

The Purification and Characterization of two  
Alkaline Phosphatases from  
*Pseudomonas aeruginosa* H103

by

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A thesis submitted to the  
Faculty of Graduate Studies  
The University of Manitoba  
in partial fulfilment  
of the requirements for the degree of  
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## ABSTRACT

Two alkaline phosphatases (AP) from *Pseudomonas aeruginosa* H103 were purified from culture supernatants grown under conditions of phosphate limitation, using a combination of ammonium sulphate precipitation, DEAE-Sephacel ion exchange chromatography and Sephadex G-75 gel filtration chromatography. These two proteins were called L-AP and H-AP corresponding to monomeric molecular weights of 39,500 and 51,000 daltons, respectively, as determined by SDS-PAGE. Both enzymes are phosphomonoesterases, as determined by their abilities to catalyse the hydrolysis of the chromogen substrate *p*-nitrophenylphosphate. The purified alkaline phosphatases were characterised for pH optimum using three different buffer systems (CHES, CAPS, Tris), in which maximum monoesterase activity for both was found using CHES buffer. Only L-AP exhibited a phosphodiesterase activity, as determined by its ability to catalyse the hydrolysis of bis-*p*-nitrophenylphosphate. Amino-terminal amino acid sequencing showed a 42% homology between H-AP and *Escherichia coli* AP. No sequence homologies were found between L-AP and H-AP nor between L-AP and *E. coli* AP. Polyclonal antibodies to L-AP, H-AP and *E. coli* enzyme were raised in New Zealand white rabbits for use in Western immunoblotting studies. Western immunoblot analyses revealed that antibodies specific for H-AP cross react with *E. coli* AP but not with L-AP. L-AP specific

antibodies only reacted with either H-AP or *E. coli* AP when used in excess. The same is true for antibodies specific for the *E. coli* AP which does not react with either L-AP or H-AP except when used in excess. Time course studies using polyclonal antibody to L-AP and H-AP indicated that L-AP was excreted from the cell into the culture supernatant after 7.5 hours of incubation under phosphate limiting conditions only, whereas H-AP was produced constitutively in both phosphate sufficient and deficient conditions. Immunoblot analyses of the periplasmic and extracellular proteins of members of the Family Pseudomonadaceae, grown in phosphate sufficient and deficient conditions, demonstrated the presence of proteins possessing AP activity which were cross reactive with *P. aeruginosa* L-AP and H-AP. This is especially true of Group I members of this Family whereby members produced active L-AP and H-AP cross reactive proteins under at least one set of conditions with the exception of *P. fluorescens* (only H-AP) and *P. syringae* (produced both but had no activity). Other species that produced both active and cross reactive proteins included *P. cepacia* (Group II) and *P. testosteroni* (Group III) while *P. maltophilia* (Group V) constitutively produced an active enzyme which cross reacted with *P. aeruginosa* L-AP. Preliminary results from immunoblot analyses using sera from Cystic Fibrosis patients revealed that L-AP but not H-AP is produced by patients known to be colonized with *P. aeruginosa*.

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**INTRODUCTION AND LITERATURE REVIEW**

## Chapter 1

### Introduction and Literature Review

#### 1.1 Introduction

Pseudomonads are commonly designated as rod shaped gram negative, non sporulating, polarly flagellated bacteria (Palleroni, 1984). They comprise a vast and heterogenous group of organisms. Of all the species, *Pseudomonas aeruginosa* has been most extensively studied and is the principle strain used in this thesis. *P. aeruginosa* can be isolated using blood agar plates where it forms large flat colonies with a ground glass appearance surrounded by a zone of hemolysis. On protease peptone 2 plates, the colonies tend to spread and give off a characteristic grapelike odour. This organism is oxidase positive and utilises glucose oxidatively in oxidative-fermentative medium (Finegold *et al.*, 1978). Physiologically, *P. aeruginosa* is superficially similar to the other gram negative bacteria especially the enteric organisms, for example, possessing the ability to grow at high temperature such as 43°C (Vasil, 1986). However, *P. aeruginosa* is non fermentative, usually obligately aerobic. It can grow in an anaerobic environment if nitrates are available instead of molecular oxygen to act as a terminal electron acceptor. If arginine is available, *P. aeruginosa* can convert it to ornithine anaerobically by way of the arginine dihydrolase enzyme pathway and generates adenosine

triphosphate (ATP) by substrate level phosphorylation (Vasil et al., 1986). In addition, the ability of *P. aeruginosa* to utilise alcohols, organic acids and sugars as sources of carbon and energy, enables it to survive in environments as diverse as soil, insects, plants and traps in the sinks of hospitals (Vasil, 1986; Liu, 1974).

*P. aeruginosa* can use a wide variety of carbon sources like succinate, amides, butyramide (Iglewski, 1989) and grows well in simple minimal media containing phosphates, ammonium salts, magnesium, traces of iron and calcium (Palleroni and Doudoroff, 1972). The production of proteases and the anaerobic exploitation of *P. aeruginosa* end products like arginine, may enable *P. aeruginosa* to initiate infection in parts of the body where little or no molecular oxygen is available (Vasil, 1986).

*P. aeruginosa* is also an opportunistic pathogen that increasingly has been implicated in nosocomial infections especially in immunocompromised patients (Husson et al., 1989). A high mortality rate is generally associated with a *P. aeruginosa* infection in patients already weakened by causes such as leukemia, cystic fibrosis, severe burns, cancer or major surgery (Finegold et al., 1978). This is due in part to the high natural resistance this organism has for many antibiotics.

*P. aeruginosa* is resistant to kanamycin, tetracycline, and sulphonamides but susceptible to the aminoglycosides,

gentamicin, tobramycin and amikacin which are the drugs of choice for the treatment of serious *Pseudomonas* infections. However, significant resistance to gentamicin and tobramycin has been reported by Centre of Disease Control, Atlanta and a burn unit in England (Finegold *et al.*, 1978). This is due to the diverse mechanisms of genetic exchange by *P. aeruginosa* including transformation, transduction and conjugation which help in the adaptation to changing conditions (Jacoby, 1986). Many strains of *P. aeruginosa* are also sensitive to carbenicillin and ticarcillin (synthetic penicillin) which are recommended for therapy primarily as an adjunct to aminoglycosides in serious infections (Marks, 1981). These organisms are also inhibited by the polymyxin antibiotics, polymyxin B and colistin, but these drugs are less useful as they are poorly distributed in the body and can be inactivated by pus and other organic matter (Finegold *et al.*, 1978). R plasmids of *P. aeruginosa* (Holloway, 1978) confer resistance to aminoglycosides by coding for enzymes which modify them. Examples of modifications are N-acetylation, O-phosphorylation and O-nucleotidylation. The acetylating enzymes use acetyl coenzyme A as a co-factor whereas the nucleotidylating enzymes and phosphorylating enzymes use ATP as substrates. The reactions catalysed by these enzymes may occur at one or several amino or hydroxyl groups on aminoglycosides (Hardy, 1983).

*P. aeruginosa* also excretes many virulence factors into



the surrounding environment. These include: exotoxin A, two hemolysins (phospholipase C and a heat stable glycolipid), alkaline protease, elastase, leukocidin, alkaline phosphatase and alginate (Iglewski, 1989). Many of these factors will destroy host tissues and allow this organism to scavenge the released nutrients for cell growth under nutrient limiting conditions. These factors will be described in greater detail subsequently. Antibiotic therapy for cystic fibrosis patients with *P. aeruginosa* colonisation often results in decrease of antibody titres against exotoxin A, elastase and phospholipase C; antibody titers to exotoxin A and elastase decrease to normal levels while the decrease is only marginal for phospholipase C. In contrast, levels of antibody to alkaline protease remain elevated with the continued presence of the bacteria. As the antibody response to these exoproteins differs after antibiotic treatment, this implies that these exoproteins may play different roles in chronic colonisation and active infection (Granstrom *et al.*, 1984). Which one of these exoproteins initiating infection is affected most by the antibiotic or is involved in prolonging colonization is still unclear.

The purpose of my project is to purify one of the virulence factors from *P. aeruginosa* H103, alkaline phosphatase (AP), for biochemical and immunochemical analysis. Though alkaline phosphatase is not a toxin, it is nevertheless essential in the acquisition of phosphate for cell growth

under phosphate limiting conditions and in conjunction with the 2 hemolysins contributes to the destruction of host tissues. Polyclonal antibodies to the purified alkaline phosphatase (AP) will be used to screen for AP production in time course experiments under phosphate sufficient and limiting conditions. Immunoblotting studies will be carried out to examine the expression of AP by other Pseudomonads and related organisms grown under phosphate sufficient and limiting conditions. Finally, the serum from cystic fibrosis patients will be examined for cross reactivity against *P. aeruginosa* H103 alkaline phosphatases to determine if this enzyme is expressed during the infective process of this pathogenic organism.

## 1.2 Pathogenicity of *Pseudomonas aeruginosa* infection

*Pseudomonas aeruginosa* has intrinsic tolerance to commonly prescribed anti-microbial agents like penicillin, tetracycline, ampicillin and sulfonamides. This is due to: (i) the lower permeability of the *P. aeruginosa* outer membrane which is due to the low *in vivo* activity of its major porin protein, protein F. Only 100-300 of the approximately  $2 \times 10^5$  porin molecules in the outer membrane is functional at any given time which in turn reduces the rate of diffusion of hydrophilic substances into the periplasm (Nikaido and Hancock, 1986); (ii) the existence of lipopolysaccharide (LPS) associated with the outer membrane proteins which can shield the LPS-Mg<sup>2+</sup> binding site from attack by antibiotics like polymyxin B (Nikaido and Hancock, 1986); (iii) the prevalence of drug resistance R plasmids and the existence of well developed systems of gene transfer (transformation, transduction, and conjugation) allows the organism to exchange genetic information, including antibiotic resistance (Jacoby, 1986); and (iv) bacteriophage can convert the somatic (O) antigen of a strain to a new serotype thus allowing *P. aeruginosa* to escape the immune system by failing to react with antibodies directed at the O antigen prior to phage infection (Vasil, 1986). All of these factors have resulted in a dramatic rise in *P. aeruginosa* infections in recent years (Husson *et al.*, 1989).

*P. aeruginosa* causes three major types of serious infections: (i) acute localized infections, as may occur in an otherwise healthy human eye following physical damage or improper use of contact lenses (Burns *et al.*, 1990); (ii) chronic localized infections, as in the lungs of patients with cystic fibrosis (Hollsing *et al.*, 1987); and (iii) severe disseminated infections, as may occur in immunocompromised patients or those with severe burns (Iglewski, 1989).

The pathogenicity of *P. aeruginosa* is multifactorial including the following traits: (i) it can withstand a harsher environment than other gram negative opportunists like *Serratia marcescens* and *Acinetobacter calcoaceticus* (Finegold *et al.*, 1978). For example, *P. aeruginosa* is able to grow over a wide range of temperature, up to 43°C (Vasil, 1986); (ii) *P. aeruginosa* is often part of the hospital environment presumably due to its ability to survive and multiply in moist environments with minimal amounts of organic matter. The organism has been incriminated in 5-15% of all hospital acquired infections in North America (Finegold *et al.*, 1978); (iii) it can grow in distilled water and disinfectants (Vasil, 1986); (iv) it is an excellent "scavenger" of essential nutrients enabling its survival in soil, hospital sink traps or a host in which certain nutrients like iron and inorganic phosphate are limited (Vasil, 1986); (v) *P. aeruginosa* produces pyocyanin, a blue phenazine pigment which plays a role in acquiring inorganic phosphates and inhibits other

microorganisms by means of its antibiotic activity. This pigment suppresses other bacterial flora leading to their replacement by *P. aeruginosa*, a common event in a long standing environment (Liu, 1974). Pyocyanin also increases the solubility of iron accumulated through production of pyochelin and pyoverdin (secondary metabolites) which may be necessary for the acquisition of iron by *P. aeruginosa* in an infection (Cox, 1985); and (vi) it excretes virulence factors such as exotoxin A and exoenzyme S; proteases like elastase and alkaline protease; hemolysins like a heat labile phospholipase C and a heat stable glycolipid; and a leukocidin (Vasil, 1986).

### **1.3 *Pseudomonas aeruginosa* infection in Cystic Fibrosis**

#### **Patients**

Chronic respiratory tract infection, which is the hallmark of cystic fibrosis patients, is the major factor in determining the severity of the illness as well as mortality (Matthews *et al.*, 1989). The predominating pathogens isolated from the sputum of cystic fibrosis patients are *P. aeruginosa* and *Staphylococcus aureus* (Hollsing *et al.*, 1987). *S. aureus* is usually the initial pathogen followed by *P. aeruginosa* and the prognosis is commonly considered poor if this dual colonisation is allowed to persist (Hollsing *et al.*, 1987).

Isolates of *P. aeruginosa* from cystic fibrosis patients are unusual in that they are often susceptible to the

bactericidal effect of human serum. Rough lipopolysaccharide on these strains often lacks the normal O-polysaccharide side chain that protects gram negative bacteria from the bactericidal effect of human serum (Speert *et al.*, 1980). These mucoid strains from cystic fibrosis patients are not typeable with conventional antisera directed against the O polysaccharide side chain of lipopolysaccharide (LPS). In contrast, environmental isolates and clinical isolates from patients with other diseases usually have smooth LPS (O) and do not produce much mucoid polyuronic acid exopolysaccharide and thus are phenotypically non-mucoid (Matthews *et al.*, 1984). This conversion from non-mucoid with smooth LPS strain to mucoid with rough LPS strains occurs within the cystic fibrosis respiratory tract. The factors for this conversion have not yet been determined (Speert *et al.*, 1990).

Cystic fibrosis serum contains a factor (which has not been identified) that decreases the ability of alveolar macrophages to phagocytose *P. aeruginosa*. This decrease in phagocytic activity is not due to a specific defect in the immunologic defense mechanism of the host (Matthews *et al.*, 1984). The formation of immune complexes resulting from the combination of antibodies with antigens on the surface of platelets stimulate the liberation of serotonin which can cause inflammation. This inflammation in turn attracts neutrophils to ingest these immune complexes which may result in the liberation of proteolytic enzymes causing tissue

necrosis (Matthews *et al.*, 1984). The lung tissue damage in cystic fibrosis patients is caused more by repeated periods of active infection of *P. aeruginosa* rather than by the colonization of *P. aeruginosa* (Granstrom *et al.*, 1984). In cystic fibrosis, colonization and infections (ability to multiply and cause disease) by *P. aeruginosa* occur in the endobronchial space with minimal invasion of the lung tissue. The organism is deprived of the abundance of nutrients like iron and phosphate for growth. A chronic low grade infection is therefore established in which the bacteria must survive under nutrient limitation which may select for phenotypic variants of *P. aeruginosa* (Speert *et al.*, 1990). This nutritional limitation within the respiratory tract of cystic fibrosis patients may account for the rough LPS with mucoid features of these *P. aeruginosa* isolates (Speert *et al.*, 1990; Marks, 1981). Besides this nutritional limitation, Speert *et al.* (1990) are investigating the possibility that these phenotypic changes may be attributed to bacteriophage as not all strains of *P. aeruginosa* can convert to the mucoid rough lipopolysaccharide phenotype.

Mucoid strains of *P. aeruginosa* also produce the exopolysaccharide alginate which traps large numbers of bacteria thus contributing to airway dysfunction (Marks, 1981; Chakrabarty *et al.*, 1989). These mucoid strains are also resistant to phagocytosis as well as exhibit a reduced permeability to antibiotics (Speert *et al.*, 1990). Alginate-

producing strains of *P. aeruginosa* are associated almost exclusively with the cystic fibrosis lung environment (more than 80%) whereas this phenotype is found in no more than 2.5% of other infections (Vasil, 1986).

Although more than short term eradication of *P. aeruginosa* is seldom achieved, exacerbation can be brought into remission by antibiotic treatment, such as with ceftazidime or cephalosporins in combination with aminoglycosides (Nelson, 1985). Unfortunately, antibiotics like gentamicin, tobramycin, and clindamycin are toxic (Marks, 1981) and have to be administered under close supervision especially if the patient is young (Finegold et al., 1978). An important task is to find means to identify early signs of infection and the optimal time of starting anti-microbial therapy.

Identification of early infection is often difficult as regular culture and sensitivity techniques in routine microbiological laboratories often fail to recover any pathogens from sputum or bronchial washing specimens of cystic fibrosis patients once anti-microbial therapy has been initiated (Finegold et al., 1978). At times, representative sputum specimens are difficult to obtain especially in young patients as more often saliva, rather than sputum are obtained for culture and sensitivity testing (Granstrom 1984).

Serological testing for the secreted exoproteins of *P. aeruginosa* may offer another tool for diagnosis. Already,



serological assays have indicated the levels of antibodies to *P. aeruginosa* exoproteins from mucoid strains (exotoxin A, elastase, alkaline protease and phospholipase C) are found to be elevated in cystic fibrosis patients (Hollsing et al., 1987). It seems that the increase in antibodies to phospholipase C (PLC) is found in close to 100% of cases and thus could possibly be used as a marker of early *P. aeruginosa* colonization. The only exception is in those patients who chronically harbour *E. coli* where antibody titers to PLC lag behind antibody response to other factors (elastase, alkaline protease, exotoxin A). *In vivo*, *P. aeruginosa* and *E. coli* probably compete with each other for access to iron. *E. coli* probably consumes the iron more efficiently than *P. aeruginosa* thus stimulating the production of iron regulated extracellular products (elastase, alkaline protease and exoprotein A). This accounts for the increase in the antibody level to exotoxin A, elastase and alkaline protease before phospholipase C. Additionally, in patients with dual colonization by *P. aeruginosa* and *E. coli*, alkaline phosphatase released by *E. coli* will make inorganic phosphate available for *P. aeruginosa* thus leading to a decrease in the secretion of phospholipase C. Very high serum antibody levels to phospholipase C may also suggest dual colonization with *P. aeruginosa* and *S. aureus*. Patients with this dual colonization also had higher anti-teichoic acid serum titer levels than in patients harbouring *S. aureus* only (Hollsing et

al., 1987). These clinical findings (increased anti-PLC and anti-teichoic acid) mean that special efforts should be made to eradicate *S. aureus* when *P. aeruginosa* appears as conditions deteriorate rapidly once *P. aeruginosa* colonization is established (Hollsing et al., 1987).

