetic poly(Glu:Tyr) as a substrate with $\gamma^{32}\text{P}$ ATP. Labeled phosphotyrosine was observed in partial acid hydrolysates of substrates. Using poly(Glu:Tyr) as a substrate, tyrosine kinase activity was shown to be heat sensitive and time dependent and was shown to be inhibited by sulfhydryl reagents. It appears that tyrosine kinase and flagellin phosphorylation occur in several *Pseudomonas spp.*

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Chapter 1

Introduction and Literature Review

Introduction. *Pseudomonas aeruginosa* is a Gram negative, saprophytic rod, varying in length from 1 to 3 μm and in width from 0.5 to 1 μm . Most strains possess a single, polar flagellum which is responsible for the organism's motility. *Pseudomonas aeruginosa*, which was first isolated in 1887, was shown to be an animal pathogen in 1889 (Doggett, 1979).

Pseudomonas aeruginosa is recognized today as a very significant pathogen, and is capable of causing disease in vertebrates, invertebrates, and plants. It is believed that up to twenty-five percent of normal individuals carry *Pseudomonas* in their gastrointestinal tract with no clinical symptoms (Doggett, 1979). *P. aeruginosa* infects those individuals whose immune system is stressed either by the nature of their disease or injury, or by prolonged chemotherapy. Therefore, this organism is considered to be an opportunistic pathogen in humans.

The patient afflicted with severe burns is at particular risk for infection with *Pseudomonas aeruginosa*. Burn wound infections originate through surface contamination or from the patient's own lower gastrointestinal tract (Pruitt and Lindberg, 1979). Studies have shown that more than sixty percent of these patients are colonized by the fifth day post-burn (Doggett, 1979). Regardless of the source, these infections begin as localized infections at the wound site, but

once the bacterial load in the wound surpasses 10^5 cfu per gram of tissue, a rapid spread ensues, leading to bacteremia and multiple organ invasion (Holder et. al., 1973; Pruitt and Lindberg, 1979). Infections with *P. aeruginosa* have been the leading cause of mortality and morbidity in these patients for many years (Nathan et. al., 1973; McManus et. al., 1981; Linares, 1982).

The frequency of *Pseudomonas* infections is high in cystic fibrosis (CF) patients, who are predisposed to pulmonary infection. Three distinct morphological forms of *P. aeruginosa* can be isolated from the infected CF patient: rough, smooth, and mucoid. The appearance of the mucoid form in the sputum is associated with the progressive debilitation of the patient. It is believed that the mucoid material protects the organism from the opsonizing effect of antibodies and from complement-mediated lysis (Huang and Doggett, 1979).

P. aeruginosa is a very important pathogen in hospital-acquired diseases. It is now the most frequently isolated agent implicated in nosocomial pneumonia (Scheld and Mandell, 1991). This organism is also responsible for many nosocomial urinary tract infections (Kohler and White, 1979). The frequent use of catheters appears to enhance susceptibility to this type of infection; these indwelling devices can become a source of overwhelming infection and bacteremia. Infections with *P. aeruginosa* are becoming more prevalent in cancer patients, as well. These infections are found in patients with acute

leukemias and other bone marrow diseases whose treatment with radiation and/or chemotherapy results in neutropenia (Froland, 1981).

The flagellum as a virulence factor. Many exoproducts of *Pseudomonas* are known to play an important role in pathogenesis. These include proteases (Nicas and Iglewski, 1985; Morihara et. al., 1989), exotoxins (Cross et. al., 1980; Woods and Iglewski, 1983), and phospholipases (Woods, 1987). The presence of a functional flagellum is also a determinant of virulence. This has been demonstrated in studies that have shown a correlation between bacterial motility and invasive infections (Luzar and Montie, 1985; Craven and Montie, 1981). The importance of flagella in the rapid spread of infection has also been demonstrated by experiments that showed that active immunization with flagellar antigen preparations (Holder et. al., 1982; Montie et. al., 1982^a; Holder and Naglich, 1986) or passive immunization with anti-flagellar sera (Drake and Montie, 1987) inhibited the lethal spread of *P. aeruginosa* from a localized infection. To further investigate the contribution of motility to virulence, a Fla⁻ mutant was isolated and used in a burned mouse model. A significant loss of virulence was observed with the nonmotile mutant, when compared to the mortality associated with the wild type motile cell (Montie et. al., 1982^b; Holder, 1985). Using the same model, it was shown that the loss of flagellum or motility was not a factor in initial colonization of the wound, but markedly reduced

the level of colonization of the liver and the blood as compared to a motile strain (Montie, et. al., 1987; Drake and Montie, 1988).

It appears that motility is also important in the early stages of lung colonization in cystic fibrosis patients. Motility and chemotaxis studies were performed on clinical isolates of *Pseudomonas aeruginosa* from patients in varying clinical condition. It was found that while only one isolate examined from a patient in good condition was non-flagellated, 70% of the isolates from patients in poor health had no flagella (Luzar, et. al., 1985). It was hypothesized that there is progressive host selection against bacterial properties associated with invasive virulence (Luzar, et. al., 1985). A study was initiated to investigate the level of flagellar antibodies in CF patients with a clinically evident infection as compared to random outpatients. It was shown that CF patients produce large amounts of antibody against the flagella of *Pseudomonas aeruginosa* (Anderson et. al., 1989). In addition, it has been demonstrated that CF lung mucin serves as a chemoattractant for *P.aeruginosa* (Nelson et. al., 1990). These data support the conclusion that flagella and motility are vital components in the virulence scheme of *P. aeruginosa* in cystic fibrosis.

Flagellar morphology. Bacterial flagella are subcellular organelles that originate in the cell membrane and extend 15-20 μm . Although the term flagellum is used interchangeably for both eukaryotic and prokaryotic organelles, the bacterial flagellum is different in several ways. (i) The bacterial flagellum is passively driven by a motor at its

base; it does no chemomechanical work (Macnab, 1987). (ii) The prokaryotic flagellum propels the cell forward by rotating, not by bending (Berg, 1974). (iii) The flagella of prokaryotes rely on the proton motive force for energy rather than ATP (Khan and Macnab, 1980). (iv) Bacterial flagella possess a switchable motor that can rotate in the clockwise as well as the counter-clockwise direction (Berg, 1974). (v) Finally, the bacterial flagellum filament contains only one protein, flagellin, whereas the eukaryotic filament is complex and is made of numerous proteins (Macnab, 1987). The flagella of prokaryotic cells are composed of three parts: the filament, the hook, and the basal body.

The filament. The filament typically ranges from 15 to 20 μm in length and has a diameter of approximately 20 nm. It consists of thousands of copies of a single protein, flagellin. The shape of the filament is dependent upon medium conditions and the primary structure of the flagellin (Iino et. al., 1974). Work in this laboratory involved the isolation and characterization of the filament protein of several strains of *P. aeruginosa*. These studies showed that the subunit protein was specific for individual strains (Allison et. al., 1985). Based upon the molecular weight and the presence of a cross-reacting antigen, the flagellins have been designated as either a-type or b-type (Ansorg, 1978; Montie and Anderson, 1988). The b-type flagellins are homogeneous, all having a molecular weight of 53 kDa. The a-type flagellins range in molecular weight from 45 to 52 kDa.

(Allison et. al., 1985). The a-type flagellins all contain a common subtype a₀ antigen and various combinations of the subtypes a₁, a₂, a₃, and a₄ (Ansorg, 1978).

Isolated flagellar filaments can be easily dissociated by heat and acid. Repolymerization will occur under suitable conditions, resulting in a filament that is indistinguishable from the original structure (Asakura, 1970). Emerson et. al., using fluorophenylalanine and [³H]leucine, demonstrated that flagella elongate by polymerization of flagellin subunits onto the distal end of the filament *in vivo* (1970). The most logical explanation for this is that the subunits are synthesized inside the cell, and are transported down the length of the existing filament through a central cavity. This model has generally been accepted based upon experimental evidence (Silverman and Simon, 1977).

Based upon genetic mapping and peptide sequencing, it is now evident that the N and C termini of flagellin from different species, including *Pseudomonas* species, are highly conserved (Macnab, 1987; Totten and Lory, 1990). The central region is much less conserved, and is believed to be the area responsible for antigenic variability.

The hook. This structure is located at the base of the filament. It is approximately 900 nm long and has a diameter slightly greater than that of the filament. It is composed of a single polypeptide subunit with a molecular weight of 42 kDa in *E. coli* and *S. typhimurium* (Silverman and Simon, 1972). The hook protein is much more stable

than flagellin; conditions of low pH and the presence of high concentrations of urea or organic solvents have very little effect on the hook and are, therefore, used to separate the hook from the filament protein (Silverman and Simon, 1977).

Antisera prepared against hook proteins have been shown to cross-react with hook antigens from different bacteria, but not with flagella filaments (Kagawa et. al., 1973; Silverman and Simon, 1977). This cross-reactivity between species may reflect a higher degree of conservation in this structure than that observed between flagellins.

The basal body. The basal body accounts for only 1% of the mass of the organelle, yet it is the most complex portion of the structure (Silverman and Simon, 1977). It is composed of a rod joined to the hook and a set of four rings. The outer rings (L and P) are tightly bound to the lipopolysaccharide (LPS) layer of the cell. The L ring appears to be associated with the LPS, while the P ring is associated with the peptidoglycan layer. The inner rings, S (for supramembrane) and M (for membrane), appear to be in contact with one another and are probably located towards the outer face of the cell membrane (Macnab, 1987).

The function of the basal body is not well understood. The L-P ring complex may serve as a bushing through which the rod passes, and the M ring may serve as a mounting plate for components necessary for motor rotation and switching (Macnab, 1987).

Post-translational modifications. Post-translational modifications are common in eukaryotes and prokaryotes. There are several post-translational modifications reported in flagella and pili. N-methyl phenylalanine has been reported in type IV pilin of *P. aeruginosa*, and is found in related pili protein in other genera (Strom and Lory, 1991). The flagella of *Salmonella typhimurium* have been shown to contain N-methyl lysine (Joys, 1988). Phosphorylation is one of the covalent modifications that eukaryotic and prokaryotic proteins may undergo in a post-translational process.

General characteristics of phosphorylation. Phosphorylated amino acids are commonly classified into three groups (Cozzzone, 1988). (a) *O*-phosphates, or *O*-phosphomonoesters, are formed by the addition of phosphate to the hydroxyamino acids serine, threonine, or tyrosine. All three are acid resistant; phosphotyrosine, in addition, is alkali stable. The *O*-phosphates constitute the major class in terms of quantity and extent of distribution in proteins. (b) *N*-phosphates are formed by phosphorylation of the basic amino acids arginine, histidine, or lysine. They are base stable, but are hydrolyzed by acid. (c) Acyl phosphates are generated by the phosphorylation of the acidic residues aspartic acid and glutamic acid. They are extremely acid and base labile, and, therefore, difficult to detect.

