PHOSPHATE TRANSPORT ACROSS THE OUTER MEMBRANE OF PSEUDOMONAS AERUGINOSA
by
RAYMOND KEITH POOLE
B.Sc., The University of British Columbia, 1980

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
(Department of Microbiology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1986
© Raymond Keith Poole, 1986
In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of **Microbiology**

The University of British Columbia  
1956 Main Mall  
Vancouver, Canada  
V6T 1Y3

Date **April 7, 1986**
ABSTRACT

When wild-type cells of *Pseudomonas aeruginosa* were grown in a phosphate-limiting medium (0.2 mM orthophosphate) they were derepressed for the production of an outer membrane protein, designated protein P. This protein was purified to homogeneity and demonstrated to form channels in planar lipid bilayer membranes. In agreement with previous data, the channels formed by protein P were anion-specific (due to the presence of a binding site for anions in the channel) and exhibited a marked selectivity for phosphate (HPO$_4^{2-}$) over other anions (e.g. chloride). These properties were not altered in protein P preparations purified free of lipopolysaccharide.

Protein P was coinducible with the enzymes alkaline phosphatase and phospholipase C, and with a periplasmic protein of 34,000 molecular weight. Mutants of *P. aeruginosa*, constitutive or non-inducible for these constituents, were isolated. This suggested that the genes encoding these products were part of a phosphate regulon. Alkaline phosphatase and phospholipase C were demonstrated to be secreted into the external medium upon induction, although this extracellular release was specific and did not involve an increase in outer membrane permeability. The 34K periplasmic protein was purified and demonstrated to bind phosphate *in vitro* ($K_d=0.34 \text{ uM}$). Specificity studies revealed that inorganic phosphate polymers (up to P15) and
arsenate could inhibit the binding of orthophosphate to the
binding protein, although organic phosphates (e.g. glucose-
6-phosphate) could not. The ability of the phosphate-
binding protein and protein P to associate was demonstrated
in vitro, with implications concerning the means by which
phosphate crosses the outer membrane.

Two major inorganic phosphate transport systems were
identified, of low (Km=19.3 uM phosphate) and high-affinity
(Km=0.39 uM phosphate), respectively. Mutants deficient in
the phosphate-binding protein were isolated and shown to
lack the high-affinity phosphate uptake system, confirming
the role of the binding protein in high-affinity phosphate
transport in \textit{P. aeruginosa}. In addition, a role for protein
P in high-affinity phosphate transport was confirmed by the
isolation of a \textit{Tn501} insertion mutant lacking porin protein
P. This mutant exhibited a ten-fold increase in Km for
high-affinity phosphate transport. The loss of these
proteins in the respective mutants was correlated with a
growth defect in a phosphate-deficient medium.

Protein P, like most porins, was isolated as an
oligomer (trimer) in its native (functional) state,
dissociating to non-functional monomers at high
temperatures. A polyclonal antiserum specific for protein P
trimers was raised and shown to cross-react with other
phosphate-starvation-inducible outer membrane proteins of
the families \textit{Pseudomonadaceae} and \textit{Enterobactereaceae}. This
cross-reactivity was observed only with the native,
oligomeric forms of these proteins. No cross-reactivity was seen with the constitutive porins produced by these strains, indicating that the cross-reactivity of phosphate-limitation-inducible oligomeric outer membrane proteins was not due to any homologies relating to porin structure in general. Using a polyclonal antiserum specific for protein P monomers, no reactivity was observed with either the oligomeric or monomeric forms of any of the phosphate-limitation-inducible outer membrane proteins (except for protein P monomers). These data suggested that the common antigenic determinants present in these proteins were conserved in the native functional proteins only.