#### 1.4 Virulence factors of *Pseudomonas aeruginosa*

*P. aeruginosa* is predominantly opportunistic in nature and owes its invasiveness of infectivity to an altered or already debilitated host, who has been compromised by potent medication, (eg. anti-cancer drugs) or burn patients having a weakened immune system (Finegold et al., 1978). Table 1 shows the characteristics of the major virulence factors of *P. aeruginosa* (Vasil, 1986). Not all strains of *P. aeruginosa* can cause infection. Only those *P. aeruginosa* with pili to adhere to susceptible host cells can cause infectious "opportunism" (Vasil, 1986). Unlike *E. coli* and many other gram negative bacteria, *P. aeruginosa* excretes many of its virulence factors, like toxins, hemolysins and proteases into the surrounding medium to damage surrounding host tissues as well as to destroy host defense mechanisms (Coleman et al., 1987; Vasil, 1986; Iglewski, 1989). These exoproducts can produce leukopenia, acidosis, circulatory collapse, necrosis of the liver, pulmonary edema, hemorrhage and tubular necrosis of the kidney in infected patients (Liu, 1974). Some of the excreted products are: (i) toxins (exotoxin A, exoenzyme S);

(ii) hemolysins, a heat labile phospholipase C and a heat stable glycolipid (Liu and Kurioka, 1967); (iii) extracellular proteases like alkaline protease and elastase; (iv) polysaccharide slime (alginate); (v) leukocidin and (vi) alkaline phosphatase.

**Table 1. Characteristics of the major virulence factors of *Pseudomonas aeruginosa* (Vasil, 1986).**

	Composition/mechanism of action	Potential role
Exotoxin A	66 kD protein, inhibits ADP-ribosyl transferase	Invasiveness, lethality, immune suppression
Phospholipase C	78 kD protein, degradation of phospholipids	Invasiveness, scavenging of nutrients (Pi)
Elastase	39 kD protein, degradation of elastin	Invasiveness, local necrosis, destruction of IgG, IgA, C <sub>3b</sub> , C <sub>5a</sub>
Exoenzyme S	49 kD protein, inhibits ADP-ribosyl transferase	Dissemination, local tissue destruction
Cytotoxin	25-42.5 kD protein	Kills leukocytes, particularly polymorphonuclear leukocytes
Pili	Polypeptide with 15 kD mol. wt. subunit	Specific adherence
Alginate	Polymer of guluronic and mannuronic acid	Inhibition of phagocytosis adherence, immune damage

**1.4.1 Exotoxin A.** This is a 66,600 dalton single polypeptide which contains 4 disulphide bridges (Leppla, 1976; Chung and Collier, 1977). Exotoxin A is the most toxic of the extracellular proteins of *P. aeruginosa* as it can inhibit protein synthesis through the inactivation of elongation factor 2 (EF 2) and suppresses T and B lymphocytes (Konig et al., 1989). Exotoxin A works by catalysing the transfer of adenosine diphosphate-ribose (ADP-ribose) from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 in a similar fashion to the action of diphtheria toxin (Konig et al., 1989). EF 2 is a cytoplasmic protein, i.e., the site of action of exotoxin A is intracellular. The toxin must transverse the cell membrane and enter the cytoplasm before it can inhibit protein synthesis. Exotoxin A conforms to the A-B structure function model of many bacterial toxins (Avigad, 1976). The B fragment of the molecule is necessary for interaction with a eucaryotic cell receptor while the A fragment is catalytic. This mode of action is also found in pertussis, cholera, diphtheria and clostridial toxins (Avigad, 1976). The specificities of both exotoxin A and diphtheria toxin for different eucaryotic cells are distinct. This may be due to the differences in the cell receptors of different eucaryotic cells. One molecule of exotoxin A can inactivate many molecules of EF 2 and can kill a susceptible cell within 24 hours (Vasil, 1986). The mean lethal dose of toxin A in mice is about 0.2  $\mu$ g when injected intravenously (Callahan, 1976).

Production of exotoxin A by *P. aeruginosa* is not constitutive (Liu, 1973). Maximal production is achieved during the declining phase of cell growth in which there is a decrease in the concentration of iron in the media (Vasil *et al*, 1978). Toxin A is produced by 90% of clinical isolates of *P. aeruginosa* (Vasil *et al*, 1977).

**1.4.2 Exoenzyme S.** This is a 49,000 dalton extracellular protein. Like exotoxin A it is a mono-adenosine diphosphate-ribosyltransferase which catalyses the transfer of adenosine diphosphate-ribose from nicotinamide adenine dinucleotide (NAD) to eucaryotic protein like elongation factor 1 (EF 1). Exoenzyme S does not modify elongation factor 2 (EF 2) but modifies a large number of proteins in extracts of rabbit reticulocytes (immature red blood cells), wheat germ extracts and elongation factor 1 (EF 1) (Iglewski, 1978). Exoenzyme S producing cells also secrete a related enzymatically inactive 53,000 dalton protein which cross reacts immunologically with the active 49,000 dalton active form. There is no detectable immunological cross reactivity between exoenzyme S and exotoxin A. The exoenzyme S is produced by 90% of clinical and environmental isolates of *P. aeruginosa* (Igweski *et al*, 1978).

**1.4.3 Elastase.** This is a 33,000 dalton metalloenzyme containing one molecule of zinc (Moriyama and Tsuzuki, 1975).

*In vitro* experiments show that elastase can inhibit neutrophil chemotaxis and inactivates complement proteins such as C<sub>3</sub>b and C<sub>5</sub>a, thereby inhibiting opsonisation by macrophages (Vasil, 1986). Elastase is an endopeptidase which can digest proteins, including elastin, collagen, fibrin, immunoglobulin G, immunoglobulin A, complement components and transferrin (Doring *et al.*, 1981). This suggests a role for elastase to promote invasiveness of *P. aeruginosa*. Elastase thus has a tremendous potential to destroy human tissue. Production of this protein is also regulated by low levels of iron (Iglewski, 1989). Elastase activity is inhibited by heavy metal ions, reducing agents, phosphoramidon, and plasma  $\alpha$ -2 macroglobulin, but is not affected by serine protease inhibitors such as di-isofluorophosphate (Wretlind and Wadstrom, 1977). The incidence of elastase production in clinical and environmental isolates of *P. aeruginosa* is about 90% (Wretlind *et al.*, 1973).

**1.4.4 Alkaline protease.** This protein is a 48,000 dalton enzyme which requires calcium or cobalt for maximal activity (Moriyama, 1963). Enzymatic activity is inhibited by chelators like ethylenediaminetetraacetic acid (EDTA). It is produced in both complex and synthetic medium though production *in vitro* is markedly decreased by free amino acids but enhanced by calcium ions especially in elastase negative strains (Moriyama, 1964). This protein has a limited role in

the pathogenesis of *P. aeruginosa* infection. Alkaline protease activity is inhibited by serum  $\alpha_2$ -macroglobulin, thus its action is mainly restricted to the focus of infection (Vasil, 1986). Incidence of alkaline protease production in clinical isolates of *P. aeruginosa* is about 90% (Wretling et al., 1973).

**1.4.5 Heat stable hemolysin.** (2-0- $\alpha$ -L-rhamnopyranosyl- $\alpha$ -L-rhamnopyranosyl- $\beta$ -hydroxydecanoate, Jarvis and Johnson, 1949). This is a glycolipid with detergent like activity that is capable of solubilizing phosphatides of lipids and membranes (Liu and Kurioka, 1967). This glycolipid together with phospholipase C may act to cause damage especially in the lungs, where phosphatidylcholine is the major surfactant. This glycolipid has low toxicity and is regulated by phosphate levels in the growth medium. Thus the production of this glycolipid can be induced during growth in phosphate deficient medium (Gray and Vasil, 1981; Poole and Hancock, 1983). It is found in about 80% of *Pseudomonas* strains (Johnston and Boese-Marrazzo, 1980).

**1.4.6 Leukocidin.** This is a 44,700 dalton cytotoxic protein that is capable of causing lysis of lymphocytes and granulocytes from several animal species such as rabbits and mice. Concentrations as low as 20 ng/10<sup>6</sup> cells are effective (Nicas and Iglewski, 1986). It will only lyse red blood cells



and platelets when very high concentrations are used (Nicas and Iglewski, 1986). All strains of *P. aeruginosa* can produce leukocidin which is cell associated upon lysis of bacterial cells. The cytotoxic action of leukocidin is mediated by calcium-dependent systems like the metabolism of phosphoditylinositol and polyphosphoinositides resulting in the accumulation of phosphatidic acid and an increase in intracellular calcium ions. Phosphorylation of a lysosomal membrane protein then occurs by activation of a  $Ca^{2+}$ -dependent kinase (Nicas and Iglewski, 1986).

**1.4.7 Alginate.** This exopolysaccharide is one of the most important virulence factors in *P. aeruginosa* infection in patients with cystic fibrosis. It is an acetylated exopolysaccharide which is composed of  $\beta$ -1, 4-linked mannuronic acid and L-guluronic acid. This polymer is responsible for the mucoid nature of *P. aeruginosa* strains isolated from cystic fibrosis patients (Vasil, 1986). Alginate producing strains of *P. aeruginosa* are associated almost exclusively with the environment of the cystic fibrosis lung. There is a high spontaneous reversion frequency of mucoid (alginate producing) strains to the non mucoid strains upon continued culturing *in vitro* (Chakrabarty *et al.*, 1989). More than 80% of cystic fibrosis isolates produce alginate which may contribute to the pathogenicity of *P. aeruginosa* by preventing phagocytosis by host macrophages. In addition,

alginate in the presence of calcium forms a gel that surrounds the *P. aeruginosa* cells with a matrix to shelter large numbers of microorganisms from host defences (Vasil, 1986). Alginate production by mucoid strains of *P. aeruginosa* is partly regulated by environmental nitrogen or phosphate conditions. Phosphate limitation selectively favours the mucoid form of *P. aeruginosa* in continuous culture (Chakrabarty *et al.*, 1989).

**1.4.8 Phospholipase C.** (phosphatidylcholine cholinephosphohydrolase , EC 3.1.4.3.). Phospholipase C is a  $\beta$ -hemolytic heat labile 72,000 - 78,000 daltons hemolysin which gives a clear zone of hemolysis around colonies of *P. aeruginosa* grown on sheep blood agar. This hemolysin has an isoelectric point (pI) of 5.5 (Vasil, 1982). *P. aeruginosa* also produces an extracellular non-hemolytic phospholipase C (Berk, 1987).

The phospholipase that can cleave the water soluble base with the phosphate groups is designated phospholipase C (PLC). Phospholipase C has a limited substrate specificity, preferentially acting on phospholipids containing quaternary ammonium groups like phosphatidylcholine (also called lecithin), lysophosphatidylcholine and sphingomyelin which are abundant in eucaryotic cell membranes and lung surfactant (Vasil, 1990; Tai *et al.*, 1985). Phosphatidylcholine is the major phospholipid component, about 75%, of lung surfactant. The role of phosphatidylcholine is to reduce the surface tension of the alveoli and prevent atelectasis (incomplete

expansion of the lungs, i.e., collapse of portions of the lungs, Liu, 1974). In contrast, phospholipase C has little activity towards phospholipids like phosphatidylethanolamine found in procaryotic membranes (Vasil, 1990).

Based on the enzymatic specificity of *P. aeruginosa* phospholipase C, production of this enzyme could possibly contribute to the virulence of *P. aeruginosa* infection in the lung. Bronchial washings from mice can induce significant production of phospholipase C by *P. aeruginosa* suggesting that phospholipase C is produced *in vivo* (Vasil, 1981). Clinical isolates of *P. aeruginosa* producing the highest amounts of phospholipase C were poorly cleared from the lungs and actually able to multiply. In contrast, *P. aeruginosa* strains producing low amounts of phospholipase C were cleared more rapidly and not observed to multiply in the lungs (Vasil *et al.*, 1982). Vasil, (1989) also found significant association of increased phospholipase C production with isolates of *P. aeruginosa* from patients with urinary tract infection. All virulent strains of *P. aeruginosa* produce phospholipase C, however, strains isolated from sputum, lung, blood and urinary tract (Vasil and Pritchard, 1986) produce the highest levels *in vitro* (Konig *et al.*, 1989).

Intradermal studies (footpad) on mice with up to 3.6-10.8  $\mu\text{g}$  of purified PLC resulted in the appearance of erythema and dermonecrosis 3 hours post injection, which persisted for 7-14 days (Berk *et al.*, 1987). Histological studies of footpads

indicated that PLC caused marked signs of inflammation within 15 minutes post injection, as manifested by cellular infiltration of macrophages and edema. This inflammatory response is due to the release of free arachidonic acid (5'-lipooxygenase) metabolites like leukotriene B<sub>4</sub> (LTB<sub>4</sub>), leukotriene C<sub>4</sub> (LTC<sub>4</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and thromboxanes from granulocytes and macrophages as well as the hydrolysis of membrane phosphatidyl- inisitol by PLC (Konig *et al.*, 1989). LTB<sub>4</sub> is a chemoattractant that supports the accumulation of neutrophils, LTC<sub>4</sub> is part of a slow reacting substance of anaphylaxis through enhancing vascular permeability and PGE<sub>2</sub> is a potent vasodilator. These products of arachidonic acid metabolism (LTB<sub>4</sub>, LTC<sub>4</sub>, PGE<sub>2</sub>, thromboxanes) are formed from the oxidation of arachidonic acid via the lipooxygenase or cyclooxygenase pathway (Berk *et al.*, 1990).

Phospholipase C has also been implicated as a virulence factor in fleecerot dermatitis in sheep. The hemolytic form of phospholipase C from *P. aeruginosa* PL 42 when injected intradermally into the skin of sheep, elicited histopathological lesions which are virtually identical to those seen in naturally occurring fleecerot. The serum of sheep afflicted with fleecerot were also found to contain high levels of circulating antibodies to phospholipase C (Chin *et al.*, 1988).

The gene for phospholipase C is not widely found in the the Genus of *Pseudomonas*, as determined by Southern

hybridisation studies using cloned phospholipase C gene as a probe, (Vasil, 1989). However, Vasil *et al.*, (1989) found that 100% of the more than 80 *Pseudomonas aeruginosa* isolates examined carried the phospholipase C gene and produced  $\beta$ -hemolysis on sheep's blood agar. Recently Vasil, (1990) and LiPuma *et al.*, (1990) have also found the existence of the phospholipase C gene in 4% of the 120 clinical isolates of  $\beta$ -hemolytic *P. cepacia* isolated from cystic fibrosis patients.

**1.4.9 Alkaline Phosphatase.** (orthophosphoricmonoesterase phosphorylase EC 3.1.3.1; Ingram, 1971; Bradshaw *et al.*, 1981). This is a non specific phosphomonoesterase that hydrolyses all phosphate monoesters. This enzyme is widely dispersed in nature from bacteria to human intestinal brush border, placenta and bone, Coleman, (1987). Day and Ingram, (1973) found *P. aeruginosa* alkaline phosphatase to be a metalloenzyme containing 4 molecules of zinc as determined by atomic absorption analysis. Alkaline phosphatase of *P. aeruginosa* resembles other alkaline phosphatases (eg. *E. coli*) in having a Michaelis constant ( $K_m$ ) of  $6.6 \times 10^{-5}$  M for *p*-nitrophenylphosphate (Day and Ingram, 1973). The energy of activation of 5.6 Kcal/mol for *P. aeruginosa* alkaline phosphatase is close to that of the *E. coli* enzyme (5.0 Kcal/mol; Day and Ingram, 1973). Alkaline phosphatase of *P. aeruginosa* is thought to be mainly confined within the periplasmic space (Husson *et al.*, 1989; Beckwith *et al.*, 1981)

but has also been localized extracellularly (Day and Ingram, 1973). As part of the phosphate scavenging system, alkaline phosphatase is not produced until the orthophosphate levels in the medium drop below 8  $\mu\text{g/ml}$  (Ingram *et al.*, 1971). Activity of this AP is restricted to secondary organic phosphates. Primary organic phosphates are not cleaved by this enzyme (Ingram *et al.*, 1978). In a medium containing very low levels of phosphate and a high concentration of glucose, *P. aeruginosa* produces phospholipase C, alkaline phosphatase, phosphate specific porin, protein binding protein and a heat stable hemolysin. The heat stable hemolysin which is a glycolipid may solubilize phosphatides. Phospholipase C may then hydrolyse these phosphatides (sphingomyelin, lysophosphatidylcholine, phosphotidylcholine) liberating phosphorylcholine and diacylglycerol in which phosphorylcholine is hydrolysed by alkaline phosphatase (AP) to choline and free phosphate (Liu, 1967; Stinson *et al.*, 1979; Gray and Vasil, 1981). Free phosphate is then transported into the cell *via* outer membrane protein porin and phosphate binding protein (Stinson *et al.*, 1979).

Day and Ingram, (1973) have purified an alkaline phosphatase (AP) and its subunit forms from *P. aeruginosa* ATCC 9027. This AP exists in three forms: a monomer of  $M_r$  39,000; a dimer of  $M_r$  68,000; and a tetramer of  $M_r$  139,000 daltons. Amino acid analysis revealed that this protein is hydrophilic. AP activity reached a maximum after the cells entered

stationary phase which was associated with a sharp drop in pH (pH 6.8 to 5) in the growth medium (Day and Ingram, 1973). At low pH (pH 3.5), 2 species of AP of molecular mass 39,000 and 139,000 daltons were observed (Day and Ingram, 1973). At pH above 4.5, a single species of average molecular mass 68,000 was observed (Day and Ingram, 1973). This AP (dimer) was thought to be the native form. It is resistant to heat, and proteolysis and is dissociated by acid pH (pH 5.0). However, the monomeric form of AP (39,000 daltons) is unstable to heat and is sensitive to proteolytic enzyme like trypsin (Bhatti and Ingram, 1982). Day and Ingram (1973) thus concluded that alkaline phosphatase of *P. aeruginosa* exists as a dimer in its functional state when the pH is above 4.5, having a pH optimum of 10.5 (Day and Ingram, 1973). Preliminary studies on the excretion of this alkaline phosphatase suggested that it is excreted as a complex with lipopolysaccharide and phosphatidylethanolamine (Ingram *et al.*, 1971; Cheng *et al.*, 1973). Poole and Hancock (1983) disputed this finding as they found that alkaline phosphatase is excreted in an uncomplexed form and the excretion did not result in any perturbation in the permeability of the outer membrane. Poole and Hancock (1983) also observed that the periplasmic activity of alkaline phosphatase increases prior to the appearance of any extracellular enzyme thus suggesting that the periplasmic localization may be a step in the export process of alkaline phosphatase in *P. aeruginosa*.

*E. coli* also has several enzymatically active isozymes which are encoded by a single *phoA* gene (Shinagawa *et al.*, 1987). The isozymes arise as a result of a point mutation in the *phoA* gene, with the various isomers numbered as 1, 2 and 3 in terms of electrophoretic mobility, with 1 being the slowest (Shinagawa *et al.*, 1987). *E. coli* AP isozymes 1 and 3 are identical or very similar with regards to optical rotatory dispersion, heat stability, substrate specificity, and pH optimum (pH 7.4). However, the steady-state rate of substrate hydrolysed at pH 5.5 that is catalysed by isozyme 1 is almost twice that of isozyme 3. Isozyme 2 is thought to be a hybrid of isozymes 1 and 3 (Shinagawa *et al.*, 1987). Sequence analysis of the amino terminus shows that isozyme 1 has an extra amino acid residue, arginine. Isozymes 2 and 3 are formed from the cleavage of the N-terminal arginine residue of isozyme 1 by a protease. The formation of isozyme 3 is inhibited by the addition of arginine into the culture media (Shinagawa *et al.*, 1987).