Biological significance of phosphorylation in prokaryotes. Protein phosphorylation has long been recognized as playing a vital role in the

control of different facets of eukaryotic metabolism. Recently, the occurrence of reversible phosphorylation in bacteria has also been established (Cozzone, 1988). In several cases, this modification plays a regulatory role analogous to that in eukaryotes. Examples include the NADP-dependent isocitrate dehydrogenase (IDH) of *Escherichia coli*, which modulates the partition of carbon flux between the Krebs cycle and the glyoxylate bypass. This enzyme is regulated by reversible phosphorylation (Garnak and Reeves, 1979). Similarly, the *dnaK* protein in *E. coli*, a protein involved in the heat-shock response and replication of cellular DNA, and the HPr protein of gram-positive bacteria involved in carbohydrate uptake are also phosphoproteins (Cozzone, 1988).

Phosphorylation is involved in two-component regulatory systems, as well. Chemotaxis is only one example of a widespread phenomenon in bacteria of sensing and responding appropriately to various environmental stimuli. Two component regulatory systems consist of a CheA-like protein (termed the sensor) and a CheY-like protein (termed the regulator). Substantial experimental evidence now exists that suggests a common mechanism of action in two-component regulatory systems involving autophosphorylation of a histidine residue in the sensor and subsequent transfer of the phosphoryl group to an aspartate residue in the regulator protein, thereby modulating regulator activity. The system is reset by the activity of a specific phosphatase (Hess et. al., 1991). These systems allow a bacterium to

respond to alterations such as changes in nitrogen, osmolarity, phosphate, and oxygen.

Phosphotyrosine in prokaryotes. The bacterial protein kinases examined thus far appear to phosphorylate their substrates at threonine or serine. Experiments performed on total phosphoproteins of *E. coli* labeled *in vivo* have shown that 96% of the acid-stable phosphoamino acids are phosphoserine residues (Cozzone, 1988). In contrast, the demonstration of protein tyrosine kinase activity has been a more recent endeavor. The presence of tyrosine kinase activity in *E. coli* has been suggested by a group that demonstrated phosphotyrosine in partial acid hydrolysates of ^{32}P -labeled proteins (Manai and Cozzone, 1983). Foster et. al., confirmed those results, but raised the question of whether the phosphotyrosine was a result of nucleotidylation rather than phosphorylation (1989). The authors pointed out that ^{32}P -labeled phosphoproteins isolated from cells incubated with ^{32}Pi may contain phosphodiester linkages to tyrosine residues. Subsequent partial acid hydrolysis of these phosphodiesters could produce *O*-phosphates (Martenson, 1984). Phosphorylation of tyrosine in prokaryotes remains controversial.

Several methods exist for demonstrating phosphotyrosine in bacteria. Phosphatases may be used to examine the release of ^{32}Pi from phosphoproteins or phosphopeptides. Failure to release ^{32}Pi from denatured phosphoprotein or phosphopeptides by phosphatase digestion is evidence of a diester linkage (Martenson, 1984). Anti-

phosphotyrosine monoclonal or polyclonal antibodies are commercially available, and may provide additional proof of unmodified phosphotyrosine. Perhaps the most direct assay to demonstrate true phosphorylation of tyrosine is to show labeling of the residue in an *in vitro* assay utilizing $\gamma^{32}\text{P}$ ATP.

Recently, phosphotyrosine has been positively identified in bacterial proteins utilizing the above-mentioned methods. Dadssi and Cozzzone have demonstrated protein tyrosine kinase activity in the strict aerobe *Acinetobacter calcoaceticus* (1990). Tyrosine kinase activity has also been demonstrated in *Streptococcus pyogenes* using an *in vitro* assay. Phosphorylation of eukaryotic proteins and synthetic polypeptides, as well as endogenous prokaryotic proteins was observed utilizing $\gamma^{32}\text{P}$ ATP (Chiang et. al., 1989). Several proteins and peptides have been found to be phosphorylated *in vitro* and *in vivo* in *Clostridium thermohydrosulfuricum* (Londesborough, 1986), and tyrosine kinase activity was also demonstrated in *Rhodospirillum rubrum* (Vallejos et. al., 1985).

AIMS OF THESIS WORK

Work in this laboratory has previously demonstrated the presence of phosphotyrosine in b-type flagellins. The data presented here will extend this finding to include a-type flagellins, as well. Experiments utilizing phosphomonoester specific phosphatases in

addition to anti-phosphotyrosine monoclonal antibodies will confirm the presence of free, unmodified phosphotyrosine in the flagellin protein of *P. aeruginosa*. Studies have also been undertaken that demonstrate the presence of a tyrosine kinase that phosphorylates flagellar protein and a synthetic substrate in an *in vitro* kinase assay utilizing $\gamma^{32}\text{P}$ ATP. Various inhibitors have been assayed for their effectiveness against the kinase activity.

Chapter 2

Materials and Methods

Bacterial strains, growth conditions. Strains of *P. aeruginosa* (a-type and b-type) were routinely grown in mineral salts medium [4×10^{-4} M K_2HPO_4 , 2.2×10^{-4} M KH_2PO_4 , 7.5×10^{-3} M $(NH_4)_2SO_4$, 2.0×10^{-4} M $MgSO_4$, 6.0×10^{-6} M $FeCl_3$, and 0.4% w:v sodium succinate]. The cultures were incubated for 20 h at 30°C to promote flagellar growth. For radiolabeling, the total phosphate concentration was reduced to twofold over the limiting level (0.62 mM). [^{32}P] Phosphoric acid was added (ICN Biomedical, Inc, Irvine, CA) at a concentration of 5 μ Ci per ml, and the cultures were incubated for 20 h at 30°C.

Isolation and purification of flagella filaments. The cells were concentrated by centrifugation at 5,000 x g at 4°C. The cell pellet was suspended in 10 mM phosphate buffer [10 mM KH_2PO_4 , 10 mM K_2HPO_4 , pH 7.0] 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 and at 16,000 x g for 15 min at 4°C to remove any contaminating cellular debris. The supernatant was retained, and the flagellar filaments were pelleted by ultra-centrifugation (24,000 rpm for 3 h at 14°C). The pellets were suspended in 10 mM Tris-chloride (pH 8.0), dialyzed overnight, and lyophilized.

Isolated flagellum filaments were purified primarily to remove lipopolysaccharide by gel filtration. The partially purified sample was dissolved in sample buffer [15 mM Tris chloride, pH 8.0, 0.1 mM EDTA, 0.1 mM PMSF, 1% w:v sodium azide, 0.1% w:v sodium deoxycholate] and loaded onto a Sephacryl S-300 superfine column (Pharmacia Chemicals, Piscataway, NJ) containing 30 mM Tris chloride (pH 8.0) and 2 mg deoxycholate per ml of buffer. 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 10 mM Tris chloride (pH 8.0) 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% NH₄OH to desalt the preparation.

Gel electrophoresis and immunoblots of flagella. Samples were dissolved in SDS sample buffer [75 mM Tris-HCl, pH 6.8, 2.4% w:v SDS, 150 mM β -mercaptoethanol, and 0.001% w:v bromophenol blue] and were boiled for 5 min. SDS-polyacrylamide gel electrophoresis was performed using 10% or 12% homogeneous gels utilizing a Mini-Protean II Dual Slab Cell apparatus (BioRad Labs, Richmond, CA). Following electrophoresis, the gel was fixed [40% v:v methanol, 10% v:v glacial acetic acid] for a minimum of 15 min and stained with Coomassie brilliant blue [10% v:v isopropanol, 10% v:v glacial acetic acid, 0.005% w:v Coomassie brilliant blue]. For

immunoblotting, the proteins were transferred to nitrocellulose following electrophoretic separation using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad Labs). The nitrocellulose was blocked with 3% w:v bovine serum albumin (BSA) in Tris-buffered saline [TBS, 10 mM Tris-HCl, pH 7.4, and 0.9% w:v NaCl] for 30 - 45 min at 37°C. The blots were incubated with mouse anti-phosphotyrosine (P-tyr) monoclonal antibody (MAb, Upstate Biotechnology, Lake Placid, NY), mouse anti-b-type flagella MAb or human anti-b-type flagella MAb at 5 µg per ml for 1 h at 37°C. Secondary antibody was horseradish peroxidase conjugated goat anti-mouse or goat anti-human IgG (BioRad) or ¹²⁵I conjugated goat anti-mouse IgG (ICN). The blot was incubated with secondary antibody at a concentration of 0.2 µg per ml for 1.5 h at 37°C. Blots were washed between steps with NETG wash buffer [150 mM NaCl, 5 mM EDTA, 60 mM Tris-HCl pH 7.4, 0.25% w:v gelatin, 2.5% v:v Triton X-100, and 0.5% w:v SDS]. For detection of peroxidase conjugated secondary antibodies, Enhanced Chemiluminescence detection (ECL) was employed (Amersham, Arlington Heights, IL). Reagents were added, and after an incubation time of 1 min, the blot was placed on ECL film (Amersham). Generally, the film was developed after 20 sec to 1 min. For detection of ¹²⁵I conjugated secondary antibody, blots were allowed to dry and were placed on X-OMAT x-ray film (Kodak, Rochester, NY) for autoradiography.