Examination of some of the physical properties of the phosphate-starvation-inducible outer membrane proteins (e.g. molecular weight, peptidoglycan association, detergent solubility) revealed that these proteins could be grouped into two classes, represented by protein P of P. aeruginosa and protein PhoE of Escherichia coli. Those proteins resembling protein P were identified in members of the fluorescent Pseudomonads, including P. putida, P. fluorescens, P. aureofaciens and P. chlororaphis. The purified proteins formed small, anion/phosphate-selective channels in planar lipid bilayers which were quite similar to protein P channels.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xvi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1. The Gram-negative cell envelope</td>
<td>1</td>
</tr>
<tr>
<td>a. The cytoplasmic membrane</td>
<td>1</td>
</tr>
<tr>
<td>b. The peptidoglycan</td>
<td>2</td>
</tr>
<tr>
<td>c. The outer membrane</td>
<td>3</td>
</tr>
<tr>
<td>2. The role of the outer membrane in transport</td>
<td>6</td>
</tr>
<tr>
<td>a. The LamB protein</td>
<td>8</td>
</tr>
<tr>
<td>b. The PhoE protein</td>
<td>11</td>
</tr>
<tr>
<td>c. Iron-regulated outer membrane proteins</td>
<td>13</td>
</tr>
<tr>
<td>d. Others</td>
<td>14</td>
</tr>
<tr>
<td>3. Bacterial phosphate transport - with specific reference to E. coli</td>
<td>15</td>
</tr>
<tr>
<td>4. The pho regulon of E. coli</td>
<td>18</td>
</tr>
<tr>
<td>Methods</td>
<td>22</td>
</tr>
<tr>
<td>1. Media and growth conditions</td>
<td>22</td>
</tr>
<tr>
<td>2. Bacterial strains</td>
<td>23</td>
</tr>
</tbody>
</table>
3. Cell fractionation and sodium dodecyl sulphate-polyacrylamide gel electrophoresis 23
4. Purification of protein P 28
5. Acetylation of protein P 29
6. Immunological methods 30
7. Preparation of protein P-phosphatidyl choline vesicles 30
8. Preparation of protein affinity columns 31
   a. Protein P-Sepharose 31
   b. Protein P-Affigel-10 31
   c. Phosphate-binding protein-Sepharose 32
9. Preparation of protein P-specific antisera 32
   a. Trimer-specific 32
   b. Monomer-specific 34
   c. Antiserum to protein P in phosphatidyl choline vesicles 35
10. Isolation of a protein P-deficient mutant 35
    a. Tn501 insertion mutagenesis 35
    b. Selection of a protein P-deficient mutant using a protein P trimer-specific antiserum 36
11. Phosphate transport assays 37
12. Enzyme assays 38
13. Nitrocefin permeability assay 39
14. Osmotic shock and purification of the phosphate-binding protein 40
15. Filter assay of phosphate binding 41
16. Equilibrium dialysis 42
17. Isolation of mutants lacking the phosphate-binding protein 42
18. Construction of a rabbit anti-protein P immunoadsorbant column ............... 44

19. Electrophoretic elution of protein P from SDS-polyacrylamide gels ............. 44

20. Purification of phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonads ..................... 45

21. Black lipid bilayer experiments .................... 47

22. Modified ELISA procedure for demonstrating an association between protein P and the phosphate-binding protein .............. 48
   a. Preparation of protein P ...................... 48
   b. Modified ELISA procedure ................. 49

23. Affinity chromatography method for determining an association between protein P and the phosphate-binding protein ............. 50
   a. Phosphate-binding protein-Sepharose 4B affinity column ................. 50
   b. Protein P-Affigel-10 affinity column .... 50

24. Isolation of regulatory mutants of alkaline phosphatase and phospholipase C ............. 51

25. Other assays .................... 52

Chapter One Outer membrane protein P: involvement in high-affinity phosphate transport in Pseudomonas aeruginosa ..................... 53