Multiple APases have also been isolated from *B. subtilis*. The two excreted vegetative forms of AP (AP III and IV) from *B. subtilis* differ slightly in size, and elution from a cationic exchange column at different ionic strengths, but have a 62% identity in their amino terminal sequences. Both cross react with antibody to *B. licheniformis* AP (Hulett *et al.*, 1990). Southern blot analysis of *B. subtilis* chromosomal DNA probed with degenerative synthetic oligomers suggests that



the two structural genes for these AP proteins are present in the *B. subtilis* genome (Hulett *et al.*, 1990). Comparison of the deduced primary amino acid sequence of the mature protein (AP III and IV) of *B. subtilis* and other AP's from *E. coli*, yeast and human placenta shows 25-30% identity (Hulett *et al.*, 1990). Two more APases (AP I and II) were recently isolated from the membrane of *B. subtilis* during vegetative growth (Izaki *et al.*, 1991). These APases differ from AP III and IV in that they are membrane bound rather than excreted. AP I and II appear to be two distinct enzymes possessing different molecular weight, substrate specificity, thermostability  $K_m$ , pH stability and peptide maps. This led Izaki *et al.*, (1991) to conclude that AP I and II were products of separate genes in this strain of *B. subtilis*. In *E. coli*, the 3 dimensional structure of the AP dimer as determined by x-ray diffraction studies shows a symmetric, globular protein with 3 active sites, each containing a metal ion, located 32 nm from each other (Wyckoff, 1987). The crystal structure of *E. coli* AP is thus a valuable prototype for use in protein structure and function studies. Comparison studies by Hulett *et al.*, (1991) shows that the active site and core of the *E. coli* AP are retained in both *B. subtilis* AP III and IV. This core is a 10 stranded  $\beta$  sheet structure which functions to position amino acid residues that have important catalytic activities, and metal and phosphate binding activities and is conserved among the AP of *E. fergusonii*, *S. marcescens*, and human

placenta (Hulett *et al.*, 1991).

The structural gene for *P. aeruginosa* AP (*phoA.35*) was cloned into *E. coli* by Filloux *et al.*, (1988). *P. aeruginosa phoA.35* was found to be normally regulated in *E. coli* in response to the phosphate concentrations in the growth medium, i.e., regulation was repressed in high phosphate medium (0.3% protease-peptone-glucose supplemented with 5 mM phosphate) and derepressed under phosphate limiting conditions (0.3% protease-peptone-glucose with no phosphate supplement). However, in the new host (*E. coli*), *P. aeruginosa phoA.35* expression was confined to the periplasm and is less efficient (65-70%) than wild type *E. coli* AP expression (90%) (Filloux *et al.*, 1988). In *P. aeruginosa*, AP activities are expressed in both cell supernatants as well as in the periplasm (Filloux *et al.*, 1988) whereas *E. coli* AP activities are confined to the periplasm (Lutenberg, 1987).

Filloux *et al.*, (1988) then proceeded to prove the existence in *P. aeruginosa* of a *pho* regulon similar to that of *E. coli*. Heterologous complementation studies were undertaken to isolate *E. coli phoB*- and *phoR*- like genes from the genomic DNA of *P. aeruginosa*. These *P. aeruginosa phoB*- and *phoR*- like regulatory genes were then introduced into *E. coli* mutants lacking these genes. Both *P. aeruginosa* regulatory genes were shown to correctly regulate the expression of AP by the *E. coli phoA* gene.

### 1.5 Phosphate regulation in *E. coli* and *Pseudomonas aeruginosa*

*P. aeruginosa* alkaline phosphatase is expressed when the organism is grown in conditions of phosphate limitation (Day and Ingram, 1973). With this in mind, phosphate regulation in *P. aeruginosa* will be discussed.

Phosphorus is the fifth most important element in bacteria following carbon, hydrogen, oxygen and nitrogen (Spiro, 1973). The proper maintenance of phosphate is of fundamental importance to any living cell. The phosphorus cycle is relatively simple because phosphate is assimilated only in the +5 valence state which is found naturally in phosphate and phosphonates. *P. aeruginosa* can also use phosphonates such as ciliatine (Wanner, 1987). The three principal forms of phosphate are orthophosphate, pyrophosphate and metaphosphate. Metaphosphate occurs only in highly polymeric polyphosphate structures which act as energy reserves in bacteria (Wanner, 1987). Though phosphate is relatively abundant in nature, phosphate is still one of the limiting factor for many bacteria because much of its natural supply occurs as insoluble salts (Wanner, 1987).

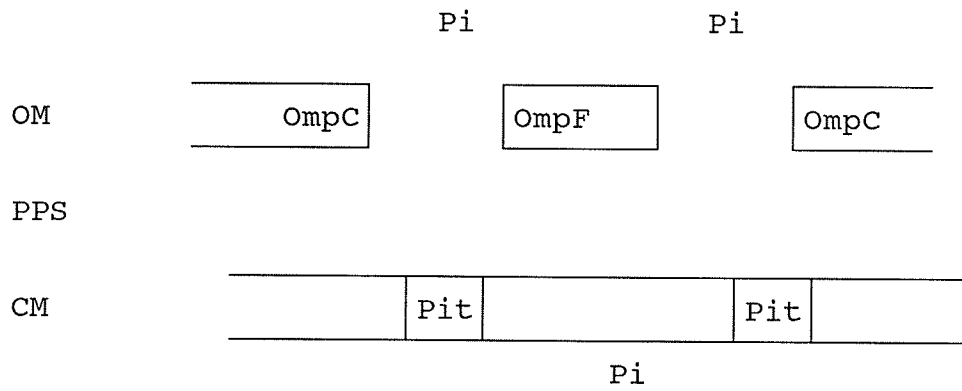
Phosphate plays two key roles in living organisms. First, it acts as a structural component in various cytoplasmic solutes like volutin and polyphosphate granules (Kulavev, 1987), nucleic acids, phospholipids and lipopolysaccharide (Lutenberg, 1987). Second, it acts in the transfer of energy

in the form of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), adenosine monophosphate (AMP), and adenosine diphosphate (ADP). The key to this phosphate requirement is established by the transport systems that brings in either inorganic phosphate or phosphate covalently linked to organic molecules such as glucose or glycerol. To cope with conditions of low phosphate, organisms like *E. coli* have developed an emergency system known as the *pho* regulon. The *pho* regulon regulates the levels of inorganic phosphate concentration by regulating levels of the phosphate scavenging protein alkaline phosphatase.

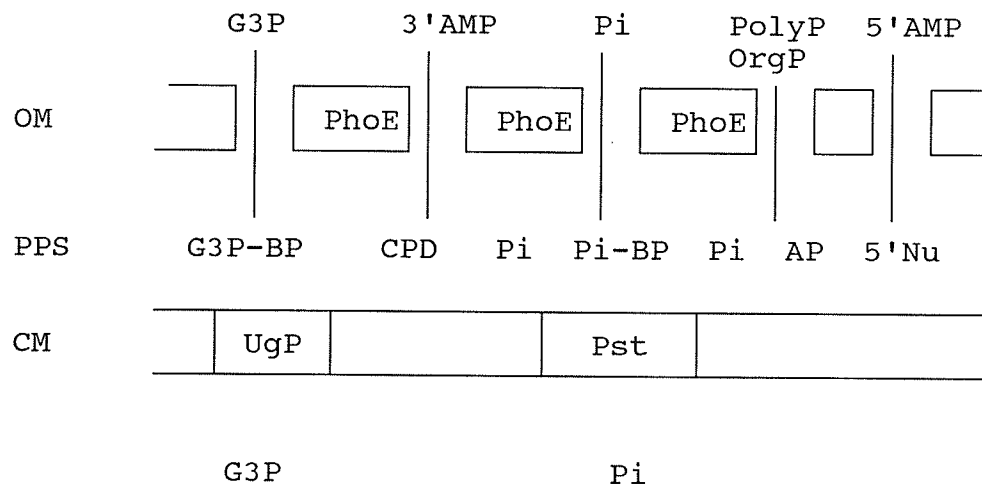
The *pho* regulon in *E. coli* is reasonably well understood. Under conditions of sufficient phosphorus, ( $>25 \mu\text{M}$ ; Rosenberg, 1987) phosphorus diffuses through the commonly produced outer membrane porin proteins (OmpC and OmpF) of *E. coli*. This phosphorus is recognised by the phosphate inorganic transport (Pit) carrier which then transports it through the cytoplasmic membrane as shown in Figure 1. Under conditions of phosphate starvation, ( $<0.16 \mu\text{M}$ ; Rosenberg, 1987), phosphate and other phosphorus containing solutes are recognized by the outer membrane porin protein (PhoE) which is derepressed under these conditions [Figure 1]. Porins are large water-filled proteinaceous pores through the outer membrane which act as molecular sieves with defined exclusion limits for hydrophilic substances (Ingraham et al., 1983). PhoE can facilitate the diffusion of  $\text{P}_i$  and  $\text{P}_i$  containing compounds through the outer

Figure 1. Uptake pathway of various phosphorus sources through the cell envelope of *E. coli* K-12 under conditions of (A) sufficient phosphate and (B) phosphate starvation. Abbreviations: AP, alkaline phosphatase; BP, binding protein; CM, cytoplasmic membrane; CPD, 2' 3'-cyclic phosphodiesterase; G3P, glycerol 3-phosphate; 5'Nu, 5' nucleotidase; OM, outer membrane; OmpC and OmpF, outer membrane pore proteins; OrgP, organic phosphate; PhoE, PhoE porin protein; Pit, low-affinity system for Pi transport; PolyP, linear polyphosphate; PPS, periplasmic space; Pst, phosphate-specific transport system; Ugp, high-affinity uptake system for G3P. (Taken from B. Lutenberg, 1987: The *pho* regulon in *Escherichia coli*).

A.



B.



membrane 6-8X more efficiently than the constitutive porins, OmpC and OmpF. Pi is then bound by the periplasmic Pi-binding protein (PiBP) and subsequently transported through the cytoplasmic membrane by the phosphate-specific transport (Pst) system. Uptake of low concentrations of glycerol 3-phosphate (G<sub>3</sub>P) is mediated by the periplasmic glycerol 3-phosphate binding protein (G<sub>3</sub>PBP) and a high affinity cytoplasmic membrane carrier (Ugp). PhoE also facilitate the passage of linear polyphosphates (PolyP) through the outer membrane. This polyphosphate is then depolymerised by the periplasmically located alkaline phosphatase leading to the release of inorganic phosphate which is then bound by the PiBP. Phosphate is removed from 5'-AMP and 3'-AMP by 5' nucleotidase (5'Nu) and 2',3'-cyclic phosphodiesterase (CpD), respectively before it can pass through the cytoplasmic membrane (Lutenberg, 1987).

When *P. aeruginosa* is grown under Pi-limiting conditions (0.2 mM, Hancock *et al.*, 1987) as with *E. coli*, a number of proteins are expressed. These include an outer membrane protein P (OprP), both periplasmic and excreted forms of alkaline phosphatase (AP), phospholipase C, a heat stable hemolysin (Vasil *et al.*, 1985) and a periplasmic phosphate binding protein (Hancock *et al.*, 1987). Repression by Pi in the medium led Liu and Kurioka, (1967) to suggest that both AP and PLC might function together to liberate Pi from phosphatides under conditions of Pi starvation (Liu and

Kurioka, 1967). The production of PLC by *P. aeruginosa* is regulated by environmental Pi levels, which makes it unique as the production of phospholipase C by other bacterial species such as *C. perfringens*, *C. novyi*, *C. sordelli* are not inhibited by Pi levels in the medium (Liu, 1974). When Pi levels decrease, PLC production increases as is seen when *P. aeruginosa* is grown in minimal media containing ammonium, potassium, and calcium ions, and a carbon source like glucose (Vasil et al., 1981; Stinson et al, 1979). The calcium ion requirement could be substituted by magnesium and strontium but not by copper, manganese, cobalt or zinc ions.  $Ca^{2+}$  or  $Mg^{2+}$  are essential for growth as well as maintaining the structure and function of the cytoplasmic and outer membrane of *P. aeruginosa* (Stinson et al, 1979). PLC excretion was also repressed by the addition of Pi but not by organic phosphate, glucose or sodium succinate. This form of repression of PLC synthesis by end products, like Pi is also found in organisms like *Bacillus subtilis* and *Micrococcus sodenesis*.

PLC is mainly found in the medium while AP is found both intracellularly and extracellularly (Ingram et al., 1982; Campbell et al., 1966). The possibility that the mechanism of excretion of PLC and AP is due to the breakdown in the permeability barrier of the outer membrane of *P. aeruginosa* was investigated by Poole and Hancock, (1983). If the excretion of AP and PLC is due to increased outer membrane permeability, then the periplasmic  $\beta$ -lactamase should be



exposed. This would lead to an increase in the rate of nitrocefin (a chromogenic substrate of  $\beta$ -lactamases) hydrolysis which was not shown to be the case. The periplasmic location of both the  $\beta$ -lactamase and a phosphate binding protein was shown to be maintained during active release of alkaline phosphatase and phospholipase C into the medium (Poole and Hancock, 1983). In this assay, Poole and Hancock, (1983) assessed the outer membrane permeability with nitrocefin. The assay measures the rate of hydrolysis of a  $\beta$ -lactam by intact cells of *P. aeruginosa* PA01 strain H103. Nitrocefin must first penetrate through the outer membrane in order to reach the enzyme,  $\beta$ -lactamase which is located in the periplasmic space underneath the membrane. *P. aeruginosa* cells, when treated with EDTA, an agent that is known to break down the outer membrane, shows a ten-fold increase in nitrocefin hydrolysis. In addition, no major outer membrane proteins or the lipopolysaccharide (LPS) specific sugar 2-keto-3-deoxyoctanate (KDO) in 50X concentrated supernatants were detected by SDS-PAGE. This further supports Poole and Hancock's (1983) theory that the excretion of AP and PLC in *P. aeruginosa* is specific and does not involve increased outer membrane permeability.

PLC, AP and heat stable glycolipid are critical for *P. aeruginosa* to "scavenge" phosphate from phospholipids (Berk et al., 1987; Vasil, 1986) or other organic compounds like tryptose (Stintson et al., 1979). Infection with *P.*

*aeruginosa* results in tissue destruction (phosphatidylcholine surrounding the lung surface) by the phosphate repressible heat stable glycolipid. PLC will then hydrolyze the release phosphatides, liberating phosphorylcholine which in turn is hydrolysed by AP to choline and free phosphate to satisfy the growth requirement of *P. aeruginosa* (Vasil,1982).

The genes responsible for mediating the phosphate limitation in *P. aeruginosa* are part of the phosphate (*pho*) regulon (Filloux *et al.*, 1988). The *E. coli pho* regulon was discovered by Torraini in 1958 (Torraini, 1958) in which alkaline phosphatase was found to be induced when cells were starved for a phosphorus source. The *pho* regulon is a system in which the production of proteins can be controlled by a single repressor substance, in this case, phosphate (Halvorson *et al.*, 1987). The *pho* regulon in *E. coli* consists of at least 25 genes (Shingawa *et al.*, 1987) scattered throughout the chromosome. The products of these genes can be found in all cellular compartments (Lutenberg, 1987). This system includes a number of genes including, *phoA*, *pstS*, *phoE* and *ugpB* which respectively code for the following: alkaline phosphatase; a phosphate binding protein; an outer membrane porin protein; and glycerol 3-phosphate binding protein. As they are all related to transport and assimilation of phosphate, they constitute a single *pho* regulon (Shinagawa *et al.*, 1978).

Figure 2 shows a schematic representation of the phosphate (*pho*) regulon in *E. coli* (Tommassen, 1987). The structural

Figure 2. Scheme of regulation of the phosphate (*pho*) regulon in *Escherichia coli*. The genes belonging to the *pho* regulon are derepressed by Pi limitation. Their expression is positively regulated by *phoB*, *phoM* and *phoR* and is negatively regulated by *phoR*, *phoS*, *pst*, and *phoU*. Abbreviations: *phoE*, outer membrane porin protein E; *phoA*, alkaline phosphatase; *phoS*, phosphate binding protein; *ugpA* and B, glycerol phosphate transport; *pstS*, periplasmic binding protein; *pstC*, periplasmic membrane protein; *pstA* and *pstB*, integral membrane proteins; *phoU* system, cytoplasmic protein. (Taken from Tommasen, 1987 and Stock *et al.*, 1989).

GENES SENSING Pi LEVEL	GENES CONTROLLING <i>phoB</i> EXPRESSION	GENES REQUIRING FUNCTIONAL <i>phoB</i>
<i>phoS</i>	<i>phoR</i> PhoR <sup>A</sup> -phosphorylates PhoB	<i>phoE</i>
	<i>phoM</i> PhoM <sup>A</sup> - <i>phoB</i> P-PhoB	<i>phoA</i>
<i>pst*</i>	<i>phoR</i> PhoR <sup>A</sup> -dephosphorylates PhoB	<i>ugpA, B</i>
*( <i>pstA, pstC, pstA, pstB, phoU</i> )		<i>phoS</i>

genes belonging to the *pho* regulon, as represented by *phoA* (structural gene for alkaline phosphatase), are activated under phosphate-limiting conditions. Their expression is positively regulated by *phoB*, *phoM* and *phoR*. Activated *phoR* protein (PhoR<sup>A</sup>) and *phoM* proteins (PhoM<sup>A</sup>) convert inactivated *phoB* protein (PhoB) to the activated form (PhoB<sup>A</sup>). High levels of PhoB<sup>A</sup>, a DNA binding protein, turn on the expression of genes like *phoE*, *phoA* and *phoS* under phosphate-limiting conditions by binding to a specific DNA sequence encoding these genes, called the Pho Box (Stock *et al.*, 1989). Their expression is negatively regulated by *phoR*, *phoS*, *pst* and *phoU*. *pst* and *phoU* constitute the *pst* operon (in order of transcription: *pstS*, *pstC*, *pstA*, *pstB*, *phoU*). This high affinity *pst* system functions to detect high extracellular phosphate and relay this information to cause the repression of *phoA*, *phoE* and *phoS*. *phoU* is a cytoplasmic protein that is not essential for phosphate uptake but is required for phosphate repression of the *pho* regulon. PhoU promotes the dephosphorylation of P-PhoB, in response to a signal that reflects the activity of the *pst* system (Stock *et al.*, 1989). PhoR, a histidine kinase appears to be a membrane receptor with an extracellular sensory domain and an intracellular signaling domain (Stock *et al.*, 1989). PhoR functions to activate *pho* expression by donating phosphoryl groups to PhoB to form P-PhoB. In high phosphate conditions, *pst* activates a PhoR dependant phosphatase that dephosphorylates P-PhoB

(Stock *et al.*, 1989). In the absence of PhoR, the *pho M* gene product (PhoM<sup>A</sup>) can activate the expression of *phoB*, independently of the phosphate levels in the environment (Tommassen, 1987). *phoM* is not part of the *pho* regulon and is not regulated by the *pst* genes (Stock *et al.*, 1989). The *pho* regulon in *E. coli* is thus an elaborate control system encompassing both positive (*phoB*, *phoR*, *phoM*) and negative (*phoR*, *phoU*) regulatory elements which respond to phosphate limitation (Beckwith *et al.*, 1981). This type of regulon is not restricted only to bacteria. In the yeast, *Saccharomyces cerevisiae*, there are 5 principal enzymes involved in the acquisition and metabolic integration of inorganic phosphate through a cyclic pathway of polyphosphate synthesis and degradation. Under conditions of phosphate starvation, genetic control of the synthesis of the enzymes (an extracellular acid phosphatase, phosphate permease, polyphosphate kinase, alkaline phosphatase and vacuolated polyphosphatase) involves positive (*PHO4*) and negative (*PHO80*) gene product effectors (Halvorson, 1987).