Preparation of membrane fractions. *P. aeruginosa* PA01 was grown in one liter of modified mineral salts medium at 37°C. Log phase cells were harvested and washed twice in 20 mM Tris-Cl, pH 7.4, containing 5 mM β -mercaptoethanol. Cells were resuspended in 40 ml of 10 mM Tris-Cl (pH 7.4), 20% w:v sucrose, and 10 mM MgCl_2 . Lysozyme was added at a concentration of 250 μg per ml, and the suspension was incubated at 4°C for one hour. Thirty ml of 1.5 mM EDTA containing 0.5 mM PMSF was added, and the cells were disrupted by repeated sonication at 4 amps. Any remaining intact cells were pelleted by centrifugation at 4,000 x g for 15 min, and the membrane fraction was obtained following centrifugation at 80,000 x g for 1.5 h. Fractions were stored at -70°C. Protein concentration was determined by the method of Goa (1953)

in vitro kinase assay. The reaction mixture contained 1 mg per ml of membrane protein, 1 mg per ml of poly(Glu:Tyr) 4:1 random copolymer (Sigma) or purified flagellar protein isolated as previously described as the substrate, 0.1 mM α or γ ^{32}P -ATP (10,000 Ci mol^{-1} , ICN), 10 mM MgCl_2 , 10 μM Na_3VO_4 , and 5 mM β -mercaptoethanol in 50 mM Tris-Cl, pH 7.4 (Wang and Koshland, 1978; Scheiven et. al., 1986). The reaction mixture was incubated at 37°C for 20 minutes, and was stopped by adding SDS sample buffer and heating at 100°C for 5 min. Gel electrophoresis was then performed. For samples containing the poly(Glu:Tyr) peptide as substrate, the reaction was stopped by the addition of unlabeled ATP (25 mM)

(Schieven et. al., 1986). BSA (15 μ l of 10 mg ml⁻¹) was added as a carrier prior to precipitation with cold 20% w:v trichloroacetic acid (TCA). Precipitates were washed twice in 20% w:v TCA, twice in ethanol, and once each in acetone and ether. For time course assays, reaction mixtures containing poly(Glu:Tyr) were prepared as described above, but reactions were done at 22°C. Aliquots (10 - 20 μ l) were taken at the specified time points, and were spotted onto Whatman #3 filter paper disks which were immediately immersed in 10% w:v TCA containing 10 mM sodium pyrophosphate (Racker, 1991). The disks were washed for one hour (changing the solution every 20 min), and were dried under a heat lamp. The disks were then assayed for radioactivity utilizing scintillation counting.

Preparation of acid hydrolysates and trypsin digest. *In vivo* labeled flagellin was hydrolyzed in 200 μ l of 6 N HCl for 45 min at 110°C. The samples were lyophilized and resuspended in 200 μ l of 0.3 N HCl. *In vitro* kinase labeled flagellar proteins were first subjected to SDS-polyacrylamide gel electrophoresis and were electroblotted onto Immobilon PVDF membrane (Millipore, Bedford, MA) for 45 min at 100 V using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). The proteins bound to the membrane were visualized by staining with India ink (Kamps, 1991). The blot was allowed to dry, and labeled proteins were detected by autoradiography. Using the exposed film as a template, the ³²P-labeled flagellin bands were excised from the Immobilon. The strips were rewetted by soaking

them for 30 sec in methanol and 30 sec in water. Excess moisture was removed, and the strips were immersed in 200 μ l of 6 N HCl.

Phosphotyrosine (2 μ l of 20 mM) was added prior to incubation at 110°C for 40 min. Following partial acid hydrolysis, the tubes were spun in a microfuge for 1 min to pellet carbon particles from the ink (Kamps, 1991). The supernatant was transferred to an Eppendorf tube and dried in a Speed-Vac. Resultant pellets were dissolved in 5 μ l of 0.3 N HCl.

For trypsin digests of *in vivo* labeled flagellin, purified protein was suspended in 0.1 M Tris chloride (pH 8.0) containing 0.1% deoxycholate at a ratio of 5:1 (w:v) minus the volume of trypsin used. After a 30 min incubation period at 70°C to denature the flagellin, trypsin was added at a ratio of 1:50 (trypsin:flagellin). The suspension was incubated for 30 min at 37°C. Following the incubation, the trypsin was inactivated by heating for 5 min at 100°C, and the suspension was lyophilized.

Phosphatase treatment of tryptic digests and flagella. For analysis of *in vivo* labeled tryptic digests, 0.06 to 0.1 units of Calf Intestinal Alkaline Phosphatase (CIP, Sigma) were added, and the suspension was incubated for 30 to 60 min at 37°C. *In vivo* labeled flagellar protein (40 μ g) was treated with 0.3 units of CIP at 37°C for 40 min.

Thin layer electrophoresis and chromatography. Approximately 15 μ l of samples and 2 μ l each of phosphoamino acid standards (each at 20

mM) were spotted on thin layer plastic backed cellulose. These were separated in a pH 3.5 buffer [10:100:1890, v:v:v:, pyridine:glacial acetic acid:water] at 18 mA and 350 volts for 2-3 h at 4°C. Chromatograms were allowed to dry in a hood and were sprayed with a ninhydrin solution [0.1% w:v ninhydrin, 95%v:v acetone, 5% v:v collidine]. After exposing to heat, ninhydrin-positive peptides and phosphoamino acid standards were visualized. Labeled peptides and amino acids were detected using autoradiography. For two dimensional thin layer electrophoresis (TLE) and thin layer chromatography (TLC), the samples were first separated by thin-layer electrophoresis as described above. For the second dimension, phospho chromatography buffer (37.5% v:v n-butanol. 25% v:v pyridine, 7.5% v:v glacial acetic acid, Boyle et.al., 1991) was used in a glass chromatography chamber equilibrated for 2 h prior to use. The solvent front was allowed to reach within 1 cm of the edge of the cellulose plate. Ninhydrin and autoradiographic detection methods were used as described above.

Chapter 3

Results

Isolation and purification of ^{32}P labeled a-type flagellin. Bacteria were labeled with ^{32}P in limiting phosphate (0.62 mM) in overnight cultures to achieve maximum labeling of flagella. Labeled flagella preparations isolated by differential centrifugation were purified by several steps of molecular sieving. Protein was passed through a Sephacryl S-300 column. The protein resolved was monitored by absorption at 265 nm, and was assayed for ^{32}P activity via scintillation counting. It has previously been shown that flagella fragments emerge at the void volume throughout the gel filtration purification process and coincide with a radioactive peak (Kelly-Wintenberg, unpublished data).

Purified labeled flagella protein was characterized by SDS-PAGE. The presence of radiolabeled b-type flagellins has been previously demonstrated (Kelly-Wintenberg et. al., 1990). A purified preparation of a representative a-type flagellin (strain PAK) labeled *in vivo* was separated by electrophoresis in 10% homogeneous gels and stained with Coomassie brilliant blue. As Figure 1A shows, the M_r of strain PAK was 45 kDa (all figures and tables are contained within the Appendix). The autoradiogram demonstrated radioactivity associated with the a-type flagellin (Figure 1B). Therefore, the phosphorylation of flagellin was characteristic of both flagellin types. The additional unresolved bands labeled above the flagellin band (Figure 1B), but not

present in the Coomassie blue stained gel (Figure 1A), corresponded to phosphate in LPS which is more tightly associated with a-type flagella.

Comparative phosphopeptide analysis of flagellins in partial acid hydrolysates. Previously, the presence of phosphotyrosine (P-tyr) in acid hydrolysates (45-60 min) of b-type flagellin was demonstrated by analysis with thin layer electrophoresis (TLE, Kelly-Wintenberg et. al., 1990). Using a 45 minute partial acid hydrolysis, the presence of P-tyr has been demonstrated in a-type flagellins. The short-term hydrolysis facilitated not only identification of P-tyr, since degradation was reduced, but also allowed comparison of phosphotyrosine-containing peptides which were incompletely hydrolyzed in both 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 2, a substantial portion of the amino acid label comigrated with phosphotyrosine in both a- and b-type flagellin which was identified by a blue-gray color following ninhydrin with collidine detection. No other phosphoamino acids were observed. The remainder of label in both types was associated with phosphopeptides which were numbered relative to the distance in centimeters they traveled from the origin (P-2, P-3, and P-4). An interesting finding was that the peptide P-8 observed on the autoradiograms of all of the b-type hydrolysates tested was not present in any of the a-type strains tested (Figure 2). Amino acid analysis has

shown that b-type flagellins contain 7 tyrosines per subunit, while a-types contain 2 to 7 tyrosines (Montie et. al., unpublished data). In view of the variable tyrosine content, this data in addition to data showing similar tryptic peptide patterns (Kelly-Wintenberg, unpublished results) suggests that certain common tyrosine residues are phosphorylated.

Phosphatase treatment of labeled flagella and tryptic digests.

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

Since the ^{32}P component in flagellin comigrated with P-tyr after partial acid hydrolysis, the presence of phosphotyrosine was suggested (Kelly-Wintenberg et. al., 1990). However, a ^{32}P labeled phosphoprotein isolated from cells grown in the presence of $^{32}\text{P}_i$ could contain phosphodiester linkages to tyrosine, and partial acid hydrolysis of these phosphodiester linkages could produce *O*-phosphates (Martenson, 1984).

To demonstrate the presence of *O*-phosphomonoester linkages, the labeled flagellins were treated with a phosphatase enzyme (Martenson, 1984). Calf Intestinal Alkaline Phosphatase (CIP) was used to treat labeled whole flagella and trypsinized samples of labeled flagellin. After treatment, these samples were compared to untreated

samples by TLE analysis. An increased enzymatic release of ^{32}Pi , which migrates further on the chromatogram, was readily observed in all cases. Representative autoradiograms of TLE's of CIP treated flagellin are shown in Figure 3. The release of ^{32}Pi from nondentaured M2 (b-type) flagellin by CIP is demonstrated in Figure 3A. In Figure 3B is shown M2 trypsinized flagellin treated with CIP. The labeled tryptic digest of M2 b-type flagellin treated with CIP released considerably more ^{32}Pi than untreated M2 flagellin. After CIP treatment a 2.5 fold decrease in peptide label and a 3.5 fold increase in Pi label over the untreated control sample were observed based on scintillation counts from chromatograms. In agreement, treatment with Bacterial Alkaline Phosphatase (BAP) demonstrated similar results as did other a-type and b-type CIP or BAP treated strains (data not shown).

Immunoblotting with anti-phosphotyrosine monoclonal antibody. To obviate problems associated with determining the form of ^{32}P existing in flagellin, a direct measure of unmodified phosphotyrosine was employed. Flagella protein isolated from non-labeled *P. aeruginosa* was blotted onto nitrocellulose and was incubated with anti-phosphotyrosine monoclonal antibodies. An enzyme detection method using goat anti-mouse IgG conjugated to horseradish peroxidase with diaminobenzidine as the substrate was initially used with some success. The need for a method with increased sensitivity prompted the adoption of an immunoblotting assay coupled to

chemiluminescent detection. The chemiluminescent blots were exposed to Enhanced Chemiluminescent (ECL) film for 30 sec to 2 min and were developed. The presence of unmodified P-tyr was confirmed in both flagellin types (Figure 4). PAK (a-type) and PA01 (b-type) flagellins both reacted positively with the anti-phosphotyrosine MAb (Figure 4B, Lanes 2 and 3, respectively). No reactivity of BSA was observed (Lane 1). To confirm these results, a replica assay was performed in which the primary antibody was incubated with 1 mM phosphotyrosine prior to use in the blotting experiment (Glenney et. al., 1988). As seen in Figure 4C, an obvious decrease in reactivity was associated with the addition of the preincubation step. The MAb no longer reacted with PAK, and its recognition for PA01 was markedly reduced.