1. Induction of protein P by phosphate limitation .......... 53

2. Co-regulation with alkaline phosphatase, phospholipase C and a 34K periplasmic protein .......... 60

3. Outer membrane permeability .................... 63

4. LPS-free protein P forms channels in planar lipid bilayer membranes ............. 69

5. Isolation of a protein P-deficient mutant ............... 73
   a. Preparation of a protein P trimer-specific antiserum ................. 73

vii
<table>
<thead>
<tr>
<th>Chapter Two</th>
<th>Role of a periplasmic phosphate-binding protein in phosphate transport in <em>Pseudomonas aeruginosa</em></th>
<th>89</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Purification and properties of the periplasmic phosphate-binding protein</td>
<td>89</td>
</tr>
<tr>
<td>2.</td>
<td>Isolation of mutants lacking the phosphate-binding protein</td>
<td>95</td>
</tr>
<tr>
<td>3.</td>
<td>Phosphate transport</td>
<td>97</td>
</tr>
<tr>
<td>4.</td>
<td>Kinetics of phosphate transport</td>
<td>100</td>
</tr>
<tr>
<td>5.</td>
<td>Growth in phosphate-deficient medium</td>
<td>103</td>
</tr>
<tr>
<td>6.</td>
<td>Physical association between outer membrane protein P and the periplasmic phosphate-binding protein</td>
<td>103</td>
</tr>
<tr>
<td>7.</td>
<td>Summary</td>
<td>109</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter Three</th>
<th>Immunological cross-reactivity of phosphate-starvation-inducible outer membrane proteins of the families <em>Enterobacteriaceae</em> and <em>Pseudomonadaceae</em></th>
<th>111</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phosphate starvation-induction of membrane proteins of the <em>Pseudomonadaceae</em> and the <em>Enterobacteriaceae</em></td>
<td>111</td>
</tr>
<tr>
<td>2.</td>
<td>Immunological cross-reactivity of phosphate-starvation-inducible outer membrane proteins</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>a. Cross-reactivity of protein oligomers in phosphate-limited cell envelopes</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>b. Identification of the cross-reactive proteins</td>
<td>124</td>
</tr>
</tbody>
</table>
c. Cross-reactivity of phosphate-starvation-induced monomers ....... 129

3. Summary ......................... 130

Chapter Four Characterization of protein P-like porins from the fluorescent Pseudomonadaceae 132

1. Purification of the phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonads ............. 132
2. Single channel experiments .................. 135
3. Ion-selectivity .......................... 142
4. Phosphate inhibition of macroscopic conductance 145
5. Summary ............................... 152

Discussion ............................... 154

1. A phosphate regulon in Pseudomonas aeruginosa . 154
2. Properties of outer membrane protein P ........ 158
3. The outer membrane of Pseudomonas aeruginosa as a permeability barrier to phosphate under limiting conditions ............. 162
4. Protein P and PhoE as members of two distinct classes of phosphate-regulated porins ........... 167
5. Conserved antigenic determinants in phosphate-starvation-inducible outer membrane (porin) proteins ............... 173

Literature Cited ........................... 176
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth of <em>P. aeruginosa</em> in a phosphate-deficient medium</td>
</tr>
<tr>
<td>2</td>
<td>Growth yield of <em>P. aeruginosa</em> as a function of the concentration of phosphate in a defined minimal medium</td>
</tr>
<tr>
<td>3</td>
<td>SDS-polyacrylamide gel electrophoretogram of purified protein P and of outer membranes and shock fluids of phosphate-deficient cells of <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>4</td>
<td>Induction by phosphate limitation and localization of alkaline phosphatase and phospholipase C of <em>P. aeruginosa</em> H103</td>
</tr>
<tr>
<td>5</td>
<td>SDS-polyacrylamide gel electrophoretogram of whole cell protein extracts and cell envelope and soluble (non-membrane) fractions of alkaline phosphatase regulatory mutants</td>
</tr>
<tr>
<td>6</td>
<td>Outer membrane permeability during growth on phosphate-deficient medium</td>
</tr>
<tr>
<td>7</td>
<td>SDS-polyacrylamide gel electrophoretogram of LPS associated with protein P</td>
</tr>
<tr>
<td>8</td>
<td>Immunoblots of electrophoretically separated <em>P. aeruginosa</em> H103 cell envelopes and purified protein P, and whole cells</td>
</tr>
<tr>
<td>9</td>
<td>SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from a protein P-deficient mutant of <em>P. aeruginosa</em> and its wild-type parent</td>
</tr>
<tr>
<td>10</td>
<td>Induction of the 34K periplasmic protein by phosphate limitation</td>
</tr>
</tbody>
</table>
11 SDS-polyacrylamide gel electrophoretogram of purified phosphate-binding protein and whole cell protein extracts of alkaline phosphatase constitutive mutants of P. aeruginosa H242 91