In *P. aeruginosa*, the phosphate starvation induced system requires the *phoB* gene for expression of OprP (equivalent to the PhoE protein of *E. coli*), the phosphate specific porin protein, which is a component of the high affinity uptake system, the alkaline phosphatase, the phospholipase C and a periplasmic phosphate binding protein. The affinity for phosphate under low phosphate conditions by OprP is at least

60-100X greater than for other anions thus demonstrating OprP substrate specificity for phosphate. In addition, OprP forms only small channels in the outer membrane which prevent enhanced susceptibility to anionic antibiotics like carbenicillin which are too large to pass through OprP channels (Hancock *et al.*, 1982).

Filloux *et al.* (1988) cloned two genes (*phoB*-like and *phoR*-like) of *P. aeruginosa* which can complement *phoB* and *phoR* mutation in *E. coli* KS-12. *E. coli* transformants carrying these genes (*phoB*-like and *phoR*-like of *P. aeruginosa*) correctly expressed *E. coli* AP. In addition, Lazundski *et al.*, (1990) had sequenced *P. aeruginosa phoB* gene and found that the translated sequence was highly homologous to the PhoB proteins of *E. coli* and *B. subtilis*. A close homology between the *phoB* protein of *P. aeruginosa*, *E. coli* and *B. subtilis* was found throughout the sequence, with the 125 amino terminal amino acids being particularly conserved. The homology was greater between *P. aeruginosa* and *E. coli phoB* proteins (59% identical and 70% similar amino acids) than between *P. aeruginosa* and *B. subtilis phoB* proteins (40% identical and 59% similar amino acids). Also, the structural gene for *P. aeruginosa* alkaline phosphatase (*phoA*) was cloned in *E. coli* and was shown to be normally regulated in response to the phosphate concentrations in the growth medium with the exception that the alkaline phosphatase protein was not excreted but remained within the periplasm suggesting that

other factors may be required for excretion to the extracellular medium (Lazundski et al., 1989). The cloned *P. aeruginosa* gene *phoA* insert was not found to harbor any regulatory genes from *P. aeruginosa*. Thus, the *phoA* promoter must be regulated by *E. coli* regulatory factors like *phoB* and *phoR* (Lutenberg, 1987). Conversely, cloned *phoB* and *phoR* regulatory gene products from the genomic DNA of *P. aeruginosa* have been shown to correctly regulate the expression of the *E. coli phoA* gene (Filloux et al., 1988). The presence of analogous phosphate regulation in *P. aeruginosa* and *E. coli* is quite significant as phylogenically, these 2 gram negative organisms belong to two relatively distant species (Lazundski et al., 1989). In addition, the presence of a well conserved *E. coli pho* box in the regulatory protein of *phoB* in *P. aeruginosa* emphasizes the close relationship in phosphate regulation among divergent organisms (Lazdunski et al., 1990).

#### 1.6 Other Pseudomonads.

The final component of this study involves the screening of various Pseudomonads for the presence of alkaline phosphatase. The Pseudomonads used in our experiments are sub-divided into 5 groups based on the level of rRNA homology as measured by the competition technique in rRNA/DNA hybridization experiments (Table 2) (Palleroni, 1972).

Group I includes both fluorescent (fluoresce in UV light at wavelengths below 260 nm) and non-fluorescent species. *P.*



Table 2. Classification of *Pseudomonas* species into ribosomal RNA groups.

rRNA homology group	Species
I	<i>Pseudomonas aeruginosa</i>
	<i>Pseudomonas putida</i>
	<i>Pseudomonas fluorescens</i>
	<i>Pseudomonas syringae</i>
	<i>Pseudomonas stutzeri</i>
	<i>Pseudomonas alcaligenes</i>
II	<i>Pseudomonas cepacia</i>
III	<i>Pseudomonas acidovorans</i>
	<i>Pseudomonas testosteroni</i>
IV	<i>Rhizobium leguminosarum</i>
	<i>Gluconobacter oxydans</i>
	<i>Agrobacterium radiobacter</i>
	<i>Agrobacterium tumefaciens</i>
V	<i>Pseudomonas maltophilia</i>
Miscellaneous	<i>Pseudomonas oxalaticus</i>
	<i>Pseudomonas rubescens</i>

*aeruginosa*, *P. fluorescens*, *P. putida* are the fluorescent species, including the sub-group, *P. syringae* which is also a plant pathogen. Members of this group can be isolated from soils and plants. *P. alcaligenes* is also commonly isolated from swimming pool water. *P. fluorescens* is commonly associated with spoilage of food (eggs, cured meats, fish and milk; Palleroni, 1972) as well as from clinical specimens from respiratory tract, urinary tract, wounds and contaminated blood bank blood. *P. fluorescens* can grow at refrigerator temperature (Finegold et al., 1987).

Group II is exclusively composed of the pathogenic species *P. cepacia*. *P. cepacia* is a plant pathogen (rot in onions), found naturally in soil (Palleroni, 1972) and has been isolated from clinical specimens from urinary tract, and respiratory tract especially in cystic fibrosis patients (LiPuma et al., 1990). This organism has also been isolated from contaminating detergent solutions in urinary catheter kits and hospital water supplies (Finegold et al., 1987).

Group III includes all the hydrogen Pseudomonads such as *P. flava* and *P. palleroni* that have the facultative autotrophic capacity to utilise hydrogen as an energy source. This group also includes non-hydrogen oxidisers, such as *P. acidovorans* and *P. testosteroni*, that cannot grow autotrophically with hydrogen and can be isolated from soils (Palleroni, 1972).

Group IV includes species sharply different from most

*Pseudomonas* species including *Rhizobium leguminosarum*, *Gluconobacter oxydans*, *Agrobacterium radiobacter* and *Agrobacterium tumefaciens*. Both *Agrobacterium* are plant pathogens although only *Agrobacterium tumefaciens* can cause galls in plants (tomato). *Agrobacterium radiobacter* has also been isolated from clinical specimens (respiratory tract and blood culture, Finegold et al., 1987). *Rhizobium leguminosarum* forms nodules on species of *Pisum* (pea), *Lens* (lentils) and *Vicia* (vetch). *Gluconobacter oxydans* forms viscous growth in beer and worts which cause ropiness in beer (Palleroni, 1972).

Group V includes *P. maltophilia* which has been isolated from milk, frozen food, water (Palleroni, 1972) as well as clinical specimens from the respiratory and urinary tract (Finegold et al., 1987).

*P. rubescens* has been assigned to this Family but possesses characteristics that are not in accord with the generic description of *Pseudomonas* whereas *P. oxalaticus* is incompletely described (Palleroni, 1972).

**MATERIALS AND METHODS**

## Chapter 2

### MATERIALS AND METHODS

#### 2.1 BACTERIA.

*Pseudomonas aeruginosa* PA01 strain H103 was obtained from R.E.W. Hancock (University of British Columbia, Vancouver, Canada). This strain was used exclusively for the production of alkaline phosphatases.

*Pseudomonas syringae* ATCC 11043, *Pseudomonas fluorescens* PIC, *Pseudomonas putida* ATCC 12633, *Pseudomonas cepacia* NCTC 10661, *Pseudomonas alcaligenes* ATCC 14909, *Pseudomonas acidovorans* NCTC 10683, *Pseudomonas maltophilia* NRC 5005, *Pseudomonas oxalaticus* ATCC 11883, *Pseudomonas rubescens* ATCC 12099, *Pseudomonas stutzeri* NCTC 10475, *Pseudomonas testosteroni* NCTC 10698, *Gluconobacter oxydans* ATCC 621-1, *Rhizobium leguminosarum* ATCC 876, *Agrobacterium radiobacter* ATCC 6467 and *Agrobacterium tumefaciens* ATCC 4452 were obtained from the bacteria culture collection, Microbiology Department, University of Manitoba, Winnipeg. These members of the Family Pseudomonadaceae were used for screening with antibodies for the expression of L-AP and H-AP under phosphate sufficient and phosphate deficient conditions.

#### 2.2 Bacterial Culture Media.

*Pseudomonas aeruginosa* H103 was maintained on 1% (w/v) Protease Peptone No. 2 (Difco Laboratories, Detroit,

Michigan). Phosphate limiting conditions were obtained by growth of this strain in Tris magnesium minimal media (TMM; Day and Ingram, 1973). Bacto-Agar (Difco Labs.) was used at 2% (w/v) to solidify agar plates.

LB broth was used for growing *P. aeruginosa* H103 in experiments requiring a phosphate sufficient media.

Nutrient broth (0.8% w/v) was used to grow *P. syringae*, *P. fluorescens*, *P. putida*, *P. alcaligenes*, *P. acidovorans*, *P. maltophilia*, *P. oxalaticus*, *P. rubescens*, *P. stutzeri*, *P. testosteroni*, *Gluconobacter oxydans*. For *Rhizobium leguminosarum*, *Agrobacterium radiobacter* and *Agrobacterium tumefaciens*, a different formula for the nutrient broth (described in Appendix) was used. For *P. cepacia*, the media used, 0.8% (w/v) nutrient broth and TMM broth were modified by the addition of 22 mM glucose as the carbon source (Cuskey et al., 1985))

### 2.3 Alkaline phosphatase purification.

Alkaline phosphatase was purified from the cell culture supernatants of *P. aeruginosa* H103 grown in TMM using a modification of a previously described protocol for the purification of phospholipase C (Stintson and Hayden, 1979). In short, bacteria from a 12 litre culture grown in TMM for 24 hours, at 30°C, were pelleted by centrifugation at 9000 x g for 20 minutes. Proteins (.029 mg/ml) in the resulting supernatant were precipitated with 70% (w/v) ammonium sulfate

for 16 hours, at 4°C. After centrifugation at 9000 x g for 20 minutes, at 4°C, the precipitate was resuspended in 10mM Tris-HCl, pH 7.2 and dialysed for 16 hours, at 4°C in the same buffer. The dialysate (approximately 300 ml) was concentrated into 50 ml using an Amicon macroconcentrator (Amicon, Danvers, MA) with a 62 mm PM30 Diaflo ultrafiltration membrane (Amicon). The concentrated dialysate was again centrifuged at 7000 x g, at 4°C for 20 minutes to remove unwanted precipitated pigments. The concentrated dialysate was then loaded onto a DEAE Sephacel ion exchange column (24 x 240 mm, Bio-rad Laboratories, Missisagua, Ont.) previously equilibrated with 10 mM Tris-HCl, pH 7.2. After washing with the equilibration buffer to remove unbound proteins, the bound proteins were eluted with a linear NaCl gradient (0.1 - 1.0 M NaCl in 10 mM Tris-HCl, pH 7.2). Fractions (3.0 mls) with alkaline phosphatase activity (hydrolysis of *p*-nitrophenylphosphate, see Appendix) were pooled and dialysed for 16 hours at 4°C against 10 mM Tris-HCl, pH 7.2. The dialysate was applied to a Sephadex G-75 gel filtration column (20 x 800 mm; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. Fractions (3.0 mls) with alkaline phosphatase activity were pooled, concentrated 50 fold using an Amicon Ultrafiltration apparatus (PM-30 filter, Amico Div., Danvers, Mass.) and analysed by one dimensional sodium dodecyl sulphate polycrylamide gel electrophoresis (see Figure 6).

#### 2.4 Alkaline phosphatase activities and pH optima.

Column fractions were assayed for the presence of alkaline phosphatase by mixing in microtitre wells (Nunc Intermed, Roskilde, Denmark), 50  $\mu$ l aliquots with 50  $\mu$ l of a 2 mg/ml *p*-nitrophenylphosphate solution (Sigma Chemical Co.) in 0.1 M Tris-HCl, pH 8.5. Relative alkaline phosphatase activities were determined by measuring the  $A_{410}$  using an Easy Reader EAR 400AT microplate reader (SLT Laboratories, Salzburg, Austria). Quantitative alkaline phosphatase activities for the purified enzymes were measured spectrophotometrically by following the rate of hydrolysis of *p*-nitrophenylphosphate (1mg/ml in 0.1 M Tris-HCl, pH 8.5) at 410 nm (Milton Roy Spectronic 601 uv-vis spectrophotometer, Rochester, N.Y.). Activity is expressed as  $\mu$ moles substrate hydrolysed/min/mg protein. The pH optimum was determined using three buffer systems: 0.1 M Tris, 0.1 M CHES (3-[cyclohexylamino] -1-propanesulphonic acid, Sigma Chemical Co.) and 0.1 M CAPS (2-[cyclohexylaminoethane] sulphonic acid, Sigma Chemical Co.) at pH range of 7.5 - 11.0. Phosphodiesterase activity was also determined spectrophotometrically at  $A_{410}$  by following the rate hydrolysis of bis-*p*-nitrophenylphosphate (Sigma Chemical Co., 1 mg/ml in 0.1 M Tris-HCl) over the pH range of 7.5 - 11.0. In all cases, protein concentration was determined at  $A_{280}$  using the following Warburg - Christian equation :

$$\text{Protein concentration (mg/ml)} = 1.55 A_{280} - 0.76 A_{260}$$

(Warburg and Christian, 1942).



### 2.5 Inorganic phosphorus determination.

Inorganic phosphorus content of *Pseudomonas aeruginosa* H103 culture supernatants was determined according to the procedure of Fiske and Subbarow, 1925. All glasswares were washed in chromic acid and rinsed in deionised water prior to use. Culture samples were removed at various times in the growth curve and the cells were pelleted by centrifugation at 7000 x g for 20 minutes at 4°C. 500 µl aliquots of the resultant supernatants were spectrophotometrically assayed at 660 nm for inorganic phosphorus content as described by Fiske and Subbarow, (1925) using acid molybdate and Fiske-Subbarow reducer solutions (Sigma Chemical Co.). Phosphorus concentrations were determined from a calibration curve obtained from a series of phosphorus standards (Sigma Chemical Co.).

### 2.6 SDS-PAGE and immunoblot procedures.

Electrophoresis was carried out using the discontinuous buffer system of Laemmli (1970) in a Model V16 Vertical gel electrophoresis apparatus (BRL, Gaithesburg, MD.). The separation gel was 1 mm thick and consisted of a 12% running gel and a 4% stacking gel. Prestained high molecular weight protein markers (BRL) and samples were boiled in a heating block (Lab. Line Instruments, Melrose Park, ILL.) for 10 minutes before loading. The amount of protein applied to the gels ranged from 20 to 100 µg. The gels were run at 50 volts

for 30 minutes followed by 3 hours at 150 volts until the tracking dye was about 1 cm from the bottom of the gel. The standard protein markers include: bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000),  $\beta$ -lactoglobulin (18,400) and lysozyme (14,300). All molecular weights are in daltons (See Appendix for further details).

Immunoblot procedures were performed as in the Appendix. Rabbit antibodies used in these studies were produced using as immunogens the two different isolates of *P. aeruginosa* alkaline phosphatase (L-AP and H-AP) and commercially prepared *E. coli* alkaline phosphatase isozyme III (Sigma Chemical Co.) following a procedure described in the Appendix. Antisera raised against the *P. aeruginosa* enzymes were adsorbed with *P. aeruginosa* H103 grown in Protease Peptone No. 2 to remove any antibodies to other possible immunogens such as lipopolysaccharide.

### **2.7 Osmotic Shock Procedure.**

This is a procedure specific for *P. aeruginosa* designed by Hoshino and Kageyama, (1980). Cells were grown overnight in appropriate media (eg. TMM), centrifuged at 4°C, at 9000 x g, for 10 minutes. The cell pellet was then resuspended in 2 ml of 0.05 M Tris-HCl pH 7.3 in 0.2 M magnesium chloride solution. Samples were incubated at 30°C for 10 minutes, followed by chilling in a ice water bath, with agitation for 15 minutes. This hot/cold treatment was repeated one more

time. Samples were then centrifuged at 9000 x g, at 4°C for 10 minutes. The supernatant (6 ml) containing the periplasmic contents was concentrated using a Microsep centrifugal microconcentrator (Filtron Technology Corporation, Northborough, MA) with a 30,000 MW cutoff filter. The periplasmic fluid (1 ml) was then dialysed overnight at 4°C against 10 mM Tris-HCl pH 7.2 with 2 changes of dialysing buffer. The dialysed samples were then further microconcentrated to approximately 500  $\mu$ l.

### **2.8 Time course of alkaline phosphatase production.**

To determine when alkaline phosphatase is expressed after the inoculation of *P. aeruginosa* H103 into phosphate limiting conditions, the organism was grown in TMM, at 30°C up to 34 hours. Samples were taken at various times during this incubation and assayed for alkaline phosphatase activity, inorganic phosphate content, total protein, and culture density ( $A_{620}$ ) using procedures described previously in Sections 2.4 and 2.5.

To determine at which point in the growth curve the two alkaline phosphatase species were expressed, *P. aeruginosa* H103 was grown in phosphate rich media and harvested by centrifugation at mid log phase ( $A_{620} = 0.6$ ). The cell pellets were washed in TMM, resuspended in several 500 ml flasks containing the same media at an  $A_{620}$  of 0.2, and incubated at 30°C. At various times, a 500 ml culture was removed from the

incubator, and cells harvested by centrifugation (10,000 x g, 10 min, at 4°C). Extracellular proteins in the resultant supernatants were precipitated with ammonium sulphate as described in Section 2.3, concentrated 20 fold, and dialysed against 10 mM Tris-HCl, pH 7.2. Cell pellets were subjected to the cold shock procedure of Hoshino and Kageyama, (1980) to release periplasmic proteins (Section 2.7). Periplasmic and extracellular proteins were examined for the presence of either type of alkaline phosphatase by SDS-PAGE and immunoblot procedures using the appropriate antibodies.

#### **2.9 Amino terminal amino acid sequence determination.**

Alkaline phosphatase protein samples were subjected to SDS-PAGE and electroblotted onto PVDF Immobilon-P membranes (Millipore Corp., Bedford, Mass.). Protein bands were visualised by staining for 5 min with 0.2% (w/v) Ponceau S (Sigma Chemical Co.) in 3% (w/v) Trichloroacetic acid. Membranes were destained with 5% (v/v) acetic acid and rinsed in distilled water prior to use. This is described more fully in the Appendix. Automated amino-terminal sequencing was performed by S. Kielland (University of Victoria, Victoria, B.C.).