A second assay was employed to confirm these findings. Goat anti-mouse IgG conjugated to ^{125}I was used as the secondary antibody. Following exposure to x-ray film and development, the presence of unmodified P-tyr was demonstrated in both a- and b-type flagellins using this assay. Figure 5 is representative of results obtained. As demonstrated in Figure 5B, PA01 (b-type) flagellin reacted positively with the anti-phosphotyrosine MAb. The degree of reaction obtained was proportionate to the amount of flagellin present. Note in Figure 5C, that anti-flagellin MAb was substituted for anti-phosphotyrosine MAb, and gave a positive reaction. This confirmed the presence of flagellin in these bands.

In vitro labeling of flagellin. Experiments were designed to demonstrate the phosphorylation of flagella filament protein by gamma labeled ATP in extracts, the most direct measure of protein kinases. Purified flagella protein was supplied as substrate with cell envelope protein as the enzyme source. Other components included $MgCl_2$ to serve as a divalent cation, sodium vanadate as an inhibitor of phosphotyrosine phosphatases (Swarup et. al., 1982), and β -mercaptoethanol. The reaction mixture was incubated for 30 minutes, and was assayed on polyacrylamide gels. The major stained band for the b-type flagella standard appeared at 53 kDa with a weak band visible at 45 kDa (Figure 6A, Lane 5). However, in the *in vitro* kinase samples, a major band appeared at 45 kDa both in the stained gel and in the autoradiogram (Figure 6A and 6B, Lane 1). No flagella bands were observed in the lane that contained no exogenous flagella as substrate (Figure 6A and 6B, Lane 2). Genistein was included in one sample at 74 μM (Akiyama, et. al., 1987), but no inhibition of activity was observed. The assay was repeated utilizing $\alpha^{32}P$ ATP; no radiolabeling was observed (Figure 6C and 6D).

An immunoblotting assay was performed to verify that both the 45kDa and the 53 kDa bands were flagellar protein. The results of the Western blot utilizing human monoclonal antibody directed against b-type flagellin are shown in Figure 7. As seen in Panel B, both bands reacted strongly with the monoclonal (Lane 2). We have attributed the appearance of the lower band to the action of a small amount of protease activity. Flagella samples had been left in a denatured state,

but the samples had not been boiled. This appears to have enhanced the susceptibility to the protease cleavage.

The assay was repeated with greater care to avoid protease degradation. As seen in Figure 8, PA01 flagella protein was specifically labeled by $\gamma^{32}\text{P}$ ATP, but not by $\alpha^{32}\text{P}$ ATP. This figure shows that the 53 kDa PA01 flagella protein was phosphorylated *in vitro* upon incubation with $\gamma^{32}\text{P}$ ATP (lane 6). Lane 2 shows that the flagellar protein was not labeled by $\alpha^{32}\text{P}$ ATP. Experiments were conducted in the absence of exogenously added flagella as substrate (Lanes 1 and 5) and confirm that the major labeled band obvious in lane 6 is, in fact, resultant from the phosphorylation of the flagella substrate, and not due to phosphorylation of a co-migrating membrane protein. No labeling of flagella was detected in samples incubated with cytoplasmic fraction as the source of enzyme (data not shown).

In vitro labeling of a 42 kDa membrane protein. In Lanes 5 and 6 of Figure 8, a labeled protein of approximately 42 kDa is apparent. This protein is present in the reaction sample containing flagellar protein as substrate, but is also evident in the sample which only contains endogenous membrane proteins as substrates. The band is not obvious in the stained gel. This labeled band was not apparent in Figure 6, however, its absence could be due to previously explained protease activity.

This finding prompted an *in vitro* kinase assay containing a higher concentration of membrane proteins in order to facilitate the

visualization of this labeled band. This assay was performed at 4°C for 10 min. Figure 9 shows the results of this study. In both experimental samples, the 42 kDa protein is labeled (Lanes 4 and 5). Close examination of the stained gel revealed the presence of a faint band which corresponded to the labeled protein (Lane 2).

Two-dimensional thin layer electrophoresis and thin layer chromatography of *in vitro* labeled PA01 flagella. Samples of *in vitro* labeled flagella were subjected to SDS-polyacrylamide gel electrophoresis and subsequently electroblotted onto Immobilon PVDF membrane. The proteins bound to the membrane were visualized by staining with India ink, and the labeled proteins were detected with autoradiography. Using the exposed film as a template, the ³²P-labeled flagellin bands were excised from the Immobilon and were subjected to partial acid hydrolysis. The hydrolysate was separated in the first dimension by TLE, as described above. Thin layer chromatography was utilized to separate in the second dimension. Phosphoamino acid standards and peptides were identified with ninhydrin plus collidine, and labeled amino acids and peptides were detected by autoradiography. Figure 10 shows that phosphotyrosine was the only phosphoamino acid detected. Phosphotyrosine was consistently seen as a blue-gray spot. Significant amounts of labeled material remained near the origin. This most likely reflects the partial acid hydrolysis conditions used and

overloading of the chromatogram which was necessary to ensure detection of phosphotyrosine.

in vitro labeling of poly(Glu:Tyr) co-polymer. Synthetic random polymers have been shown to serve as excellent substrates for many protein kinases. A polymer of glutamic acid and tyrosine at a ratio of 4:1 is a substrate for all protein tyrosine kinases thus far tested (Racker, 1991). It has been proposed that an important part in recognition of substrates by tyrosine kinases is charge distribution, and that the distance between a tyrosine and a charged amino acid, such as glutamate or aspartate, signals a protein kinase (Abdel-Ghany et. al., 1989).

There are several advantages to using a synthetic polymer in an *in vitro* kinase assay. First, the K_m value of these polymer substrates may be orders of magnitude lower than those of small consensus sequence peptides (Racker, 1991). These polymers are also easily synthesized, and many are commercially available at a low cost. In some systems, it is advantageous to utilize a tyrosine polymer as a substrate in crude extracts so that there is no interference from protein-serine kinases (Racker, 1991). A disadvantage of the polymers currently available is that they are quite heterogeneous in molecular weight, therefore excluding SDS-PAGE as a tool for analysis.

An *in vitro* kinase experiment was designed that contained a random copolymer of glutamic acid and tyrosine as the substrate in

the place of flagellar protein and employed thin layer electrophoresis for analysis. Figure 11 illustrates that poly(Glu:Tyr) was phosphorylated at tyrosine following a 20 min incubation with the membrane fraction and $\gamma^{32}\text{P}$ ATP as assayed by TLE of partial acid hydrolysates (Lane 2). When $\alpha^{32}\text{P}$ ATP was employed, several radiolabeled spots were detected, however, no phosphotyrosine was seen (Lane 1). Experiments were performed that included $\alpha^{32}\text{P}$ ATP with the membrane fraction alone, and displayed a similar pattern of radiolabeled spots (data not shown). The spots that appeared in Lane 1 most likely represent non-kinase phosphorylation of membrane proteins.

Time-course of *in vitro* phosphorylation of poly(Glu:Tyr). The *in vitro* kinase reaction was repeated with the co-polymer as substrate, and 30 μl aliquots were withdrawn from the reaction vial at 0, 5, 10, 20, and 30 minutes. The aliquots were spotted onto Whatman filter disks which were immediately immersed in 10% TCA containing 10 mM sodium pyrophosphate (Racker, 1991). After rigorous washing, the disks were assayed for radioactivity. As evident in Figure 12, the phosphorylation of poly(Glu:Tyr) increased with time, further proof of an enzymatic addition of phosphate.

An additional assay was performed which also included membrane without co-polymer. Figure 13 shows that there is a slight increase in membrane phosphorylation with time, but that the levels of ^{32}P activity are substantially below those of the experimental sample

containing poly(Glu:Tyr). For example, at 10 min, the level of membrane phosphorylation was only 57% of the level associated with the poly(Glu:Tyr) sample. This experiment substantiated the evidence presented in Figure 12; the co-polymer was indeed a suitable substrate for the tyrosine kinase in the membrane. The radioactivity levels observed in Figure 12 were not due solely to phosphorylation of membrane proteins or to autophosphorylation of the kinase.

The assay was also performed in the absence of membrane to observe background levels of radioactivity. Figure 13 shows a comparison between the experimental assay with poly(Glu:Tyr) and membrane proteins and a reaction mixture that does not contain membrane proteins as a source for enzyme. The sample without membrane proteins maintains a steady, low level of radioactivity while the sample containing enzyme increases with time to substantially higher levels of radioactivity. Another assay was performed in which the membrane was boiled for 5 minutes prior to use in the *in vitro* kinase assay. The kinase activity was reduced to 21% of the activity of experimental values.

Time-course assay with kinase inhibitors. It has been reported that 42 eukaryotic protein tyrosine kinases contain a conserved cysteine residue within subdomain XI of their catalytic domain (Hanks and Quinn, 1991). It is not known if this residue is essential for kinase activity. To our knowledge, no prokaryotic tyrosine kinases have been sequenced as yet; therefore, it is not known if this cysteine is

also conserved in bacterial systems. A study was initiated in an attempt to inhibit the kinase with a sulfhydryl agent. *N*-ethylmaleimide (NEM) has been shown to inactivate enzymes by interfering with thiol groups of cysteine residues and mercury is known to inhibit via metal binding (Riordan and Vallee, 1972). The time course assay was performed, assaying for the effectiveness of NEM. The results of this study are shown in Figure 14. An obvious decrease in kinase activity was associated with the inclusion of NEM. This assay was repeated several times with similar results. When mercuric chloride was assayed, a significant level of inhibition was also observed (Table 1). Addition of 200 μ M HgCl₂ resulted in a 51% inhibition of kinase activity after 10 min.

Several inhibitors of eukaryotic tyrosine kinases have been reported. Quercetin, was the first protein tyrosine kinase blocker discovered (Glossman et. al., 1981; Graziani et. al., 1981; Graziani et. al., 1983). Synthetic derivatives of quercetin, such as the isoflavone genistein, have been shown to be more selective (Akiyama et. al., 1987). These protein tyrosine kinase blockers have been found to be ATP competitors (Levitzki, 1992). Both quercetin and genistein were assayed for inhibition of the tyrosine kinase in the *in vitro* reaction with poly(Glu:Tyr), but neither was effective. A similar finding was reported for quercetin when assayed in *Acinetobacter calcoaceticus* (Dadssi and Cozzone, 1990).