12 Scatchard plot of phosphate-binding activity 93

13 Phosphate uptake in P. aeruginosa 99

14 Kinetics of phosphate uptake in P. aeruginosa 102

15 Growth of a phosphate-binding protein-deficient mutant in a phosphate-limited medium 105

16 SDS-polyacrylamide gel electrophoretogram of cell envelopes prepared from phosphate-deficient and phosphate-sufficient grown strains of the families Pseudomonadaceae and Enterobacteriaceae 116

17 Interaction of protein P trimer-specific or monomer-specific antiserum with Western blots of purified protein P and P. aeruginosa PA01 strain H103 cell envelopes 120

18 Interaction of protein P trimer-specific antiserum with Western blots of cell envelope preparations of different bacteria grown under phosphate-deficient or sufficient conditions 122

19 Two-dimensional (unheated x heated) SDS-polyacrylamide gel electrophoretogram of purified protein P and cell envelopes prepared from phosphate-limited strains of the Pseudomonadaceae and the Enterobacteriaceae 126

20 SDS-polyacrylamide gel electrophoretogram of purified phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonadaceae 134
21 Strip chart recordings of stepwise increases in the conductance of an oxidized cholesterol membrane caused by the phosphate-starvation-inducible outer membrane protein of *P. putida* 138

22 Histogram of the conductance fluctuations observed with membranes of oxidized cholesterol in the presence of the phosphate-starvation-inducible outer membrane protein of *P. putida* 140

23 Average single channel conductance of the phosphate-starvation-inducible porin protein of *P. aureofaciens* as a function of the KCl concentration in the aqueous solution bathing an oxidized cholesterol membrane 147

24 Phosphate inhibition of chloride flux through protein P channels 151
<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Bacterial strains</td>
<td>24</td>
</tr>
<tr>
<td>II Measurements of LPS associated with conventionally purified and electroeluted protein P</td>
<td>71</td>
</tr>
<tr>
<td>III Functional properties of conventionally purified and electroeluted protein P in planar lipid bilayer membranes</td>
<td>72</td>
</tr>
<tr>
<td>IV Plasmids tested for utility in transposon insertion mutagenesis of <em>P. aeruginosa</em></td>
<td>77</td>
</tr>
<tr>
<td>V Kinetics of high-affinity phosphate transport in a protein P-deficient mutant strain and its wild-type parent</td>
<td>84</td>
</tr>
<tr>
<td>VI Growth of a protein P-deficient mutant and strains wild-type for protein P in a phosphate-limited medium</td>
<td>86</td>
</tr>
<tr>
<td>VII Substrate specificity of the phosphate-binding protein</td>
<td>94</td>
</tr>
<tr>
<td>VIII $^{32}$P-orthophosphate binding by periplasmic extracts of wild-type and mutant strains of <em>P. aeruginosa</em></td>
<td>96</td>
</tr>
<tr>
<td>IX <em>In vitro</em> association of the phosphate-binding protein and outer membrane protein P</td>
<td>107</td>
</tr>
<tr>
<td>X Properties of the phosphate-starvation-inducible membrane proteins of the <em>Enterobacteriaceae</em> and the <em>Pseudomonadaceae</em></td>
<td>113</td>
</tr>
<tr>
<td>XI Channel-forming properties of affinity-purified and electroeluted phosphate-starvation-inducible outer membrane oligomers of the fluorescent Pseudomonads</td>
<td>141</td>
</tr>
</tbody>
</table>
XII  Single channel conductance of phosphate-starvation-inducible porin proteins of the fluorescent Pseudomonads in salts of varying anion and cation size 144

XIII  Binding affinities of phosphate-starvation-inducible porin proteins of the fluorescent Pseudomonads for chloride and orthophosphate 148
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;405&lt;/sub&gt;/A&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Absorbance at 405/600 nm</td>
</tr>
<tr>
<td>Cb</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>KDO</td>
<td>2-keto-3-deoxy octulosonic acid</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>Kn</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NPPC</td>
<td>para-Nitrophenyl phosphorylcholine</td>
</tr>
<tr>
<td>P15</td>
<td>phosphate polymer comprising 15 phosphate units</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pNPP</td>
<td>para-Nitrophenyl phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tc</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Tp</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>XP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate-p-toluidine</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Firstly, I thank Bob Hancock, who should already know that he has my warmest appreciation for the guidance, encouragement and friendship he has shown me. As well, my labmates, past and present, have my enduring thanks for their camaraderie and fellowship, without which this experience would have been sorely lacking.