#### **2.10 Screening for L-AP and H-AP expression in members of the Family Pseudomonadaceae.**

*P. syringae*, *P. fluorescens*, *P. putida*, *P. cepacia*, *P.*

*alcaligenes*, *P. acidovorans*, *P. maltophilia*, *P. oxalaticus*, *P. rubescens*, *P. stutzeri*, *P. testosteroni*, *G. oxydans*, *R. leguminosarum*, *A. radiobacter* and *A. tumefaciens* were incubated overnight, at 28°C, in 8 ml of nutrient broth for phosphate sufficient and TMM for phosphate deficient conditions. For *P. cepacia*, both phosphate sufficient and phosphate deficient media were supplemented with 20 mM glucose. The next day, these cultures were subcultured into 200 ml of the respective media (nutrient broth or TMM) and incubated at 28°C overnight. Cells were harvested at 9000 x g at 4°C for 20 minutes. Excreted proteins (concentration varies for each species) in the supernatant were precipitated with 70% (w/v) saturation ammonium sulphate. The precipitated proteins were then spun at 10,000 xg at 2°C for 20 minutes, then resuspended in 3 ml of 10 mM Tris-HCl pH 7.2. The samples were dialysed overnight at 4°C against 10 mM Tris-HCl pH 7.2 with 2 changes before concentrating six fold in a microconcentrator (Filtron Technology) with a 30,000 MW cutoff filter. The pelleted cells were washed once with 10 mM Tris-HCl, pH 7.2 to remove traces of supernatant proteins. The washed cells were then subjected to Hoshino and Kageyama (1980) osmotic shock procedure. The periplasmic shock fluids were then dialysed and microconcentrated to approximately 500 µl. Both osmotic shock fluid and supernatant samples were: 1) electrophoresed, Western blotted onto nitrocellulose transfer membrane (Micron separations Inc., Westboro, MA) and probed

with antibody to L-AP and H-AP, and 2) assayed for the presence of alkaline phosphatase activity.

### **2.11 Screening for antibodies to L-AP and H-AP in cystic fibrosis sera.**

This is a pilot study performed in collaboration with Dr. V. Chernick (Dept. of Pediatrics, U. of Manitoba). 0.5-1.0 ml of whole blood were taken intravenously from 2 cystic fibrosis patients known to be colonized with *P. aeruginosa* and 1 cystic fibrosis patient who was not colonized with this organism. The samples were spun at 3000 x g and the sera removed. Purified enzymes (L-AP and H-AP) were electrophoresed on SDS-polyacrylamide gels, Western blotted and incubated with a 1:100 dilution of each patient's serum, followed by a second antibody specific for human IgG antibodies conjugated to peroxidase (Sigma Chemical Co.). Immunoenzymatic detection of proteins were carried out as described in the Appendix.

**RESULTS**

## Chapter 3

### RESULTS

#### 3.1 Time course of alkaline phosphatase expression.

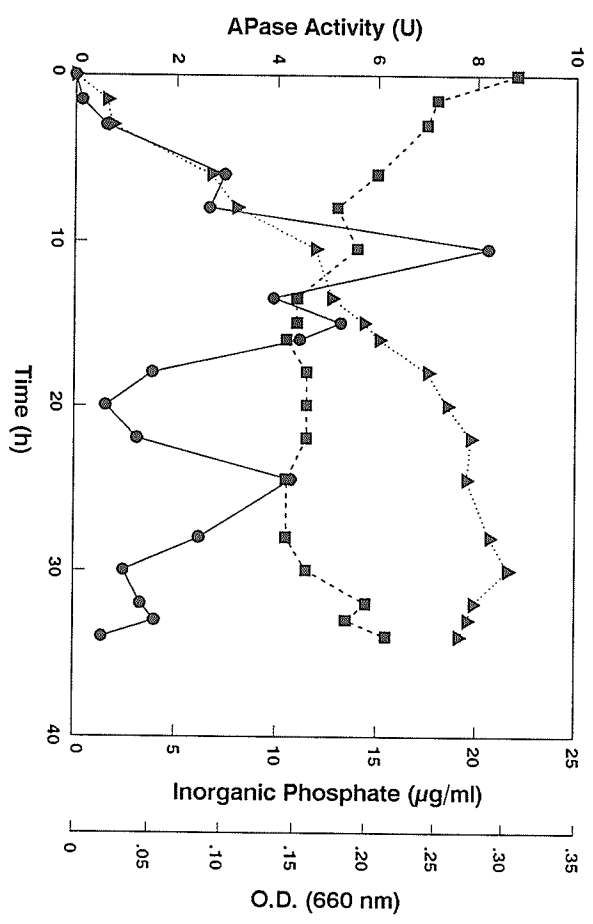
An experiment was done to determine the time of excretion of functional alkaline phosphatase by *Pseudomonas aeruginosa* H103 grown in phosphate-limiting conditions. A time course for culture density, free inorganic phosphate levels and extracellular alkaline phosphatase activity was performed using a 12 litre batch culture. The results are presented in Figure 3. Alkaline phosphatase activity increased steadily and reached a maximum at approximately mid-log phase, after 10.5 hours of incubation. This increase corresponds to a steady decrease in free inorganic phosphate levels in the medium, from 22  $\mu\text{g/ml}$  at time zero to 10.5  $\mu\text{g/ml}$ , after 16 hours of incubation. Alkaline phosphatase activity decreased once inorganic phosphate levels stabilised (late log phase), after 30 hours of incubation. A second peak of alkaline phosphatase activity, followed by an increase in inorganic phosphate levels was detected in stationary phase. The increase in inorganic phosphate is due to AP activity and may also reflect the release of intracellular phosphate into the medium due to cell lysis.

#### 3.2 Alkaline phosphatase purification.

The excreted proteins in the supernatant from a 12 litre batch culture of *Pseudomonas aeruginosa* H103 grown in phosphate



Figure 3. Relationship between free inorganic phosphorus levels, growth and alkaline phosphatase production of *Pseudomonas aeruginosa* H103 in phosphate limited media (TMM). Symbols: ■, free inorganic phosphorus levels as determined by Fiske and Subbarow method; ▲, cell density as determined at  $A_{620}$ ; ○, alkaline phosphatase activity, determined by hydrolysis of *p*-nitrophenylphosphate.



limited media (TMM) (16 hours) were precipitated with a 70% (w/v) saturation solution of ammonium sulphate, resuspended in 10 mM Tris-HCl, pH 7.2, and dialysed. The proteins were concentrated 50 fold, loaded into a DEAE Sephacel ion exchange column and eluted with a linear gradient of 0.1-1 M sodium chloride in 10 mM Tris HCl at pH 7.2. Column fractions were tested for alkaline phosphatase activity ( $\mu$ moles substrate hydrolysed/mg protein/min). Figure 4 shows that the majority of the proteinaceous material exhibiting alkaline phosphatase activity bound to the column and was eluted by the sodium chloride gradient. The alkaline phosphatase-positive fractions were then pooled and further separated on a Sephadex G-75 gel filtration column. The elution profile of the Sephadex column of protein concentration and alkaline phosphatase activity is shown in Figure 5. Two peaks of alkaline phosphatase activity are evident in Figure 5. Fractions corresponding to these peaks were pooled, concentrated separately and analysed by SDS-PAGE. Coomassie Brilliant Blue stained gels revealed that these two pools of alkaline phosphatase activity are actually two enzyme species having different monomeric molecular weights (Figure 6). The monomeric molecular weights of these two alkaline phosphatases, as determined by SDS-PAGE are shown in Figure 7. The first alkaline phosphatase peak has a molecular weight of 51,000 daltons while the second alkaline phosphatase peak was shown to be 39,500 daltons, by comparing with a series of protein

Figure 4. Elution profile of *Pseudomonas aeruginosa* H103 alkaline phosphatase activity from a DEAE Sephacel ion exchange column. Symbols: o, protein concentration at  $A_{280}$ ;  $\Delta$ , APase activity.

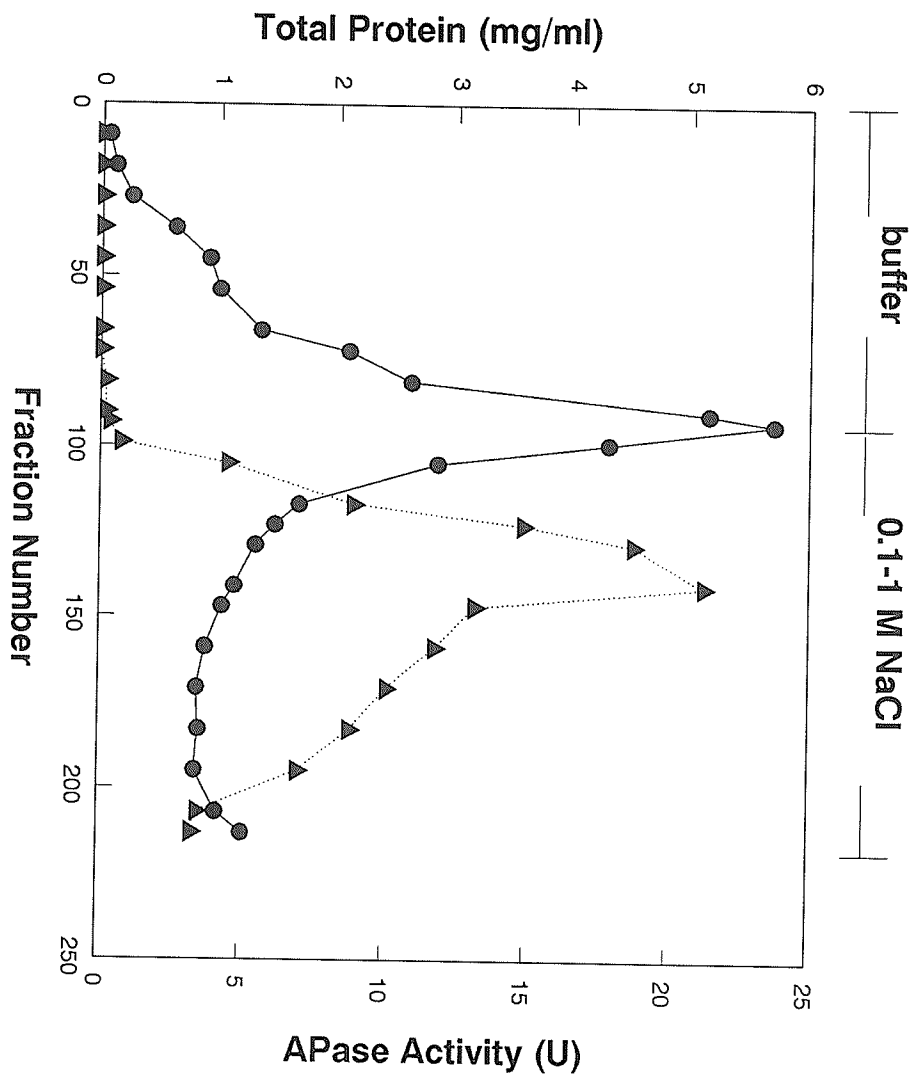


Figure 5. Elution profile of *Pseudomonas aeruginosa* H103 alkaline phosphatase from a Sephadex G-75 gel filtration column. Symbols: o, protein concentration at  $A_{280}$ ; ▲, alkaline phosphatase activity.

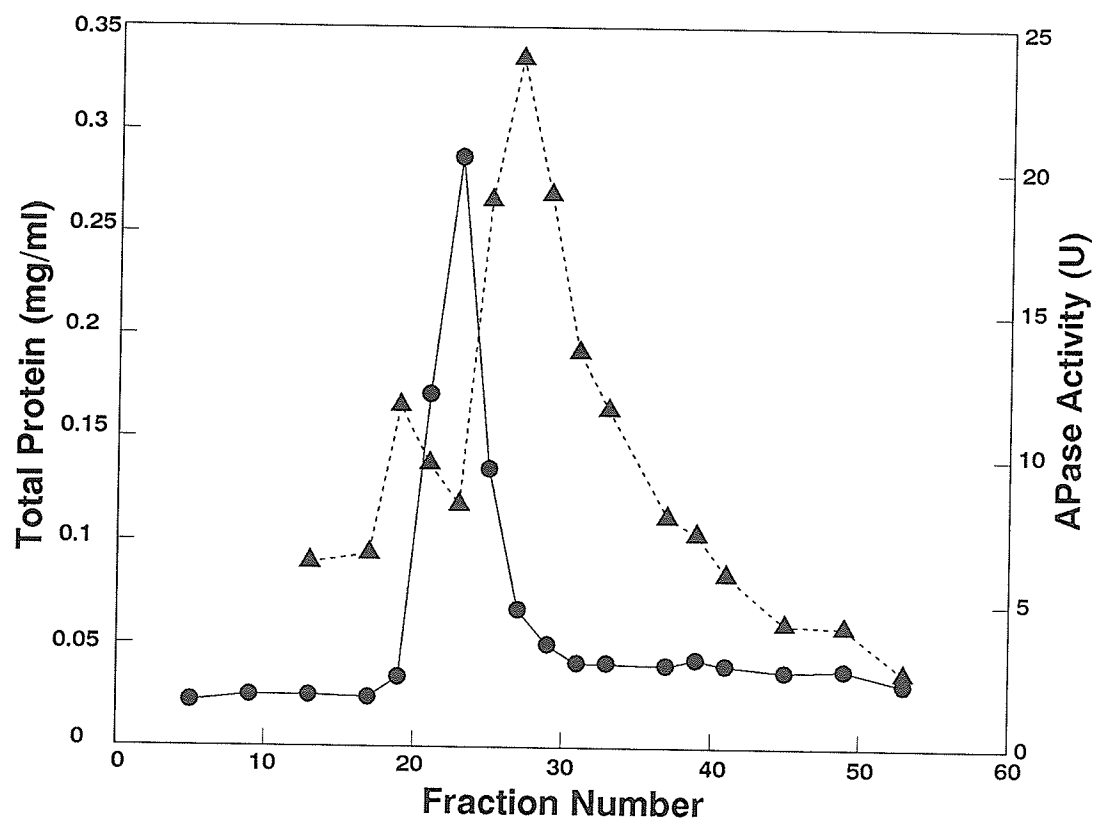


Figure 6. Coomassie Brilliant blue stained SDS-polycrylamide gel of L-AP and H-AP fractions that were eluted from a Sephadex G-75 column. Lane 1, L-AP (second APase peak from Figure 6); Lane 2, H-AP (first APase peak from Figure 6); Lane 3, *E. coli* AP isozyme III (Sigma Chemical Co.). Molecular weights are indicated as  $\times 10^3$  daltons.



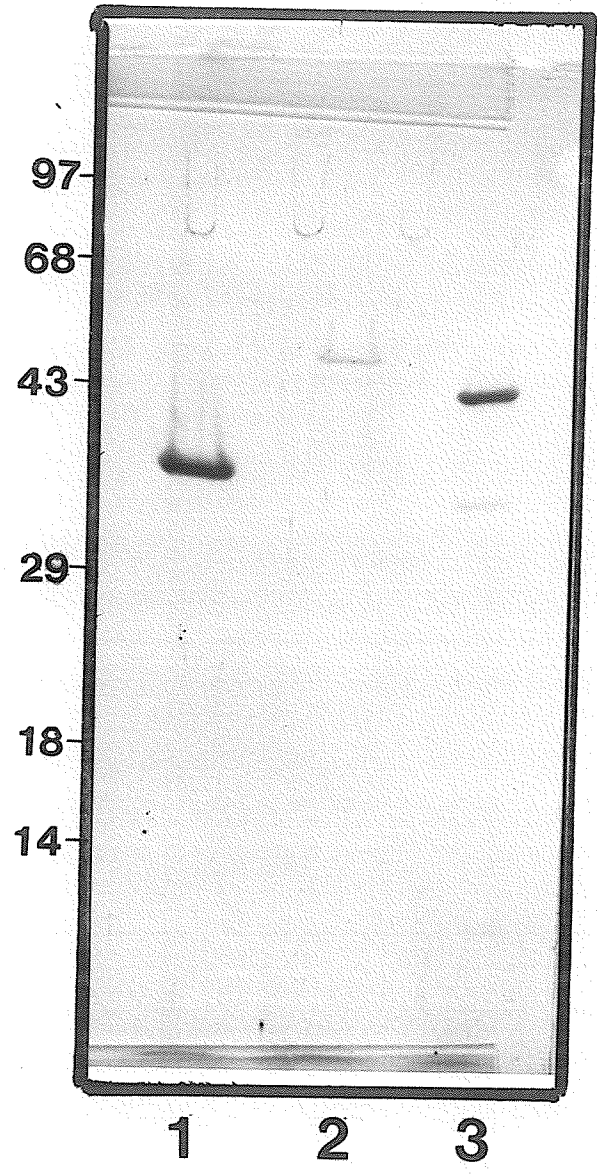
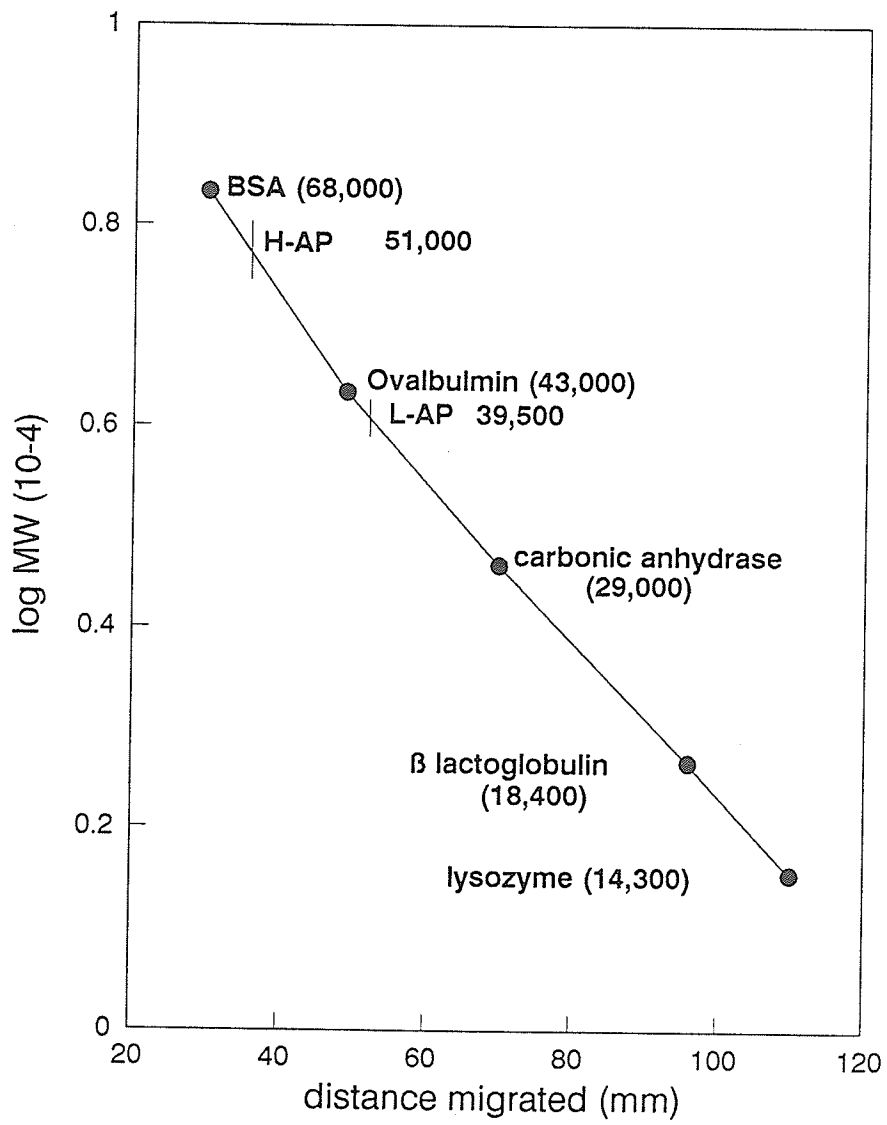


Figure 7. The molecular weight of *Pseudomonas aeruginosa* H103 alkaline phosphatases L-AP and H-AP, as determined by their mobility on Coomassie Brilliant Blue stained SDS-polyacrylamide gels.