Chapter 4

Discussion

The presence of phosphotyrosine in both antigenic types of flagellin has been confirmed. In agreement with an earlier report investigating b-type flagellin (Kelly-Wintenberg et. al., 1990), a-type flagellin also exhibits phosphotyrosine and not phosphothreonine nor phosphoserine. Phosphoserine has been implicated as a component of *Campylobacter* flagellin (Logan et. al., 1989). Since the two straight chain phosphoesters of phosphoserine and phosphothreonine are more stable in acid than phosphotyrosine (Cooper et. al., 1983), they should have been observed if present under the partial acid hydrolysis conditions employed for characterization of phosphotyrosine. Further evidence showed that the flagellin reacted with anti-phosphotyrosine monoclonal antibodies using two sensitive assays. This data is consistent with the existence of phosphate as an unmodified monophosphoester in the flagellin.

The origin of the phosphotyrosine is important to establish, because phosphotyrosine in bacteria has been considered unlikely (Foster et. al., 1989). Phosphotyrosine isolated in acid or alkaline hydrolysates of cytoplasmic proteins was shown to originate primarily from nucleotidylated glutamine synthetase in *E. coli* (Foster et. al., 1989). Glutamine synthetase of *Pseudomonas* is also adenylated (Meyer and Stadtman, 1981). To answer this question, *in vivo* labeled flagellar protein and tryptic peptides were treated with two types of

phosphomonoester-specific alkaline phosphatases. In both cases, a significant release of ^{32}P i was observed following treatment with the phosphatase. Failure to have released ^{32}P i utilizing these enzymes would have indicated phosphodiester linkage. The release of ^{32}P i was more easily visualized in the sample digested with trypsin, since proteins in general are poor substrates for these phosphatases. This is especially true if the molecular weight is greater than one million (Martenson, 1984), as is the case with flagella. An interesting observation was that two of the tryptic spots were not as susceptible to the phosphatase treatment as the other spots.

As a direct demonstration of enzymatic addition of phosphate to the flagellin, $\gamma^{32}\text{P}$ ATP was employed in an *in vitro* kinase assay with cell envelope proteins as the enzyme source. Results showed that flagellin is labeled with $\gamma^{32}\text{P}$ ATP, and not with $\alpha^{32}\text{P}$ ATP, indicative of a kinase reaction. Two-dimensional analysis demonstrated that the flagellin is labeled at tyrosine, in agreement with previously reported data. This is the first protein of known function in bacteria that has been shown to be specifically labeled by a tyrosine kinase.

A recent report detailed tyrosine kinase activity associated with the cytoplasmic membrane of *Pseudomonas solanacearum* (Atkinson et. al., 1992). Results presented here are consistent with that finding. In that report, an 85 kDa membrane protein of unknown function was identified as the major substrate and appeared to be autophosphorylated. We have noted that a 42 kDa protein is phosphorylated at 4°C which may suggest autophosphorylation.

Further experiments are necessary in order to support this hypothesis. The 42 kDa protein may be the tyrosine kinase; it remains to be seen if these two proteins are related.

In the same report, a 45 kDa protein in the cytosolic fraction was shown to be weakly labeled (Atkinson, et. al., 1992). The 45 kDa protein falls in the molecular weight range reported for *Pseudomonas* flagellins (Allison et. al., 1985). In this regard, the flagellin of another species of *Pseudomonas*, *Pseudomonas fluorescens*, is labeled by ^{32}Pi *in vivo* (South and Montie, unpublished data).

Although the properties of the tyrosine kinase remain to be investigated in detail, it would be of value to identify the substrate sequence in the flagellin. Utilization of the PAK flagellin, which is sequenced, will aid in this regard, since there are only two tyrosines per monomer (Totten and Lory, 1990). The published sequence showed that PAK flagellin contained two tyrosines located centrally at positions 172 and 244 in the variable region. One of these tyrosines (Tyr¹⁷²) is interesting because it is adjacent to a phenylalanine (Phe¹⁷³). It has been suggested that an aromatic amino acid adjacent to the phosphorylated tyrosine may be important as a structural determinant for phosphorylation (Geahlen and Harrison, 1990). This tyrosine is also flanked by an asparagine (Asp¹⁶⁹) and a lysine (Lys¹⁷⁴). In addition, there are several acidic residues further removed on the N-terminal side. These are elements common to a phosphorylated tyrosine peptide in the nicotinic acid acetylcholine receptor subunits, beta and delta (Wagner et. al., 1991; Swope et. al.,

1992). The second tyrosine is also a possible target, since it fits the general scheme for tyrosine kinase substrates that contain adjacent acidic glutamate residues (Glu²⁵⁸ and Glu²⁶⁰) in the N-terminal direction with several basic residues on the carboxyl side (Geahlen and Harrison, 1990). The broad specificity for tyrosine kinase substrates and the indication that conformational elements contribute to site specificity, however, preclude a more definitive prediction (Geahlen and Harrison, 1990).

The phosphorylation of the synthetic peptide substrate poly(Glu:Tyr) indicates that there is some similarity of the *Pseudomonas* kinase to others reported, including bacterial (Chiang et. al., 1989), animal (Ben-David et. al., 1991) and yeast tyrosine kinases (Schieven et. al., 1986; Racker, 1991). Using this assay, it was found that membrane tyrosine kinase activity was eliminated by heating and that the polymer was phosphorylated in a time-dependent manner. Synthetic tyrosine-containing peptides have been used extensively in the isolation of tyrosine kinases by providing a means of detecting activity during purification (Wong and Goldberg, 1984; Zioncheck et. al., 1986). The use of this substrate should prove helpful in isolation and further characterization of the *P. aeruginosa* kinase.

Kinase activity was shown to be inhibited by the sulfhydryl-binding agents *N*-ethylmaleimide and mercury. Although little is known about prokaryotic tyrosine kinases, eukaryotic kinases display an apparent -SH sensitive site (Hanks and Quinn, 1991). Further characterization of this property will await purification of the kinase.

A similar finding was reported with the tyrosine kinase of *Acinetobacter calcoaceticus* (Dadssi and Cozzone, 1990). The *P. aeruginosa* kinase and the kinase of *Acinetobacter* were relatively insensitive to known the eukaryotic tyrosine kinase inhibitor, quercetin. Genistein was shown to have no inhibitory effect on *Pseudomonas* kinase activity with either flagellar protein or with poly(Glu:Tyr) as substrate.

Although the purpose of flagellin phosphorylation by a tyrosine kinase is not as yet understood, several possibilities can be postulated. Results showing that the two tyrosines are found in the variable region of the PAK flagellin (Totten and Lory, 1990) suggest that this modification is probably not required for export because sequences common to all flagellins at the N-terminal and C-terminal ends have been primarily implicated in this process (Kuwajima et. al., 1989). Phosphorylation may serve to protect flagellin monomer so that precursor protein is not degraded by intrinsic proteases during export. Preliminary experiments have shown that flagellin treated with alkaline phosphatase are more susceptible to proteolytic degradation (Kelly-Wintenberg, unpublished results). The positioning of the phosphotyrosine in the exposed variable region of flagellin may also provide additional resistance to extracellular proteases. It is interesting that the majority of tyrosine phosphorylations so far identified in prokaryotes are found in membrane-associated proteins. There may be some evolutionary analogy between these surface-located proteins and hormone sensor complexes in eukaryotic

membranes. Since many phosphokinases generally serve as regulatory enzymes in biology, the location of a signaling system in the prokaryotic membrane should be considered.

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APPENDIX

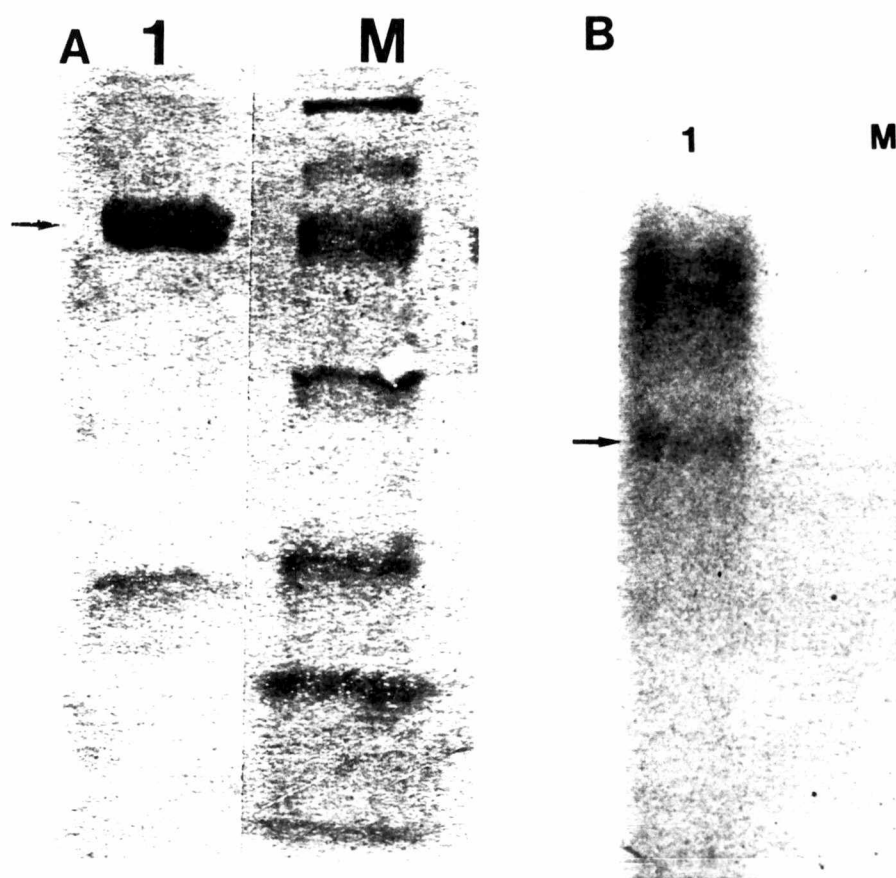


Figure 1: *in vivo* labeling of PAK a-type flagella with ^{32}P . A) Coomassie blue stained gel. (B) Autoradiogram of gel. For panels A and B, lanes: 1, PAK (10 μg); M indicates molecular weight standards of 93, 66, 45, 31, 22, and 14 kDA. The arrow represents the location of the flagellin in the autoradiogram (panel B).