To Jerri, my kindred spirit, whom I first met at the outset of this long journey, I am grateful, for helping me to maintain a semblance of sanity in the insane world that is graduate school. Without her understanding, patience and unfailing confidence in me I could not have come so far. Lastly, I thank my family, especially my mom and dad, who have always encouraged but never pushed me, and who have always shown an interest in my work, even when it was all 'Greek' to them.
INTRODUCTION

Nutrient acquisition by prokaryotic organisms necessarily involves transmembrane translocation of solute molecules. In the case of gram-negative organisms there are two membranes which must be traversed during the unidirectional movement of nutrient molecules from the environment to the cell interior. The mechanisms by which this unidirectional transport occur are specific to the membranes being traversed.

1. The gram-negative cell envelope. Electron microscopic studies have confirmed that the cell envelope of gram-negative bacteria, including Psedomonas aeruginosa, consists of three layers, the cytoplasmic or "inner" membrane, the peptidoglycan or murein layer and the outer membrane (Lugtenberg and van Alphen, 1983). In some instances, and in specific strains, a capsule and/or additional (A)-layer is also present external to the tripartite cell envelope (Glauert and Thornley, 1969; Schleytr, 1978; Troy, 1979).

   a. The cytoplasmic membrane. The membrane bounding the cytoplasm is comprised of approximately equimolar amounts of phospholipid and protein in a typical lipid bilayer (Lugtenberg and van Alphen, 1983). The hydrophobicity of this membrane (Machtiger and Fox, 1973) makes it a barrier to hydrophilic molecules although hydrophobic molecules diffuse relatively freely across it.
(Teuber et al., 1977). As such, the inner membrane functions as a highly specific permeability barrier to hydrophilic molecules, with solute translocation dependent upon the presence of specific energy-requiring transport systems in the membrane (Wilson, 1978). In certain transport systems, water-soluble binding proteins present in the space between the inner and outer membrane (the periplasm (Mitchell, 1961)) function in concert with inner membrane transport proteins in the uptake of solute molecules (Oxender, 1972; Wilson and Smith, 1978; Hoshino and Nishio, 1982; Eisenberg and Phibbs, 1982). The energy for nutrient uptake is derived from the electrochemical gradient of protons across the inner membrane (the proton motive force) generated from the primary active transport of H+ ions during respiration or ATP hydrolysis and/or from phosphate-bond energy in the form of ATP or related metabolites (Berger and Heppel, 1974; Harold, 1977; Hong et al., 1979).

b. The peptidoglycan. The peptidoglycan exists as a network of linear amino sugars (N-acetyl glucosamine and N-acetyl muramic acid) covalently linked via cross-bridges between tetrapeptides attached to N-acetyl muramic acid (Schleifer and Kandler, 1972). Originally described as a rigid monolayer present in the periplasmic space (Braun et al., 1973) the peptidoglycan of enteric organisms has recently been suggested to exist as a hydrated 'gel' which probably occupies the entire space between the outer and cytoplasmic membranes (Hobot et al., 1984). In addition,
the peptidoglycan is covalently cross-linked to the outer membrane via a protein analogous to the major lipoprotein of Escherichia coli (Braun, 1975; Lugtenberg et al., 1977). Although the peptidoglycan of P. aeruginosa is not chemically different from that of enteric organisms (Meadow, 1975), it is probably not covalently linked to the outer membrane (Hancock et al., 1981a). There is no evidence to suggest that the peptidoglycan provides a barrier to solute molecules during transport, although, together with membrane-derived oligosaccharides (MDOs) (van Golde et al., 1973) it provides fixed localized charges within the periplasm which gives rise to a Donnan potential across the outer membrane (inside negative) (Stock et al., 1977). This potential may play a role in the movement of appropriately charged substrate molecules across the outer membrane (Costerton, 1970).