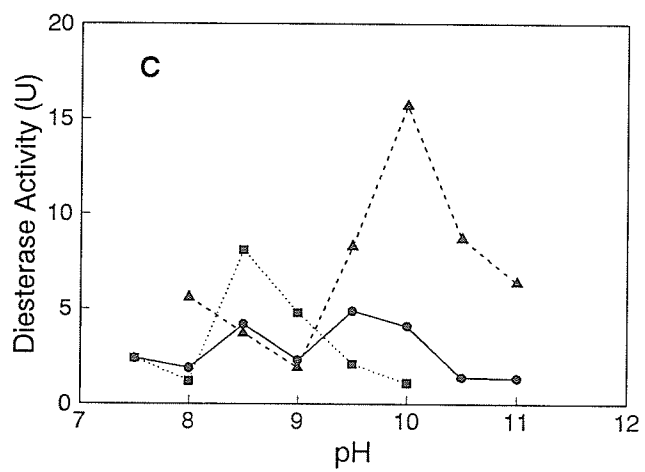
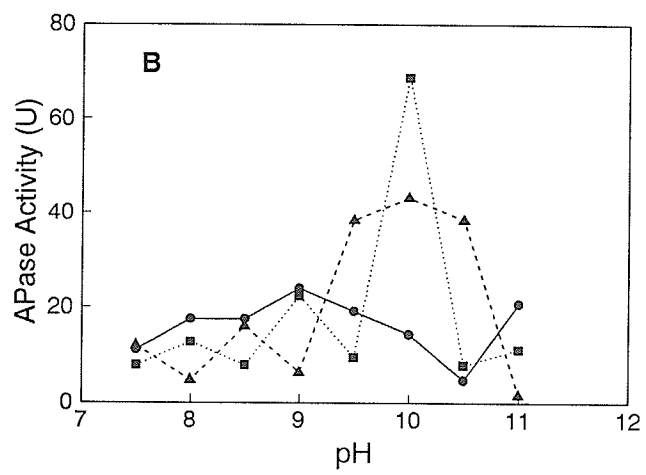
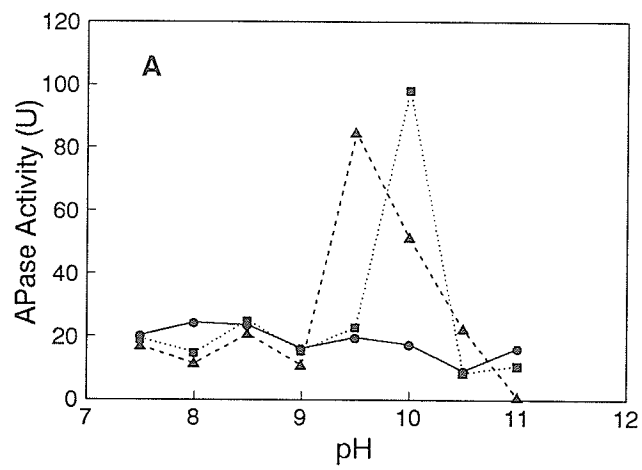


molecular weight standards. These two enzymes were named H-AP and L-AP, for the 51,000 and 39,500 daltons proteins respectively.

Note that these 2 enzymes are produced in very small quantities, as reflected by protein concentrations in Figure 4 and 5.

**3.3 Substrate specificity and pH optima of alkaline phosphatase.** To verify that the two purified enzymes, H-AP and L-AP, were both alkaline phosphatases, the rates of their respective phosphomonoesterase activities were measured using *p*-nitrophenylphosphate as the substrate in three different buffer systems, Tris, CHES, and CAPS over a pH range of 7.5-11.0. The effective pH range for Tris (pH 7.0-9.5), CHES (pH 8.6-10.0) and CAPS (pH 9.7-11.1) covers the range of pH optima for human alkaline phosphatase activities (liver, placental and bone; Stintson, 1989). The results are summarized in Figure 8. The highest activity for both alkaline phosphatases was observed in CHES buffer at pH 10.0 (98.3 U/ml for L-AP and 68.8 U/ml for H-AP). Significant differences between the fractions were also found in CAPS buffer with the pH optimum for L-AP at 9.5 (84.7 U/ml) while that of H-AP is at pH 10 (43.2 U/ml). There is also a relative reduction of 48% of phosphomonoesterase activity between CHES and CAPS buffer for L-AP and 37% for H-AP at pH 10. With Tris buffer at pH 10, a reduction of 82% and 66% against CHES and CAPS buffer

Figure 8. pH optima for the alkaline phosphatase activities of L-AP and H-AP. Panel A, phosphomonoesterase activity of L-AP; Panel B, phosphomonoesterase activity of H-AP; Panel C, phosphodiesterase activity of L-AP. Symbols: ■, CHES buffer; ▲, CAPS buffer, o, Tris buffer. Phosphomonoesterase activity was determined by the hydrolysis of *p*-nitrophenylphosphate and phosphodiesterase activity was determined by the hydrolysis of bis-*p*-nitrophenylphosphate.



respectively was found in L-AP and 79% and 60% for H-AP, showing that phosphotransferase activity in Tris is low for both L-AP and H-AP. The pH optimum in Tris buffer is 8 for L-AP and 9 for H-AP which indicates the functional differences between these two enzymes. These results also show that, overall, L-AP has significantly higher phosphomonoesterase activity than H-AP. Also, L-AP produces a low phosphodiesterase activity as determined by the rate of hydrolysis of bis-p-nitrophenylphosphate whereas no phosphodiesterase activity was detected for H-AP.

#### **3.4 Western immunoblot analyses.**

Polyclonal antisera were raised in white New Zealand rabbits using purified H-AP, L-AP, and a commercial preparation of *E. coli* alkaline phosphatase (AP) isozyme III as the immunogens. The polyclonal antibodies were then used as the primary antibody to probe Western immunoblots of these enzymes. The results are presented in Figure 9. H-AP antibodies cross reacted with the *E. coli* enzyme but not with L-AP (Panel B). *E. coli* AP-specific antibodies do not cross react with L-AP or H-AP (Panel C). Antibodies against L-AP did not cross react with H-AP and only reacted with *E. coli* alkaline phosphatase (Panel A) if an excess of this antibody was used (1:25 dilution) as shown in Figure 10.

Figure 9. Western immunoblot analysis of *Pseudomonas aeruginosa* L-AP, H-AP, and *Escherichia coli* AP. Panels A, B, and C represent identical immunoblots probed with *P. aeruginosa* L-AP, H-AP and *E. coli* AP polyclonal sera, respectively. All sera were diluted 100 fold. Lane 1, L-AP; lane 2, H-AP; lane 3, *E. coli* AP. Samples were electrophoresed and probed as described in Materials and Methods. Molecular weights are as indicated  $\times 10^3$  daltons.



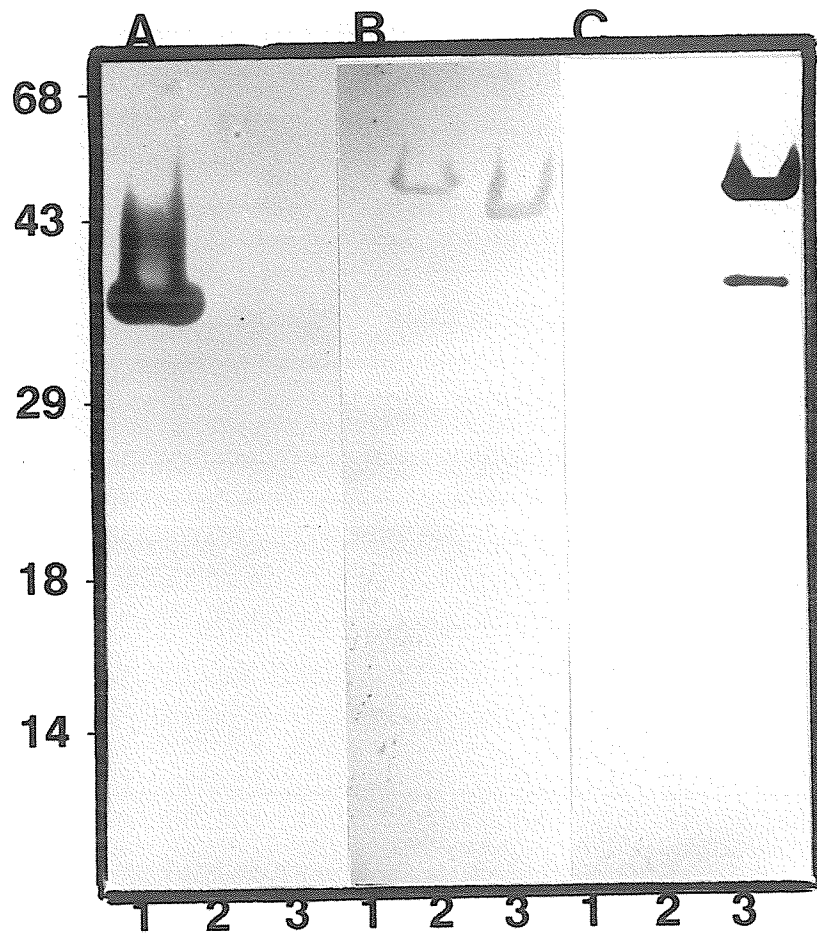
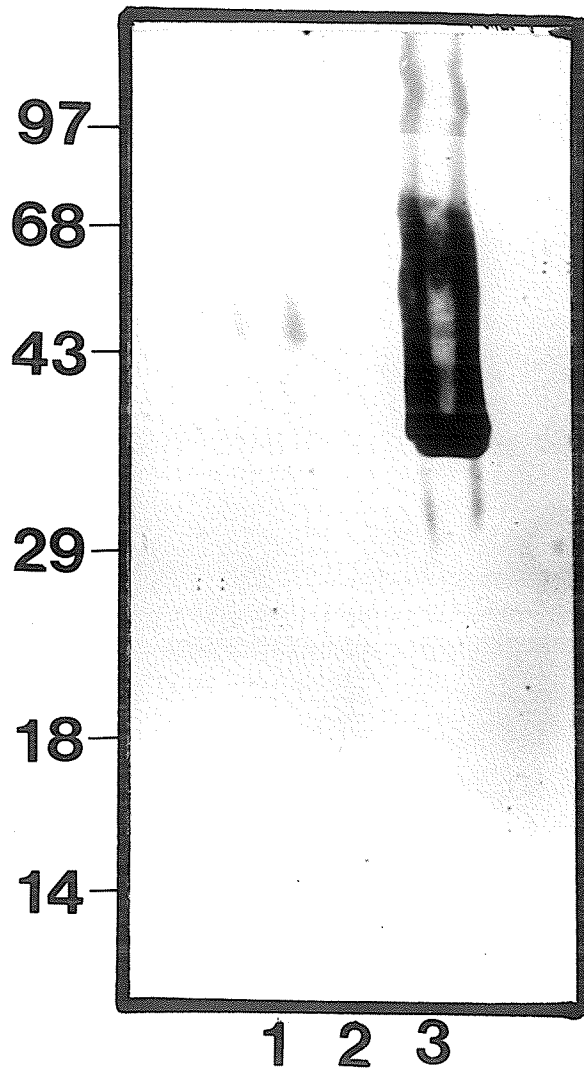


Figure 10. Western immunoblot analysis of *Pseudomonas aeruginosa* L-AP, H-AP, and *E. coli* AP. Antibody to L-AP is used in excess (1:25) dilution as the primary antibody. Lane 1, *E. coli* AP; lane 2, H-AP; lane 3, L-AP.



### 3.5 Time course of H-AP and L-AP production.

Time course experiments were also performed to determine the point at which H-AP and L-AP are expressed during growth of *P. aeruginosa* in phosphate limiting media. Cultures were removed from incubation at various times, the extracellular proteins were precipitated with 70% saturated ammonium sulphate and periplasmic proteins were released by osmotic shock treatment (Hoshino and Kageyama, 1980). The precipitated proteins were dialysed, concentrated, electrophoresed, Western blotted onto nitrocellulose transfer membrane, probed with the appropriate antibodies (L-AP and H-AP) and immunodetected with a peroxidase conjugate. Immunoblots in Figure 11 show that H-AP was detected in both cellular fractions (osmotic shock fluid and supernatant) after only 1 hour of growth, suggesting that H-AP is produced constitutively. This hypothesis was confirmed by the detection of H-AP in the culture supernatant of mid-log phase *P. aeruginosa* grown in phosphate rich media (LB) as shown by the immunoblot in Figure 12. L-AP expression, on the other hand, was only detected after 7.5 hour of incubation, in phosphate reduced conditions, as shown by the immunoblot in Figure 13. Similar results were also reported by Poole and Hancock (1983) using a different phosphate-limited medium. Initially, a larger amount of H-AP is detected in the cell supernatant than in the periplasmic contents. This is not due to any carry over from subculture as the cells are washed prior to suspension in TMM for time

Figure 11. Western immunoblot analysis of periplasmic contents and cellular supernatant from *P. aeruginosa* grown in Tris-magnesium media (TMM). Panel A and B represent identical immunoblots probed with *P. aeruginosa* H-AP specific anti-serum at a working dilution of 1:100. Panel A, periplasmic contents; Panel B, supernatant. All lanes represent time after inoculation of culture into TMM. Lane 1, 1 hr ; lane 2, 1.5 hr; lane 3, 2 hr, lane 4, 2.5 hr; lane 5, 3.0 hr; lane 6, 3.5 hr; lane 7, 4.0 hr; lane 8, 4.5 hr; lane 9, 5 hr. Samples were electrophoresed and probed as described in Materials and Method.

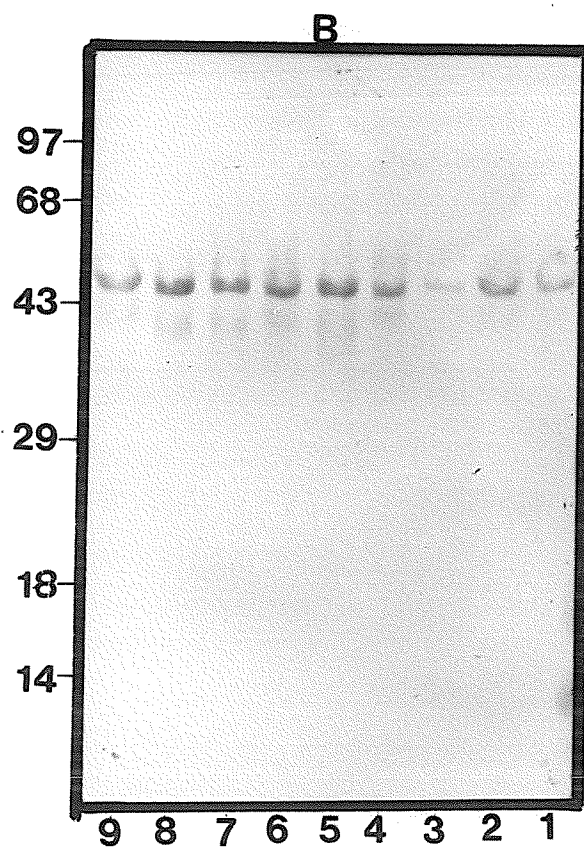
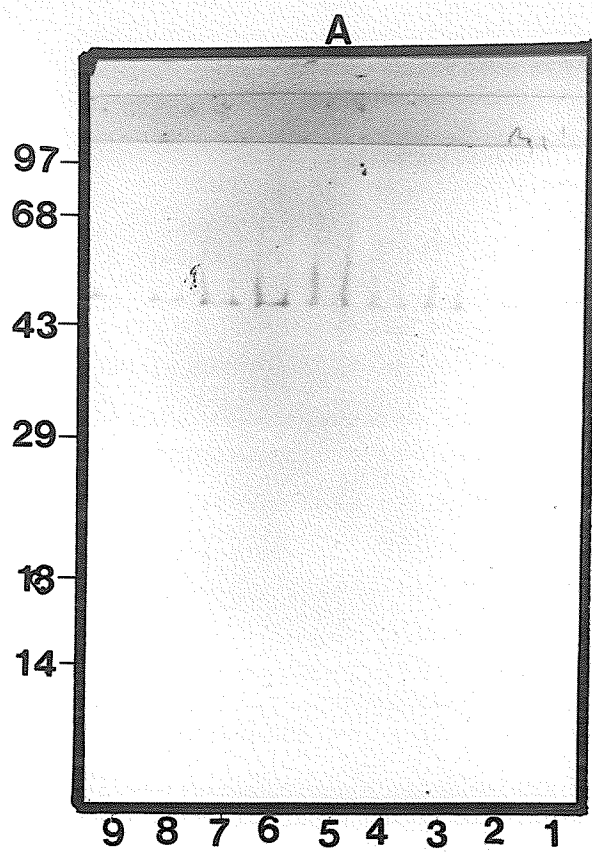


Figure 12. Western immunoblot analysis of periplasmic contents and culture supernatant from *Pseudomonas aeruginosa* H103 grown in phosphate rich media (LB). Immunoblots were probed with L-AP specific antibodies in Panel A and H-AP specific antibodies in Panel B. Antibodies were diluted 100 fold. Lane 1-3 represent periplasmic contents at, early log, mid log and late log respectively. Lane 4-6 represent culture supernatant samples taken at early log, mid log and late log respectively.