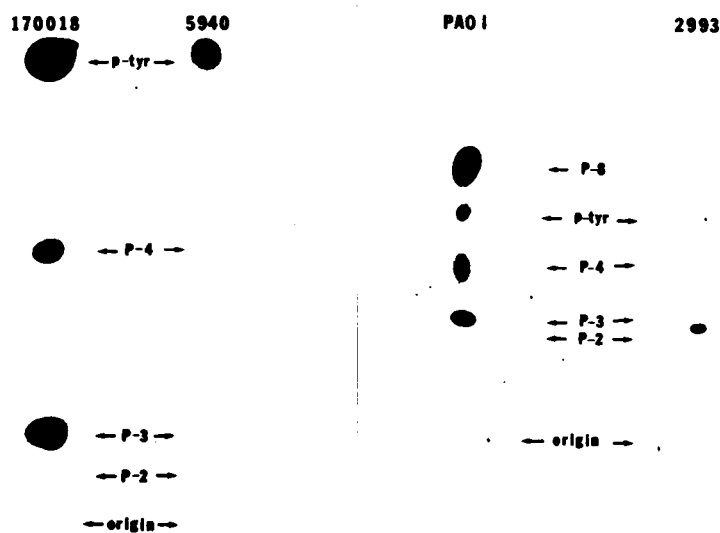


Figure 2: Autoradiogram of thin-layer electrophoresis of partial acid hydrolyzed flagellin (a-type and b-type). (A) 170018 (a-type), 5940 (a-type) (B) PA01 (b-type), 2993 (a-type) P-tyr represents phosphotyrosine. P denotes partial acid hydrolysis resistant phosphopeptides, and the number represents distance migrated (cm) from the origin.

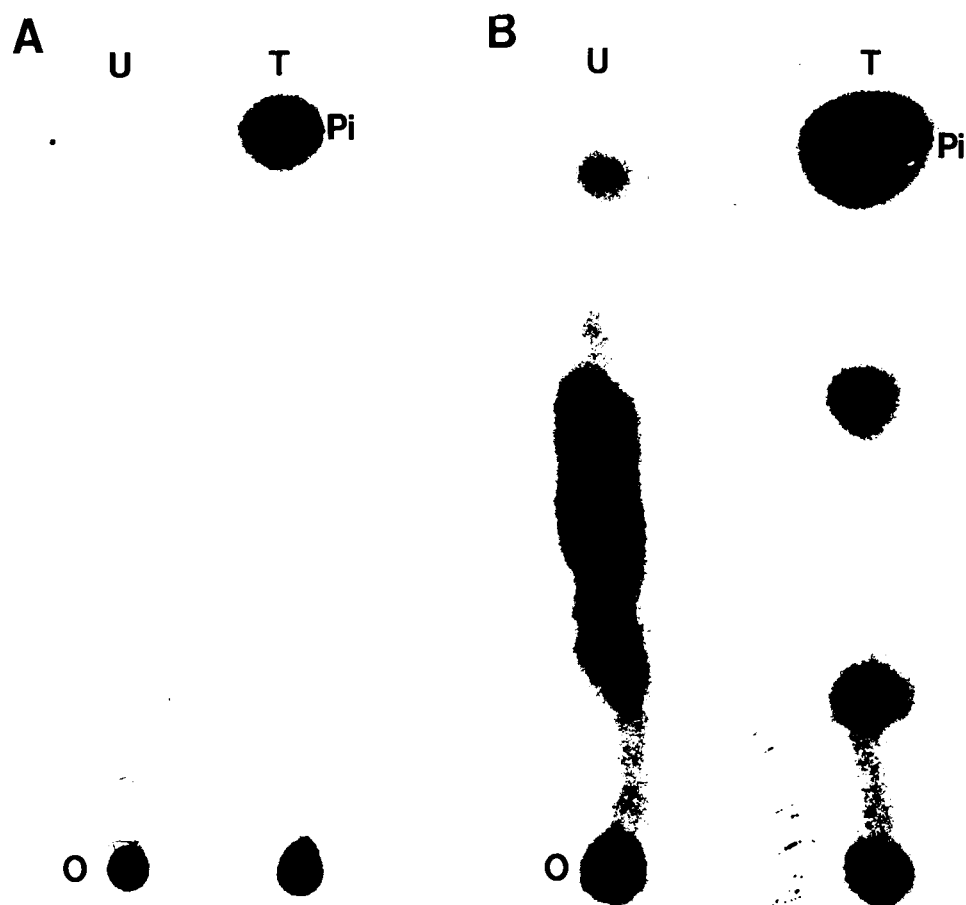


Figure 3: Release of ^{32}P i in phosphatase-treated flagellin. To demonstrate the presence of an *O*-phosphomonoester linkage, samples of M2 flagella and tryptic peptides were treated with Calf Intestinal Alkaline Phosphatase and compared to untreated samples using TLE analysis. (A) Nondenatured flagella. (B) Tryptic peptides. U denotes untreated, and T denotes treated. Pi represents free ^{32}P i and O denotes origin.

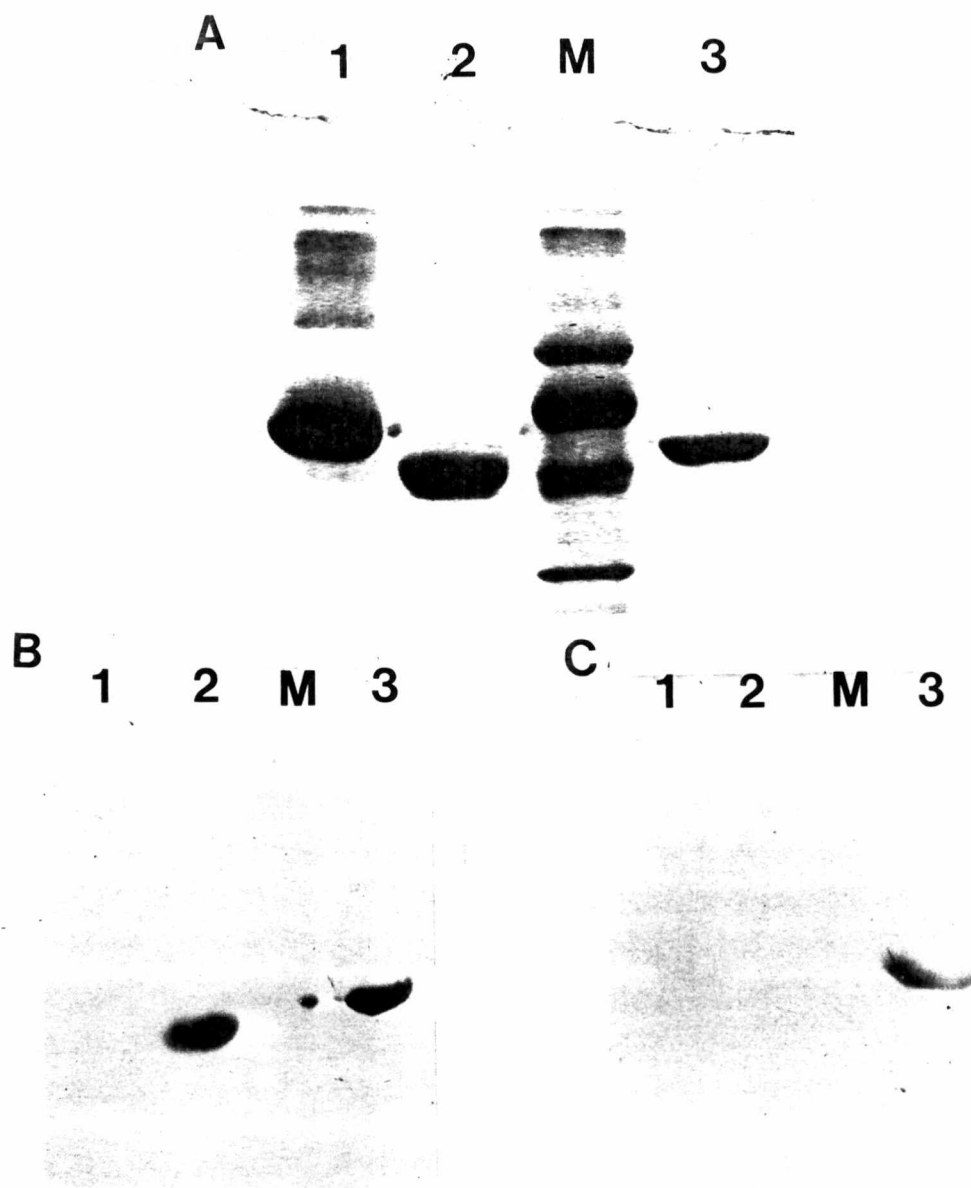
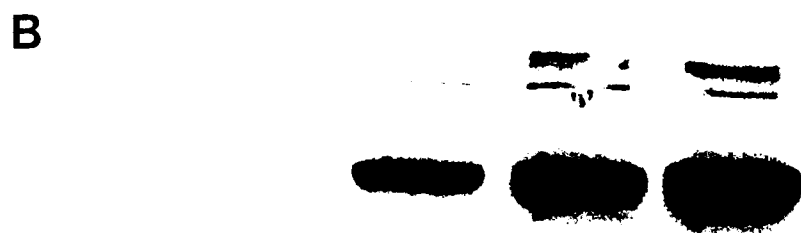
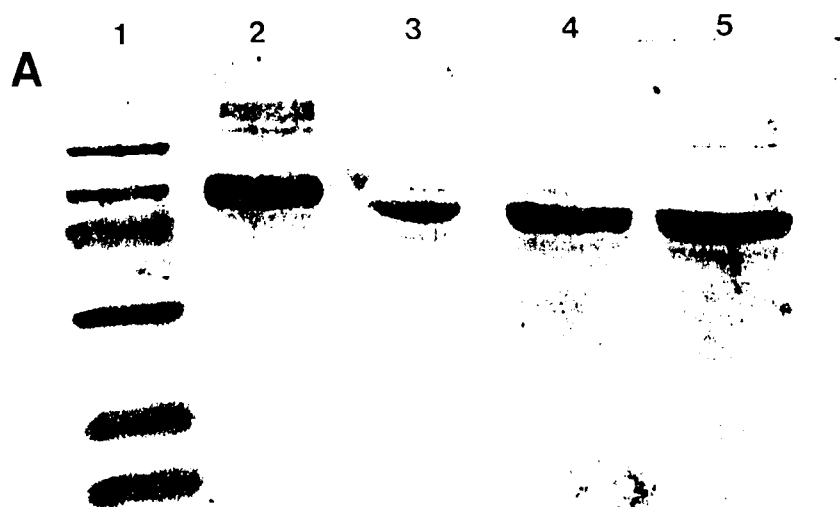


Figure 4: Immunoblotting with anti-phosphotyrosine monoclonal antibody - chemiluminescent detection. (A) Coomassie blue stained gel. (B) "Lumogram" of immunoblot probed with anti P-tyr MAb. (C) Lumogram of immunoblot probed with anti-P-tyr MAb pre-incubated with 1 mM P-tyr. Lanes: 1, BSA; 2, PAK (a-type); 3, PA01 (b-type). M represents molecular weight standards.