c. The outer membrane. The outer membrane is comprised of phospholipids, protein and lipopolysaccharide (LPS) a lipidic molecule unique to gram-negative bacteria (Lugtenberg and van Alphen, 1983). LPS is an amphipathic molecule possessing a hydrophobic portion, lipid A, embedded in the membrane, and a hydrophilic polysaccharide portion extending out from the cell surface (Luderitz et al., 1982). The distal portion of the polysaccharide, the O antigen, usually consists of repeating oligosaccharide units which exhibit wide variability even within a single species (Luderitz et al., 1982). Like the inner membrane, the outer
membrane appears as a bilayer in the electron microscope (Glauert and Thornley, 1969). However, the outer membrane is unusual in that the phospholipid is present exclusively (except, perhaps, in certain mutants) in the inner leaflet of the bilayer, while essentially all of the LPS occurs in the outer leaflet (Muhlradt and Golecki, 1975; Funahara and Nikaido, 1980). The proteins of the outer membrane occur in both leaflets and in some cases actually span the entire membrane (Lugtenberg and van Alphen, 1983). Unlike the inner membrane, the outer membrane lacks a hydrophobic uptake pathway (Nikaido, 1976), probably due to the presence of negatively charged LPS molecules on its outer surface, and functions as a non-specific permeability barrier to hydrophilic molecules (Nikaido, 1979). In the case of enteric bacteria this serves to protect the organisms from the detergent-like actions of bile salts, fatty acids and glycerides, as well as from proteolytic and lipolytic enzymes and glycosidases present in the gut (Lugtenberg and van Alphen, 1983). The \textit{P. aeruginosa} outer membrane, which has been implicated in the high intrinsic resistance of this organism to antibiotics (Angus \textit{et al.}, 1982; Yoshimura and Nikaido, 1982; Nicas and Hancock, 1983), may well serve a similar role in nature since \textit{P. aeruginosa} is commonly found in the soil where antibiotic producing organisms are also found.
Hydrophilic solute molecules below a defined molecular weight cutoff (the exclusion limit) are capable of permeating the outer membrane via a passive diffusion process (Nikaido, 1979) mediated by a class of proteins of molecular weights 35,000-45,000, called porins. These proteins form water-filled channels through the hydrophobic core of the outer membrane (Hancock et al., 1979; Nikaido, 1979). Porins exist in the outer membranes as trimers (Tokunaga et al., 1979; Angus et al., 1983; Maezawa et al., 1983), are non-covalently attached to the peptidoglycan (Lugtenberg et al., 1977; Hancock et al., 1981a) and are usually isolated in association with LPS (Furukawa et al., 1979; Schindler and Rosenbusch, 1978). LPS association is not, however, essential for porin function in vitro (Parr et al., in press) although it has been proposed to be involved in modulating in vivo porin activity (Kropinski et al., 1982).

The exclusion limit of the *P. aeruginosa* outer membrane is significantly larger than that of *E. coli* or *S. typhimurium* outer membranes (Mr 3,000-9,000 compared with 600-700) (Nakae and Nikaido, 1975; Nakae, 1976; Hancock and Nikaido, 1978), an observation consistent with the formation in model systems of larger channels by the major porin protein F of *P. aeruginosa* (2.2 nm dia.) than by proteins OmpF (1.1 nm) and OmpC (1.0 nm), the major *E. coli* porins (Benz et al., 1985). Interestingly, however, the outer membrane of *P. aeruginosa* is less permeable than that of
E. coli (Angus et al., 1982; Yoshimura and Nikaido, 1982; Nicas and Hancock, 1983), which has lead to the suggestion that only a small percentage of protein F molecules form functional pores in vivo.