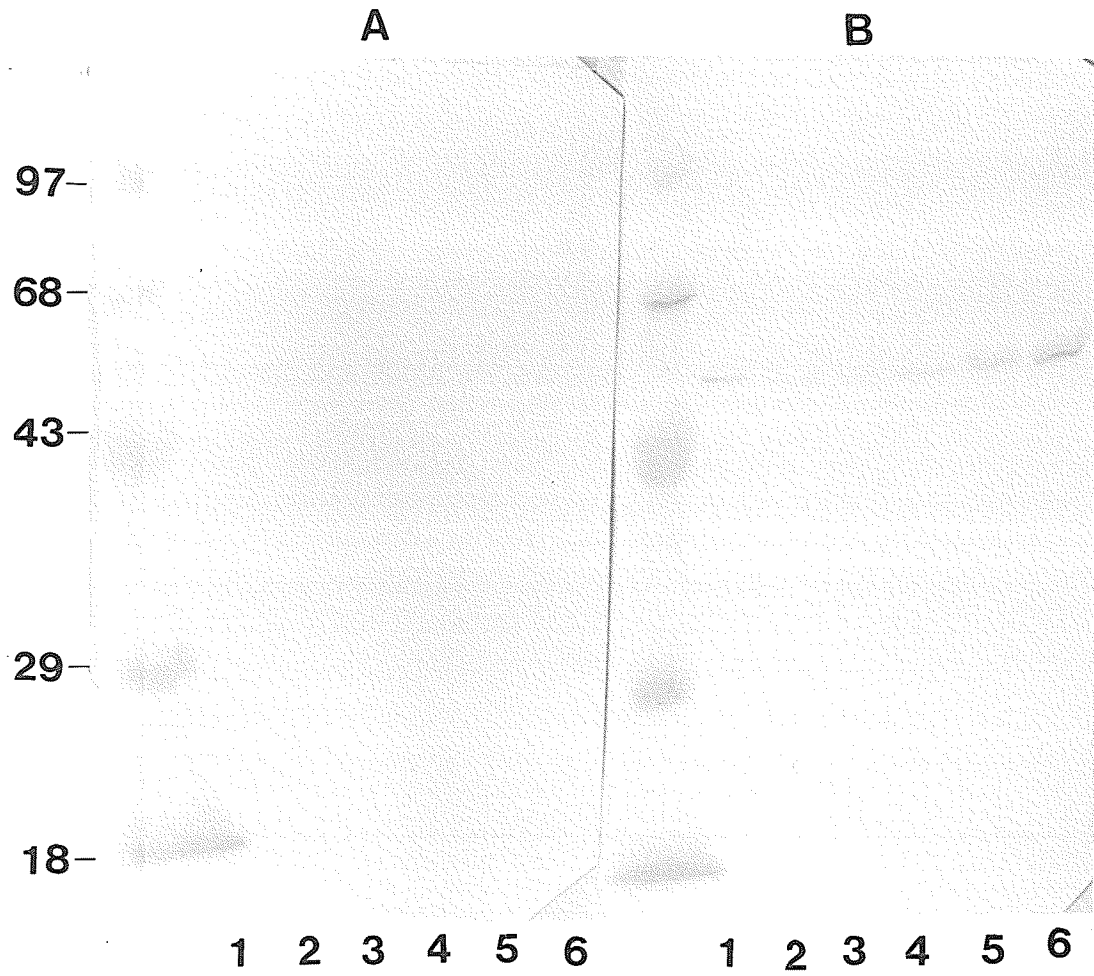
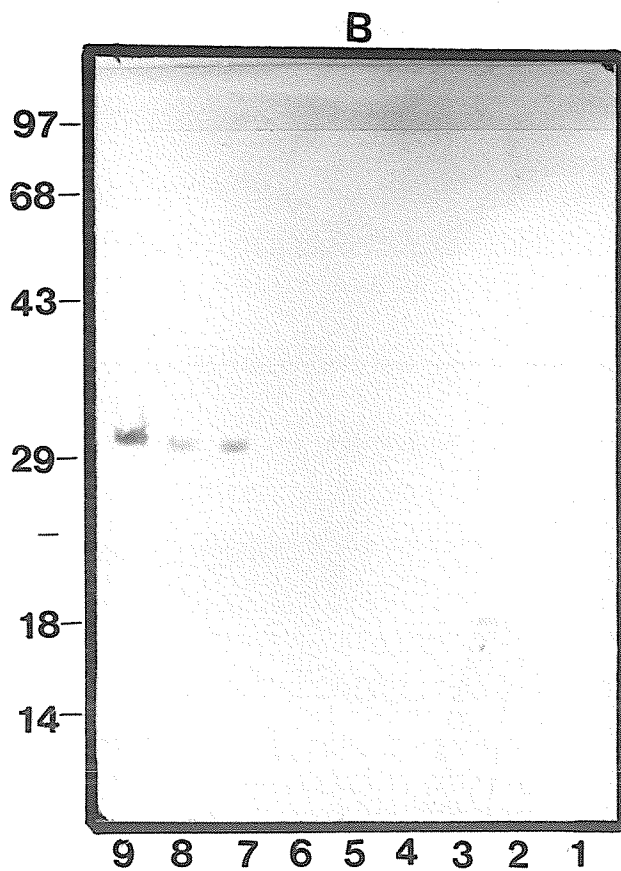
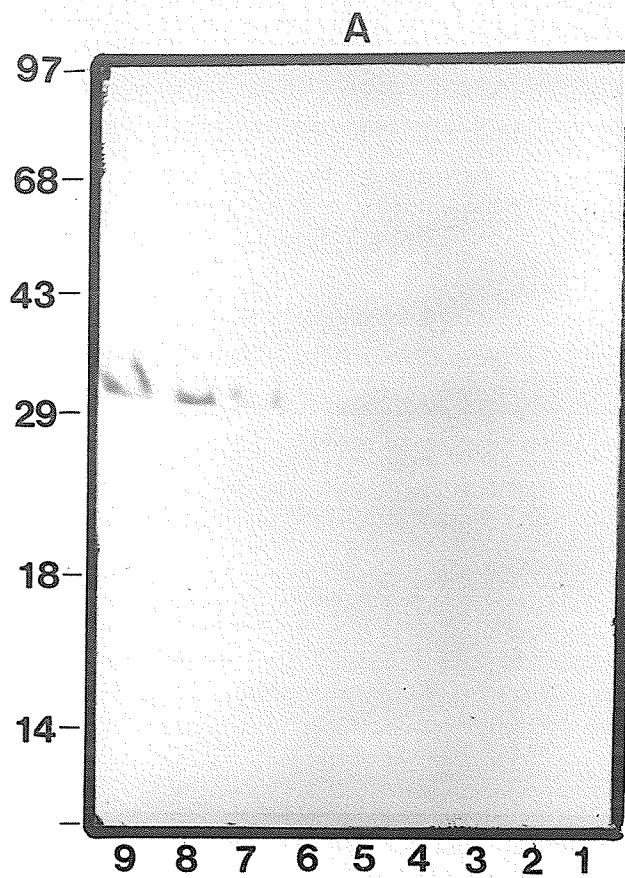




Figure 13. Western immunoblot analysis of *Pseudomonas aeruginosa* H103 periplasmic contents and supernatant grown in Tris magnesium media (TMM). Panel A and B represent identical immunoblots probed with *P. aeruginosa* L-AP specific anti-serum (1:100). Panel A, periplasmic contents; Panel B, supernatant. All lanes represent time after inoculation of culture into TMM. Lane 1, 3 hr; lane 2, 3.5 hr; lane 3, 4.5 hr; lane 4, 6.0 hr; lane 5, 6.5 hr; lane 6, 7.0 hr; lane 7, 7.5 hr; lane 8, 8 hr; lane 9, 8.5 hr. Samples were electrophoresed and probed as described in Materials and Method.



course experiments.

### 3.6 Amino terminal sequences.

The first 19 and 15 amino acids were sequenced for purified H-AP and L-AP respectively. These sequences were then aligned for visual comparison to each other and to the N-terminal sequence of *E. coli* alkaline phosphatase isozyme III (Bradshaw *et al.*, 1981). The results are presented in Figure 14. No sequence homologies were found between L-AP and H-AP, nor between L-AP and the *E. coli* alkaline phosphatase. However, a 42% homology was observed between the H-AP and *Escherichia coli* alkaline phosphatase sequences.

### 3.7 Expression of L-AP and H-AP in other Pseudomonads.

Screening for the expression of L-AP and H-AP by other *Pseudomonads* (Table 2) was conducted using immunoblot analyses with L-AP and H-AP specific antibodies of periplasmic and supernatant proteins produced by these strains grown under phosphate sufficient (nutrient broth) and phosphate deficient (TMM) conditions. Cells were grown in the respective media for 16 hours, harvested by centrifugation, washed in 10 mM Tris-HCl, pH 7.2 and subjected to the osmotic procedure of Hoshino and Kageyama, (1980). The excreted proteins present in the culture supernatants were precipitated with 70% (w/v) ammonium sulphate and collected by centrifugation. Both osmotic shock fluids and supernatant were dialysed,

Figure 14. Amino acid sequence of *Pseudomonas aeruginosa* H103 H-AP, L-AP and *E. coli* AP. Conserved or identical sequences are underlined. Conservative amino acid changes: D/E; I/L/V.

	1	5	10	15	20														
<i>E. coli</i> AP	T	<u>P</u>	<u>M</u>	<u>P</u>	<u>V</u>	<u>L</u>	<u>E</u>	<u>N</u>	<u>R</u>	<u>A</u>	<u>A</u>	<u>Q</u>	<u>G</u>	<u>N</u>	<u>I</u>	A	P	G	G
<i>P. aeruginosa</i> H-AP	Q	Q	<u>D</u>	<u>D</u>	<u>P</u>	<u>S</u>	<u>L</u>	<u>F</u>	<u>N</u>	<u>R</u>	<u>Q</u>	<u>A</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>L</u>	S	V	R
<i>P. aeruginosa</i> L-AP	V	T	G	G	G	A	S	L	P	A	E	L	Y	R					

- a. reaction with anti-H-AP specific serum.
  - b. reaction with anti-L-AP specific serum.
  - c. Molecular weights of cross reactive protein (kilodaltons).
  - d. qualitative alkaline phosphatase activity.
  - e. periplasmic proteins in osmotic shock fluids.
  - f. extracellular proteins in culture supernatants.
- N.D. non detected.
- +. positive alkaline phosphatase activity with *p*-nitrophenylphosphate in 0.1 M Tris-HCl, pH 8.5.
  - . no alkaline phosphatase activity.

Table 3. Summary of immunoblot analyses and qualitative alkaline phosphatase activities of members of the Family *Pseudomonadaceae* under phosphate sufficient conditions.

Species	H-AP <sup>a,c</sup>		L-AP <sup>b,c</sup>		AP activity <sup>d</sup>	
	PP <sup>e</sup>	EC <sup>f</sup>	PP	EC	PP	EC
Group I						
<i>P. aeruginosa</i>	51	51	N.D.	N.D.	+	+
<i>P. putida</i>	53	55	52	N.D.	-	-
<i>P. fluorescens</i>	40	N.D.	N.D.	N.D.	+	-
<i>P. syringae</i>	49	40	50	N.D.	-	-
<i>P. stutzeri</i>	49	50	35	N.D.	+	+
<i>P. alcaligenes</i>	51	N.D.	N.D.	N.D.	+	+
Group II						
<i>P. cepacia</i>	52	54	49	49	+	+
Group III						
<i>P. acidovorans</i>	N.D.	N.D.	N.D.	N.D.	-	-
<i>P. testosteroni</i>	43	N.D.	N.D.	N.D.	+	+
Group IV						
<i>R. leguminosorum</i>	N.D.	N.D.	N.D.	N.D.	-	-
<i>G. oxydans</i>	N.D.	55	N.D.	N.D.	+	+
<i>A. radiobacter</i>	N.D.	N.D.	N.D.	N.D.	-	-
<i>A. tumefaciens</i>	N.D.	N.D.	N.D.	N.D.	-	-
Group V						
<i>P. maltophilia</i>	N.D.	N.D.	33	33	+	+
Miscellaneous						
<i>P. rubescens</i>	N.D.	N.D.	N.D.	N.D.	+	+
<i>P. oxalaticus</i>	N.D.	N.D.	N.D.	N.D.	+	-

- a. reaction with anti-H-AP specific serum.
  - b. reaction with anti-L-AP specific serum.
  - c. Molecular weights of cross reactive protein  
(kilodaltons).
  - d. qualitative alkaline phosphatase activity.
  - e. periplasmic proteins in osmotic shock fluids.
  - f. extracellular proteins in culture supernatants.
- N.D. non detected.
- +. positive alkaline phosphatase activity with *p*-nitrophenylphosphate in 0.1 M Tris-HCl, pH 8.5.
  - . no alkaline phosphatase activity.



Table 4. Summary of immunoblot analyses and qualitative alkaline phosphatase activities of members of the Family *Pseudomonodaceae* under phosphate deficient conditions.

Species	H-AP <sup>a,c</sup>		L-AP <sup>b,c</sup>		AP activity <sup>d</sup>	
	PP <sup>e</sup>	EC <sup>f</sup>	PP	EC	PP	EC
Group I						
<i>P. aeruginosa</i>	51	51	39	39	+	+
<i>P. putida</i>	51	N.D.	54	54	+	-
<i>P. fluorescens</i>	54	N.D.	N.D.	N.D.	-	-
<i>P. syringae</i>	52	N.D.	N.D.	N.D.	-	-
<i>P. stutzeri</i>	N.D.	N.D.	37	N.D.	+	+
<i>P. alcaligenes</i>	47	N.D.	34	N.D.	+	+
Group II						
<i>P. cepacia</i>	50	50	49	49	+	+
Group III						
<i>P. acidovorans</i>	N.D.	N.D.	N.D.	N.D.	-	-
<i>P. testosteroni</i>	50	N.D.	33	N.D.	+	+
Group IV						
<i>R. leguminosorium</i>	48	N.D.	N.D.	N.D.	-	-
<i>G. oxydans</i>	N.D.	N.D.	N.D.	N.D.	+	+
<i>A. radiobacter</i>	49	N.D.	N.D.	N.D.	-	-
<i>A. tumefaciens</i>	49	N.D.	N.D.	N.D.	-	-
Group V						
<i>P. maltophilia</i>	N.D.	N.D.	37	38	+	+
Miscellaneous						
<i>P. rubescens</i>	47	50	57	N.D.	+	-
<i>P. oxalaticus</i>	47	50	N.D.	N.D.	+	-

concentrated, electrophoresed, Western blotted onto nitrocellulose membrane and probed with anti-L-AP or anti-H-AP antibodies. The results are summarised in Table 3 for phosphate-sufficient and Table 4 for phosphate-deficient growth conditions, respectively. The Pseudomonads in our screening were grouped according to their rRNA homology as measured by the competition technique in rRNA/DNA hybridisation experiments (Palleroni, 1972).

Under phosphate-sufficient conditions, all members in Group I and *P. cepacia* (Group II) have proteins (periplasmic and/or extracellular) that cross reacted with antibodies to *P. aeruginosa* H103 H-AP. However, only *P. fluorescens*, *P. stutzeri*, *P. alcaligenes*, *P. cepacia*, *P. testosteroni* and *G. oxydans* had any detectable alkaline phosphatase activity as shown in Table 3. In addition, *P. maltophilia*, *P. rubescens* and *P. oxalaticus* also synthesized proteins that produced a detectable alkaline phosphatase activity but failed to cross react with antibodies to *P. aeruginosa* H-AP. The other members of this Family that cross react with this H-AP specific antibody are *P. testosteroni* of Group III (periplasmic) and *G. oxydans* (extracellular).

Only Group I members, *P. aeruginosa*, *P. fluorescens* and *P. alcaligenes*; *P. cepacia* of Group II and *P. maltophilia* of Group V produced proteins which cross reacted with antibodies to L-AP. All of these also produced a detectable alkaline phosphatase activity. Additionally, proteins from *P.*

*testosteroni*, *G. oxydans*, *P. rubescens* and *P. oxalaticus* did not cross react with this antibody although AP activity was detected.

Under phosphate-limiting conditions as shown in Table 4, all members of Group I (except *P. stutzeri*), *P. cepacia* of Group II, *P. testosteroni* of Group III, all of Group IV (except *G. oxydans*) and both miscellaneous strains produced proteins that cross react with antibody to H-AP. However, not all of these cross reactive proteins had alkaline phosphatase activities. Only, *P. putida*, *P. alcaligenes*, *P. cepacia*, *P. testosteroni*, *P. rubescens* and *P. oxalaticus* produced AP activities. In addition, *G. oxydans* and *P. maltophilia* had AP activity but did not cross react with *P. aeruginosa* H-AP.

Under phosphate-limiting conditions, only *P. putida*, *P. stutzeri*, *P. alcaligenes*, *P. cepacia*, *P. testosteroni*, *P. maltophilia* and *P. rubescens* cross reacted with antibody to L-AP. All of these cross reactive species produced AP activities. AP activity was also detected for the non-cross-reactive strain *P. oxalaticus*.

### **3.8 Detection of L-AP specific antibodies in cystic fibrosis sera.**

Preliminary results of Western immunoblot analyses of cystic fibrosis sera revealed that cross reactivity existed with L-AP, but not H-AP, for the 2 patients colonized with *P. aeruginosa*. The other sera from a patient not colonized with

*P. aeruginosa* did not cross react with either L-AP or H-AP data not shown).

**DISCUSSION**

## Chapter 4

### DISCUSSION

Two alkaline phosphatase enzymes were isolated from *Pseudomonas aeruginosa* H103. They were called H-AP and L-AP, for high and low molecular weight according to their different mobilities on SDS-PAGE. H-AP and L-AP also differ in substrate specificity (L-AP has a diesterase activity while H-AP does not), pH optima, immunological cross reactivity, N-terminal amino acid sequence and time of induction in phosphate limiting conditions. Both were purified using the same procedure and both have been identified in periplasmic and extracellular locations. Although alkaline phosphatases produced by a variety of *P. aeruginosa* strains have been studied by other groups (Day and Ingram, 1973; Husson, 1989), no one has described two different alkaline phosphatases produced by the same strain of *P. aeruginosa*.

The enzyme we called L-AP, or a closely related species, has been purified by Day and Ingram, (1973). The enzyme they describe has a average monomeric molecular weight of 39,000 and is active over a broad pH range, with an optimum activity at pH 10.5. This closely resembles L-AP. They did not examine the diesterase activity, nor amino acid sequence and did not perform any immunochemical studies. Husson *et al.*, (1989) reported the production of a series of monoclonal antibodies specific for an alkaline phosphatase isolated from

the periplasm of *P. aeruginosa* which did not interact with *E. coli*. According to our immunoblot results, this enzyme could also be L-AP or a closely related species.

Although, Filloux et al., (1987) did report an alkaline phosphatase which cross reacted with antibodies to *E. coli* alkaline phosphatase while screening for *P. aeruginosa* export defective mutants, they did not further distinguish this alkaline phosphatase from the one described by Day and Ingram, (1973). Their immunoblot results correspond with our immunoblot studies where we found that antibodies specific for H-AP did cross react with the *E. coli* AP. On the other hand, we found that L-AP did not cross react with antibodies to *E. coli* alkaline phosphatase although we found that antibodies to L-AP could cross react with *E. coli* AP if an excess of antibody was used.