Figure 5: Immunoblotting with anti-phosphotyrosine monoclonal antibody - ^{125}I detection. (A) Coomassie blue stained gel. (B) Autoradiogram of immunoblot probed with anti P-tyr MAb. (C) Autoradiogram of immunoblot probed with anti-b-type flagellin MAb. For panels A, B, and C, lanes: 1, Molecular weight standards; 2, BSA (5 μg); 3, PA01 (2 μg); 4, PA01 (4 μg); 5, PA01 (6 μg). Samples were not boiled prior to electrophoresis; therefore, the weaker bands above the flagellin bands represent undissociated flagellin.



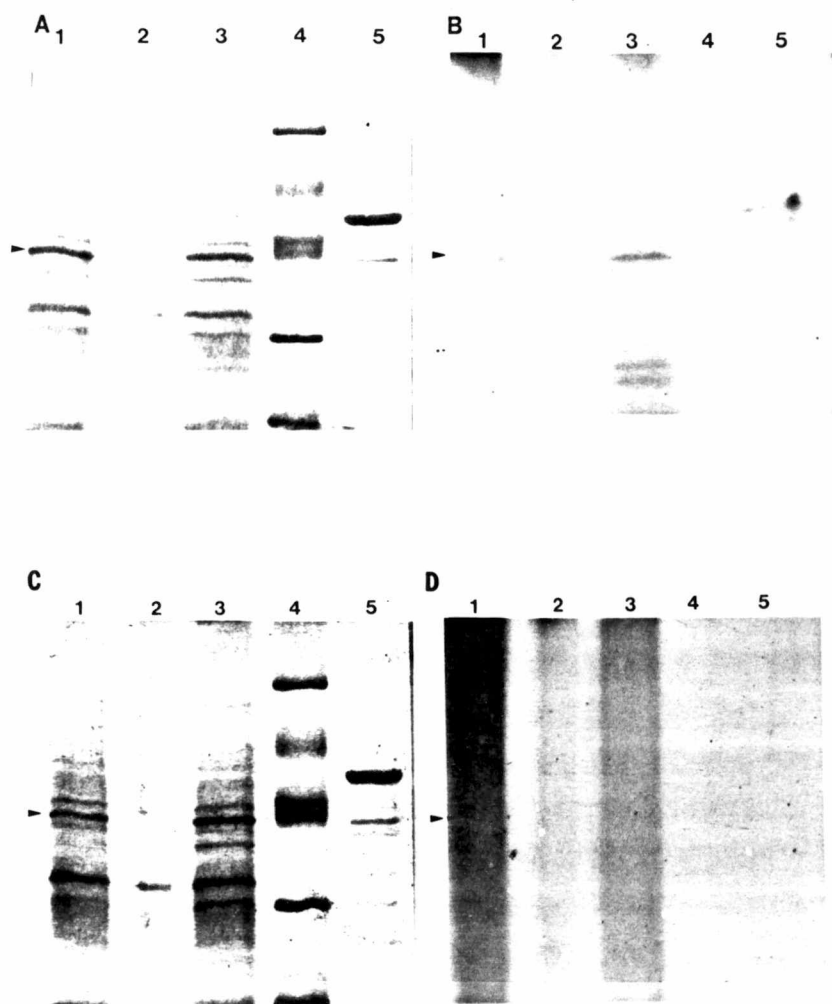


Figure 6: *in vitro* phosphorylation of PA01 (b-type) flagella with $\gamma^{32}\text{P}$ ATP. PA01 flagella was incubated with cellular envelope proteins in the presence of alpha or gamma ^{32}P ATP. (A) Coomassie blue stained gel, $\gamma^{32}\text{P}$ ATP sample. (B) Autoradiogram of gel in A. (C) Coomassie blue stained gel, $\alpha^{32}\text{P}$ ATP sample. (D) Autoradiogram of gel in C. Lanes: 1, flagella as substrate; 2, no exogenous flagella added; 3, flagella plus 74 μM genistein; 4, molecular weight standards; 5, unlabeled PA01 flagellin.

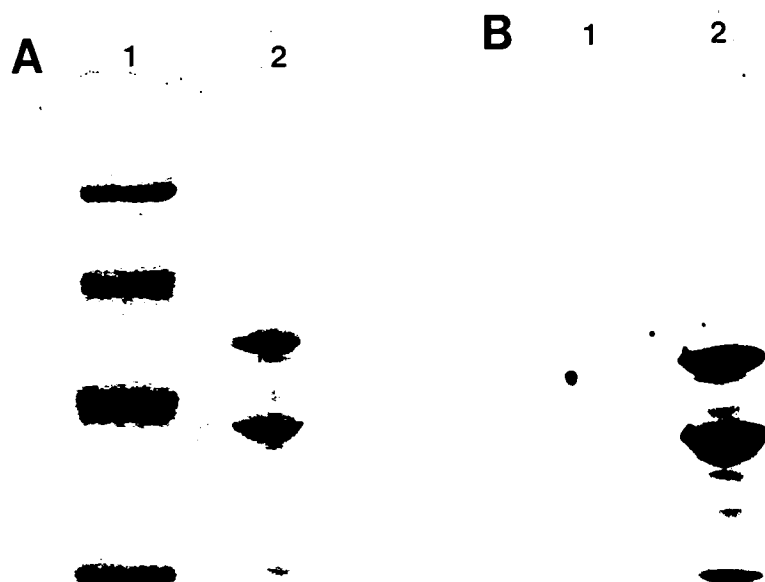


Figure 7: Demonstration of flagellin in *in vitro* labeled 45 kDa protein by cell envelope fraction. (A) Coomassie blue stained gel (B) lumogram of immunoblot probed with human anti-b-type flagellin MAb employing ECL detection. For both, lanes: 1, molecular weight markers; 2, unlabeled PA01 (10 µg) pre-incubated in SDS sample buffer.

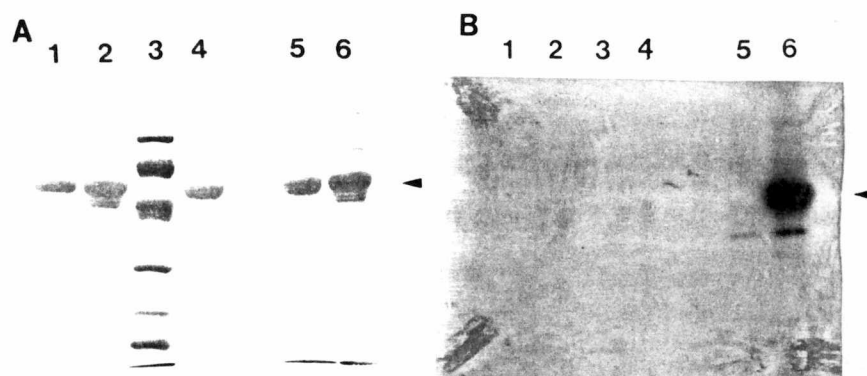


Figure 8: Repeat of *in vitro* phosphorylation of PA01 flagella with $\gamma^{32}\text{P}$ ATP. PA01 flagella was incubated with cellular envelope proteins in the presence of $\alpha^{32}\text{P}$ ATP or $\gamma^{32}\text{P}$ ATP. (A) Coomassie blue stained gel. (B) Autoradiogram of gel. Lanes: 1, $\alpha^{32}\text{P}$ ATP, no exogenous flagella as substrate; 2, $\alpha^{32}\text{P}$ ATP with flagella as substrate; 3, Molecular weight standards; 4, unlabeled PA01 as standard; 5, $\gamma^{32}\text{P}$ ATP with no exogenous flagella as substrate; 6, $\gamma^{32}\text{P}$ ATP with flagella as substrate. Note: in Lanes 1 and 5, unlabeled PA01 flagella was added to the samples after the reaction was stopped.

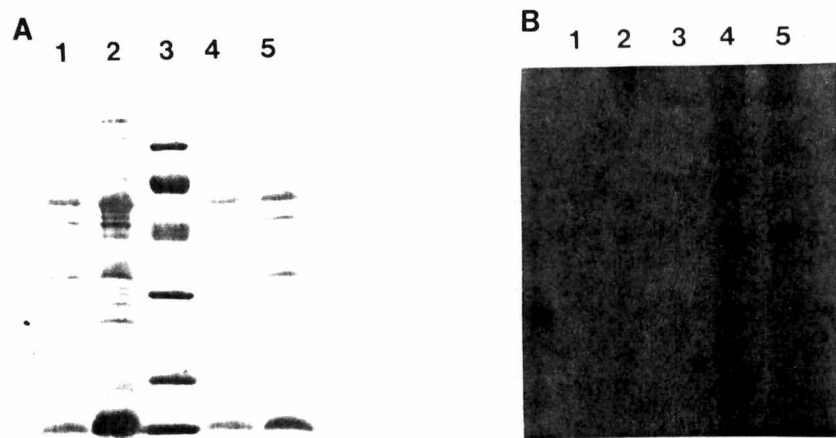


Figure 9: *in vitro* labeling of a 42 kDa membrane protein with $\gamma^{32}\text{P}$ ATP. Membrane proteins at 1 mg/ml and 2 mg/ml were incubated with $\gamma^{32}\text{P}$ ATP for 10 min at 4°C. (A) Coomassie blue stained gel (B) Autoradiogram of gel. Lanes: 1, unlabeled membrane (10 μg); 2, unlabeled membrane (30 μg); 3, molecular weight standards; 4, $\gamma^{32}\text{P}$ ATP labeled membrane used at a concentration of 1 mg/ml in the kinase reaction; 5, $\gamma^{32}\text{P}$ ATP labeled membrane used at a concentration of 2 mg/ml in the kinase reaction.

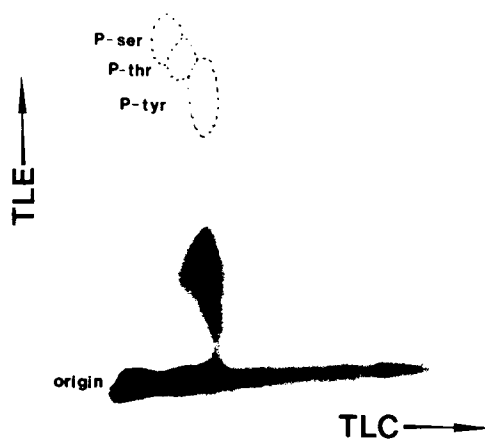


Figure 10: Two-dimensional thin layer electrophoresis and chromatography demonstrating phosphotyrosine in *in vitro* labeled PA01. Following *in vitro* labeling and gel electrophoresis, proteins were transferred onto Immobilon. Immobilon containing labeled flagellin was excised and was subjected to partial acid hydrolysis. The hydrolysate and phosphoamino acid standards were separated by TLE followed by TLC. P-tyr: phosphotyrosine. P-ser: phosphoserine. P-thr: phosphothreonine.

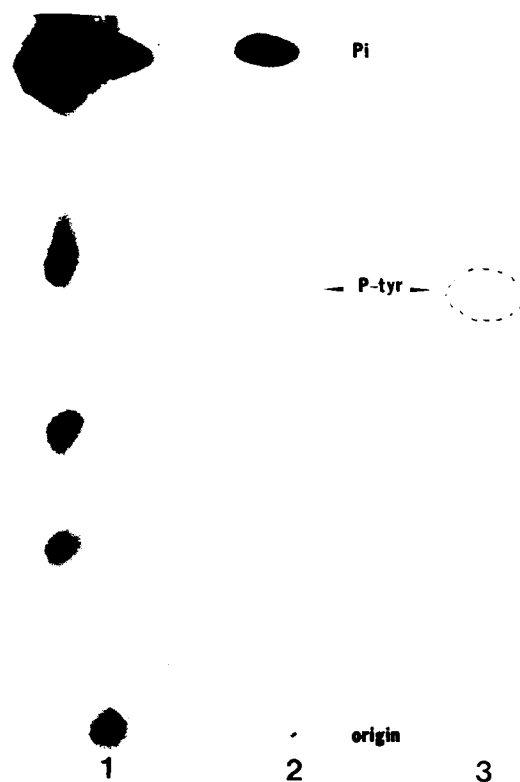


Figure 11: Thin-layer electrophoretic analysis demonstrating *in vitro* phosphorylation of poly(Glu:Tyr). Poly(Glu:Tyr) was used as substrate and was precipitated with TCA following a 20 min incubation with membrane proteins and $\alpha^{32}\text{P}$ ATP or $\gamma^{32}\text{P}$ ATP. The co-polymer was subjected to partial acid hydrolysis (45 min) and was separated by TLE. Lanes: 1, $\alpha^{32}\text{P}$ ATP; 2, $\gamma^{32}\text{P}$ ATP; 3, P-tyr standard. P-tyr: phosphotyrosine. Labeled spots in the $\alpha^{32}\text{P}$ ATP lane most likely represent non-kinase addition of phosphate to membrane proteins.

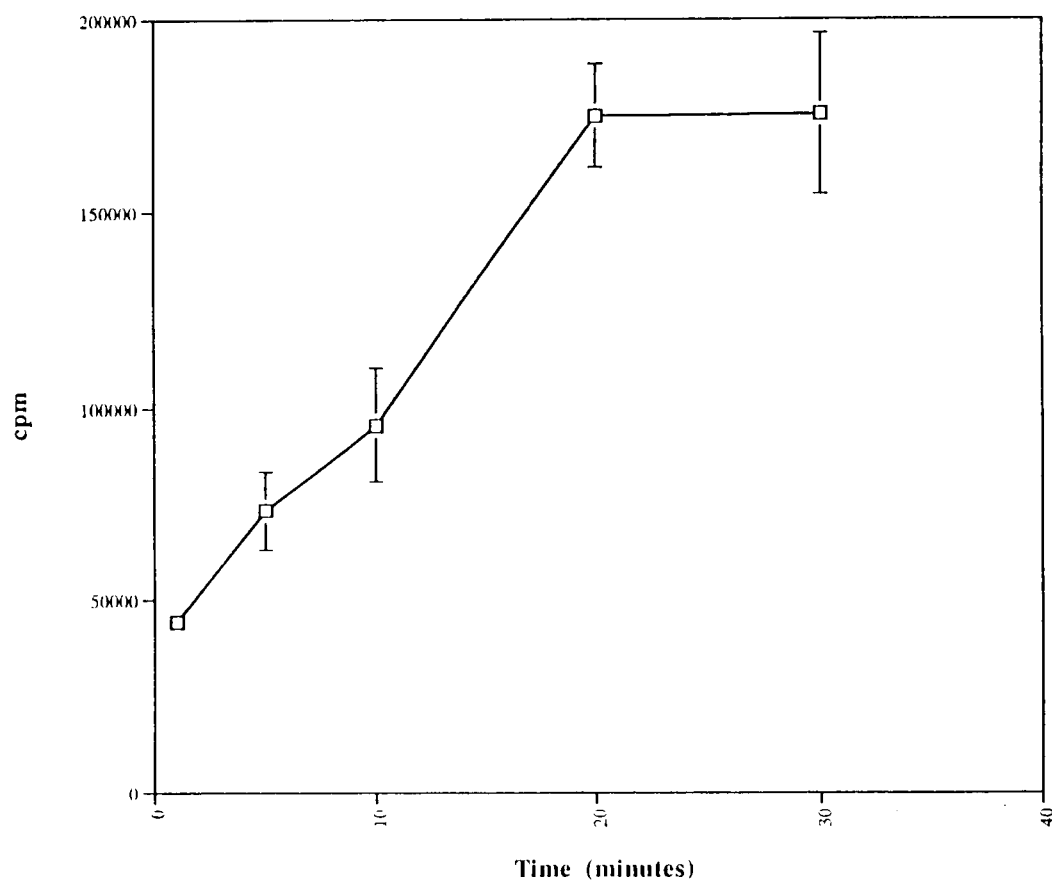


Figure 12: Time-dependent phosphorylation of poly(Glu:Tyr) with cell envelope fraction. Samples (30 μ l) were withdrawn from an *in vitro* assay at 0, 5, 10, and 20 minutes, and were assayed for radioactivity. Points represent means from triplicate samples. Error bars represent standard error.

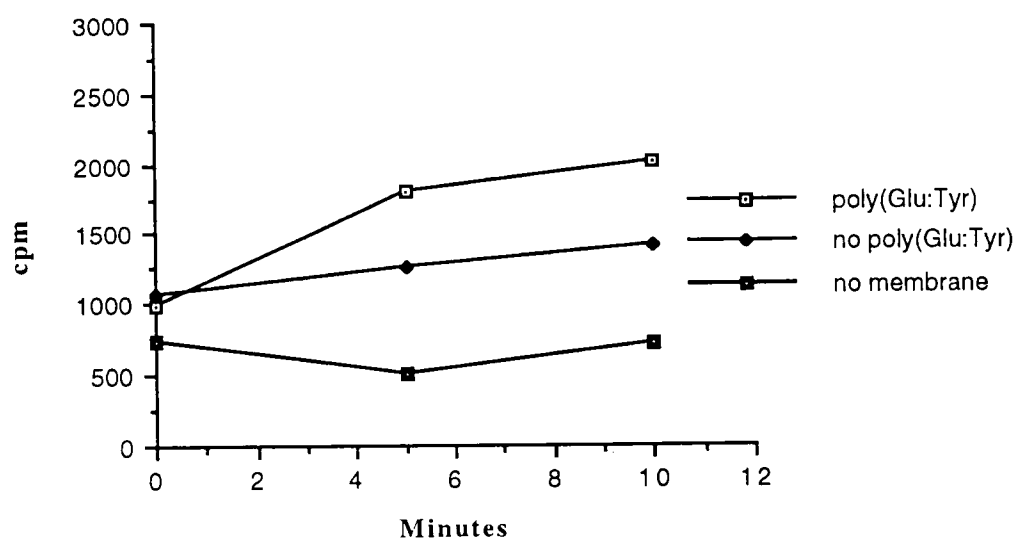


Figure 13: *in vitro* time-course assay with membrane proteins as substrate. Time course assay was repeated in order to compare the level of membrane phosphorylation with that of the poly(Glu:Tyr) sample. Aliquots were withdrawn at 0, 5, 10, and 20 minutes.

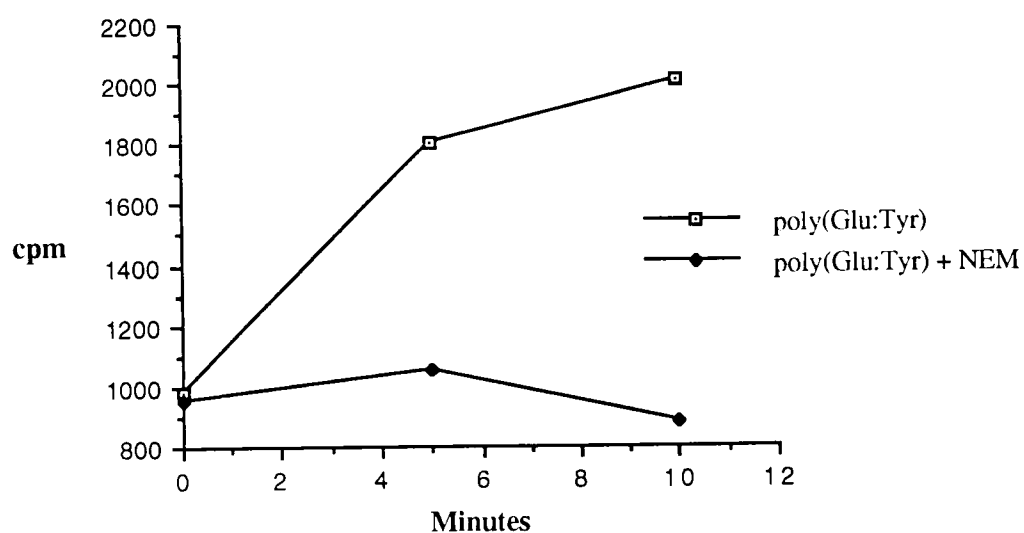


Figure 14: Effect of *N*-ethylmaleimide on *in vitro* phosphorylation of poly(Glu:Tyr). An *in vitro* assay was performed in the presence of 50 mM NEM. An obvious decrease in kinase activity was associated with the addition of the NEM.

Table 1: Effect of mercuric chloride on *in vitro* kinase assay^a

Experiment	cpm	Percent Inhibition
Control	165225	-----
100 μ M HgCl ₂	96035	42
200 μ M HgCl ₂	80818	51

a: Samples were assayed after 10 min at 22°C.

VITA

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