We found that a low level of H-AP was produced constitutively while L-AP was only induced when *P. aeruginosa* was grown in phosphate poor media. In analysing these results, one must keep in mind our procedure for visualising the presence of these two enzymes. Prior to electrophoresis and Western immunoblot, cellular fractions are concentrated up to 50 fold, whereas, the alkaline phosphatase activity was determined using unconcentrated samples [Figure 3]. No alkaline phosphatase activity was detected in the initial unconcentrated cell supernatants taken for time course experiments. Likewise, Day and Ingram, (1973) probably did

not expect that any AP activity existed or decided residual activity was too low to be significant. However, H-AP but not L-AP was readily detected in immunoblot experiments once these samples were concentrated [Figure 11]. H-AP was also detected in cells grown in phosphate rich media (LB) as shown in Figure 12. This indicates that very low levels of H-AP appear to be produced constitutively by this organism. The induction of L-AP on the other hand, appears to coincide with the large peak of alkaline phosphatase activity produced by *P. aeruginosa* when free phosphate levels drop [Figure 3]. It is possible that low levels of H-AP are constitutively produced and excreted by *P. aeruginosa* to sustain the phosphate scavenging process while the more versatile L-AP is reserved for conditions of severe phosphate deprivation.

Filloux *et al.*, (1988) have cloned the structural gene for alkaline phosphatase (*phoA.35*) of *P. aeruginosa*. When cloned into *E. coli*, this gene is regulated normally as the expression of the gene product, AP is repressed in high phosphate medium and derepressed under phosphate limiting growth conditions. Lazundski *et al.*, (1990) have recently reported the cloning of a second AP gene (*phoA.45*) which produces an alkaline phosphatase which strongly cross reacts with antibodies to *E. coli* alkaline phosphatase and runs with the same mobility as H-AP. Preliminary experiments indicate that these two genes appear to map at different places on the *P. aeruginosa* chromosome. Multiple structural genes for



alkaline phosphatase have also been cloned from *Bacillus licheniformis* (Dubose, 1990) and *Bacillus subtilis* (Hulett et al., 1991). The genes for the two *B. subtilis* enzymes have been cloned and the gene products characterized. These 2 enzymes (*phoAIII* and *phoA IV*) differ slightly in subunit molecular weight and substrate specificity but have very similar N-terminal amino acid sequences and antibody cross reactivities. In humans, as well, there are at least 4 genes encoding alkaline phosphatases in the APase family: intestinal, placental, placental-like, and bone/liver/kidney (Hulett et al., 1991). The evolution of this family of genes, has presumably involved the duplication of a primordial gene for bone/liver/kidney APase to generate the gene for intestinal AP, with a subsequent additional duplication that generates the gene for placental AP (Chung and Shih, 1990). Thus, the placental AP in human represents the most recently mutated form (Chung and Shih, 1990).

The immunological cross reactivity and sequence homology between *P. aeruginosa* H-AP and *E. coli* alkaline phosphatase seems to reflect the overall similarity between the high affinity phosphate transport systems found in these two organisms. The two systems share the presence of similar regulatory components (Hancock et al., 1988; Filloux et al., 1988), periplasmic phosphate binding proteins (Torraini, 1987), the outer membrane porins (which are induced by low phosphate but have different specificities) and an alkaline

phosphatase (Filloux *et al.*, 1988). The differences between the two systems are also notable, being the presence in *P. aeruginosa* but not in *E. coli* of extracellular phosphate scavenging molecules like alkaline phosphatase, phospholipase C, a heat stable hemolysin (Liu, 1979) and specificity of the porin (OprP) (Poole and Hancock, 1983).

The differences between the phosphate transport systems of *E. coli* and *P. aeruginosa* probably reflect the differential evolution of the two systems. In bacteria, gene transfer may account for the relative ease with which wide taxonomic gaps can be crossed, eg. *Yersinia* - *Alcaligenes* - *Pseudomonas* - *Xanthomonas*. Thus, in *E. coli*, the absence or deletion of the signal sequence encoding for protein excretion across the outer membrane at NH<sub>2</sub>- or COOH- termini of AP as compared with the presence or insertion of a signal peptide for protein excretion in *P. aeruginosa* (Hartley, 1984) probably reflects the environment in which they survive. *E. coli* are facultative anaerobes while *P. aeruginosa* are generally aerobes suggesting that *E. coli* has a wider growth environment, thereby outgrowing and crowding out *P. aeruginosa* when the two are found together. Presumably *E. coli* also has a more complex *pho* regulon, thus, it can scavenge for phosphate from the environment more efficiently than *P. aeruginosa*. *P. aeruginosa* thus had to evolve a different system to scavenge for nutrients through excretion of enzymes which can reach a wider area for nutrients.

In the *pho* regulon of *E. coli*, the positive regulator protein PhoM is constitutively produced irrespective of the external phosphate levels when the major negative regulator *phoR* is absent. PhoM will activate *phoB* to produce PhoB protein which in turn activates a number of genes belonging to the *pho* regulon (*psi*, *phoE*, *phoA*, *phoS*, *ugpA*, *ugpB*; Torraini-Gorini, 1987). Activated *phoA* will then express alkaline phosphatase which will in turn hydrolyse phosphorylcholine to choline and inorganic phosphate. PhoR, however will positively or negatively activate the expression of *phoB* protein and consequently the expression of *phoA* and other genes of the *pho* regulon depending upon environmental signals (phosphate sufficient or deficient, Figure 2). In *P. aeruginosa* H103, the gene regulating the production of H-AP may be equivalent to *phoM* of *E. coli*. Conversely, the regulatory protein that influences the production of L-AP under phosphate-limiting condition has its equivalence in *phoR* and *phoB* of *E. coli*, as demonstrated by Filloux *et al.*, (1988). Thus, having the two enzymes encoded by separate genes would provide *P. aeruginosa* with a back up system allowing for growth in phosphate poor systems if either L-AP or H-AP becomes non functional.

The presence of strong analogies between the components of *E. coli* and *P. aeruginosa* *pho* regulons led to the analysis of the members of the Family *Pseudomonodaceae* for the presence of a similar phosphate regulated system. Similarities between

these species was assayed qualitatively using as criteria the ability to produce proteins capable of cross reacting with antibodies specific for L-AP and H-AP, and the presence of an alkaline phosphatase activity. L-AP and H-AP have been found to exist in both the periplasm and culture supernatants, thus both cellular fractions were examined for all the species in question. A great range of results were obtained from both assays, none particularly consistent with the ribosomal RNA homology groups into which the strains were placed. In many instances, a cross reactive protein was observed yet no AP activity was detected and vice versa. In the cases (e.g., *G. oxydans* grown in phosphate deficient conditions) where AP activity was detected but no cross reactive protein was detected could be explained by assuming either too little of that enzyme was produced or that no epitopes were shared with the *P. aeruginosa* H-AP or L-AP. Likewise, when a cross reactive, yet non-active enzyme was detected, one could speculate that shared epitopes were just a coincidence. The use of polyclonal sera can cause these types of problems, however, monoclonal antibody technology was not available at the time of this study.

Major consistencies were found largely within Group I, that including *P. aeruginosa*. All members produced active L-AP and H-AP cross reactive proteins under at least one set of conditions with the exception of *P. fluorescens* (only produced H-AP) and *P. syringae* (produced both APases but had no

activity). The reasons for these discrepancies still eludes us.

Without looking at each species individually, the organisms of note are: 1) *P. cepacia* (Group II) which produces proteins that cross react with both L-AP and H-AP and exhibits activity in the supernatant and periplasmic contents. This organism is a human pathogen and is probably under constant phosphate stress, thus produces both enzymes constitutively for maximal scavenging efficiency; 2) *P. testosteroni* also produces both forms and probably should be placed into Group I as it has a very similiar profile to *P. aeruginosa*; and 3) *P. maltophilia* which produces an active L-AP constitutively. Phosphate deficient conditions, in general tend to produce the greatest number of cross reactive and active species in all strains examined. This observation could be the basis of a broad generalization in that all of these species have developed a phosphate scavenging system. However, how closely the various systems are to that of *P. aeruginosa* or *E. coli* will require more in depth analyses.

Preliminary results of Western immunoblot analyses for the presence of L-AP and/or H-AP in sera of cystic fibrosis patients (3 samples) revealed the following results: 1) sera from 2 patients colonized with *P. aeruginosa* interacted with L-AP, but not with H-AP while the sample from the uncolonized patient did not interact with either L-AP nor H-AP. As the sampling size is too small, no firm conclusions can be made at

this stage. However, one could speculate that since L-AP is detected, it must be produced in large enough quantities to stimulate an antibody response. This leads one to speculate that the environment in the cystic fibrosis host is phosphate limiting. One could also assume that H-AP is produced in too low a concentration to be immunogenic. Further analyses of a large number of samples will undoubtedly shed more light on our theories.

In conclusion, this study describes two APases produced by *P. aeruginosa* H103. They are encoded by two different structural genes (Filloux *et al.*, 1988 and Ladzunski *et al.*, 1990), however only the DNA sequencing of these two structural genes can confirm the relationship between the 2 enzymes and the other related enzymes. Future studies could also include the production of monoclonal antibodies to these two APases to use to study the evolutionary conservation of this protein in members of the Family Pseudomonodaceae.

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**APPENDIX**

## APPENDIX

**(A) MEDIA****(1) Tryptose Magnesium Minimal Media (TMM)**

120 mM Tris HCl pH 7.2	120 ml of 1 M Tris pH 7.2
0.1% (w/v) tryptose	1.20 g
20 mM ammonium sulphate	2.66 g
1.6 mM calcium chloride	0.24 g
10 mM potassium chloride	0.76 g
50 mM glucose	9.10 g
0.01% (w/v) magnesium sulphate	0.10 g

Make up to 1000 ml with DDW; x 12 for large scale preparation of *Pseudomonas aeruginosa* H103.

**(2) LB broth/LB agar plates**

1% (w/v) tryptone	10 g/L
0.5% (w/v) yeast extract	5 g/L
0.5% (w/v) sodium chloride	5 g/L

2% (w/v) Bacto-Agar if making LB agar plates.

**(3) Nutrient broth**

(*R. leguminosorum*, *A. radiobacter* and *A. tumefaciens*)

mannitol	5.0 g/L
yeast extract	2.0 g/L
K <sub>2</sub> HPO <sub>4</sub>	0.5 g/L
MgSO <sub>4</sub>	0.2 g/L
NaCl	0.1 g/L, pH to pH 6.8-7.0

**(B) One-Dimensional Sodium Doecyl Sulphate Polycrylamide Gel electrophoresis (SDS-PAGE) (Hames, 1981)**

**(1) Running Buffer**

Tris base	62 g
Glycine	288 g
SDS	20 g

Make up to 5 litres with DDW.

**(2) Reduction Mixture**

	volume (ml)
0.5 M Tris HCl pH 6.8	1.25
Glycerol	1.00
DDW	1.45
10% (w/v) SDS	0.10
bromophenol blue	0.001 g

**(3) 11% SDS-PAGE running gel**

	volume (ml)
Acrylamide:bis-acrylamide (44:0.8)	4.71
DDW	8.265
1.5 M Tris pH 8	4.688
1% (w/v) ammonium persulphate	0.45
10% (w/v) SDS	0.375
5 M sodium chloride	0.315
TEMED (N,N,N',N'-tetramethylethylenediamine)	0.075

<b>(4) 4% (w/v) SDS-PAGE stacking gel</b>	volume (ml)
Acrylamide:bis acrylamide (30:0.8)	0.5
DDW	3.08
0.5 M Tris pH 6.8	1.25
1% (w/v) ammonium persulphate	0.12
10% (w/v) SDS	0.05
TEMED	0.03

**(5) Coomassie Blue stain for SDS-PAGE**

Coomassie Brilliant Blue R250	8.0 g
Isopropyl alcohol	500 ml
Glacial acetic acid	200 ml

**(6) Destaining solution**

Methanol	800 ml
Glacial acetic acid	300 ml
Water	2900 ml

Mix thoroughly. Use when freshly made.

**(7) Preparation of SDS-PAGE samples**

High MW prestained standards: (BRL, Gaithersburg, MD)  
 5  $\mu$ l standard + 5ul reduction mix. Samples (30 ul) + 10 ul  
 reduction mix + 1 ul 2  $\beta$ -mercaptoethanol. Both standards and  
 samples were momentarily centrifuged using a microcentrifuge  
 and then heated in a 100°C dry heating block for 10 minutes.  
 They were then loaded onto a 0.8 mm thick SDS- polycrylamide

gel (15 x 17 cm) gel slab mounted on a Model V16 Vertical gel electrophoresis apparatus (BRL, Gaithersburg, MD). The gel is run at 50 volts using a Bio Rad Model 200/2.0 power unit (Bio Rad Lab., Missasagua, Ont) for 30 minutes to allow the proteins to concentrate in the stacking gel. The voltage is increased to 150 volts and the gel is run for a further 3 hours. At the end of the electrophoretic run, remove stacking gel and immerse running gel in a container of staining solution. After 30 minutes of staining, the stained gel is destained for a further 12 hours with 3 changes of destaining solution until no background is observed. The gel is then wrapped with a sheet of cellophane membrane (Bio Rad, Missasaguana, Ont) mounted on a glass plate and dried overnight at room temperature.

**(C) Preparation of Sephadex G-75 gel filtration column**

Weigh out 20 g of dry gel ( Pharmacia, Uppsala Sweden) into 200 mls of 10 mM Tris-HCl pH 7.2 and allow to swell in the excess solvent for 24 hours at 4°C. The gel slurry is then placed in a boiling water bath to remove air bubbles. As a further precaution, the gel slurry is deaerated under vacuum for 60 minutes. Pour column as directed in Pharmacia instructions. If not in use, run volume of eluant with 0.02% (w/v) sodium azide through column before storing at 4°C.

**(D) Immunization Protocol (Harlow, E. and Lane, D., 1988).**

I/M (intramuscular) injection of female white New Zealand rabbit:

**DAY 1**

500  $\mu$ l of antigen ( approximately .25 mg/ml) in 10 mM Tris-HCl pH 7.2 is mixed with 500  $\mu$ l of Freund's Complete Adjuvant (Difco Lab., Detroit, Michigan) until a thick suspension is produced. 500  $\mu$ l of this solution is injected in 2 sites on the hind legs, using a disposable 22G hypodermic needle (Becton Dickinson, Mississauga, Ont.).

**DAY 10**

Injected sample = 500  $\mu$ l of antigen (.25 mg/ml) in 10 mM Tris-HCl pH 7.2 mixed with 500  $\mu$ l of Freund's Complete Adjuvant

**DAY 31**

Injected sample = 250  $\mu$ l of antigen (.25 mg/ml) in 10 mM Tris-HCl pH 7.2 mixed with 250  $\mu$ l of Freund's Incomplete Adjuvant (Difco Lab., Detroit, Michigan)

**DAY 38**

50-100  $\mu$ l of whole blood is removed from the rabbit's ear as a test bleed. 0.4 ml of Atravet (Ayerst laboratories, Montreal, Que) containing 10 mg/ml of acepromazine maleate which has both vasodilating and sedating effect was injected into the rabbit's hind leg using a 22G hypodermic needle. After 3-4 minutes, a 22G hypodermic needle was used to collect 600-900  $\mu$ l of whole blood from the dilated inner peripheral vein.

**DAY 43**

Injected sample = 250  $\mu$ l antigen mixed with 250  $\mu$ l of Freund's Incomplete Adjuvant.

**DAY 46**

Bleed the rabbit (100 ml of whole blood) by cardiac puncture.

**(E) Immunoblotting (Western blot) (Harlow, E. and Lane, D. 1988)**

Run identical samples and standards on duplicate SDS-polyacrylamide gels. Coomassie Blue stain one gel and electrophoretically transfer protein from the other gel to nitrocellulose transfer membrane (Micron Separations Inc., Westboro, MA) at 10 mA overnight at room temperature using a Bio-Rad Trans-blot cell (Bio-Rad Laboratories). The blotting setup is described in Section F of this Appendix.

**(1) Western Blotting Buffer**

DDW	2400 ml
Methanol	600 ml
Tris-Base	5.6 g
Glycine	43.25 g

Dissolve the components and pH to 8.3 with 4N sodium hydroxide. Store at 4°C. Can be used repeatedly (3-4X) until solution becomes cloudy.



**(2) PBS (Phosphate buffered saline)** 1000 ml

Sodium chloride	8 g
Potassium dihydrogen phosphate	0.2 g
Disodium hydrogen phosphate	2.9 g
Potassium chloride	0.2 g pH to 7.4 with conc. HCl.

**(3) Immunoenzymatic detection of proteins (Harlow, E. and Lane, D., 1988)**

Soak Hybridization transfer membrane (Micron Separations Inc., Westboro, MA) in 20 ml of 1% (w/v) skim milk (Difco, Detroit, Michigan)/ PBS pH 7.4 for 30 minutes with agitation at room temperature to saturate sites on the membrane which do not contain protein. Rinse off the skim milk with PBS pH 7.4. Add a diluted antibody (ranging from 1:25 to 1:200 dilution) to 20 ml of 1% (w/v) skim milk/PBS, pH 7.4 and agitate for at least 60 minutes. Wash off the antibody with PBS and add in 20 ml of 1/1000 dilution of anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma Chemical Co.,) and further agitate for 60 minutes. Wash off the second antibody solution with PBS and add 20 ml of peroxidase substrate.

**(4) Peroxidase Substrate**

Solution A --- 30 mg of 4-chloro-1-naphthol (Sigma Chemical Co.) in 10 ml of methanol.

Solution B --- 50 ml of PBS pH 7.4 + 30  $\mu$ l of 30 % hydrogen

**(2) Blotting buffer**

300 ml of 10X stock (CAPS buffer)

300 ml of methanol

2400 DDW

**(G) Alkaline phosphatase assay (Day and Ingram, 1978)**

AP substrate : 2 mg/ml of p-nitrophenylphosphate

Mix 1 tablet of substrate (5 mg/2.5 ml; Sigma Chemical.) into 2.5 ml of 0.1 M Tris-HCl pH 8.5. Mix equal volumes (175  $\mu$ l) of substrate and sample. Immediately, record the absorbance reading ( $A_{410}$ ) at time zero (initial) in a Milton Roy spectronic 602 spectrophotometer and record absorbance reading at 30 seconds intervals hereafter, for a total of 3 minutes. The reaction is then allowed to proceed to completion and the final absorbance is then taken.

Extinction coefficient =  $\frac{\mu\text{moles substrate hydrolysed}}{A_{410 \text{ final}} - A_{410 \text{ initial}}}$

Enzyme activity =  $\frac{\Delta A_{410} / \text{time (minutes)} \times \text{Ext. coefficient}}{\text{mg/ml protein}}$

=  $\mu\text{moles substrate hydrolysed/min/mg protein}$

Protein conc (mg/ml) =  $1.55 A_{280} - 0.76 A_{260}$  (Warburg and Christian eqn, 1942).