2. The role of the outer membrane in transport. The permeability of the outer membrane, mediated by porins, provides a pathway for the entry of nutrient molecules (e.g. sugars, amino acids, nucleosides, ions). Because porins in general are non-specific, exhibiting only weak ion-selectivity in reconstituted planar bilayer membranes (Benz et al., 1985), nutrient molecules cross the outer membrane down their respective concentration gradients. The rate of diffusion (V) of solute molecules across the outer membrane can be described by the equation $V = P \times A \times [C_o - C_i]$ (Fick's first law of diffusion) where $P$ is the permeability co-efficient, $A$ is the area of the membrane and $C_o$ and $C_i$ represent the concentration of substrate outside (in the external medium) and inside (in the periplasm) the cell, respectively (Yoshimura and Nikaido, 1982). The presence of high-affinity periplasmic binding proteins, and of cytoplasmic membrane transport systems of low $K_m$, functions to maintain low periplasmic concentrations of these solutes so that, in the presence of sufficient extracellular concentrations of these nutrients, this gradient is usually sufficient to transport levels of nutrients which are not limiting for transport or growth. However, sufficiently low
external concentrations of nutrient molecules or a decrease in outer membrane permeability resulting from, for example, the porin-deficiency of mutant strains (Von Meyenburg, 1971; Lutkenhaus, 1977; Nicas and Hancock, 1983), have been demonstrated to decrease the rate of solute movement across the outer membrane such that the overall rates of transport (Lutkenhaus, 1977) and growth (Von Meyenburg, 1971) are limited by diffusion across the outer membrane. In the case of permeability mutants, reduction of outer membrane permeability increases the Km of the overall transport process despite the fact that upon diffusion across the outer membrane a substrate is transported across the cytoplasmic membrane via a specific system with a very low Km. Nonetheless, the effect of porin-deficiency on transport and growth is seen only at lower concentrations of substrate since it is theoretically (from Fick's first law) and practically (Von Meyenburg, 1971; Lutkenhaus, 1977) possible to restore a non-limiting rate of diffusion across the outer membrane of porin-deficient mutants simply by increasing the external substrate concentration.

Pseudorevertants of porin-deficient mutants which express novel porin proteins (Henning et al., 1977; Von Meyenburg and Nikaido, 1977; Van Alphen et al., 1978; Pugsley and Schnaitman, 1978; Chai and Foulds, 1979) restore the cell's ability to transport non-limiting concentrations of solute across the outer membrane, in the presence of sufficient external concentrations of nutrient molecule.
Similarly, the rate-limiting diffusion of nutrient molecules across the outer membrane resulting from low extracellular nutrient concentrations is compensated for in some bacteria by the synthesis of inducible outer membrane proteins which function in the facilitated uptake of the limiting nutrient. Examples include the phosphate-starvation-inducible PhoE proteins of the Enterobacteriaceae (Overbeeke and Lugtenberg, 1980; Sterkenburg et al., 1984; Bauer et al., 1985) and the iron regulated outer membrane proteins found in many bacterial species (Ernst et al., 1978; Braun and Hantke, 1982; Sciortino and Finklestein, 1983; Williams et al., 1984; Brown et al., 1984). In addition, novel membrane proteins are sometimes produced in cases where the nutrient molecule permeates the outer membrane poorly via the major porin proteins. Such proteins include the maltose/maltodextrin LamB (Ferenci and Boos, 1980) and the Tsx nucleoside transport (Hantke, 1976) proteins of E. coli.

a. The LamB protein. Although inducible in E. coli strains grown in non-limiting concentrations of maltose (Braun and Krieger-Bauer, 1977), the LamB protein, which functions as the phage lambda receptor, appears to be an essential component of maltose transport at low (< 10 uM) - but not at high (> 1 mM) concentrations of maltose (Szmelcman and Hofnung, 1975; Szmelcman et al., 1976). Studies involving the reconstitution of the purified protein into liposomes have demonstrated that the LamB protein indeed exhibits a marked preference for maltose over other
disaccharides, facilitating the diffusion of maltose into liposomes 40 times faster than, for example, sucrose (Luckey and Nikaido, 1980a). The LamB protein also serves as an efficient channel for the uptake of maltodextrins (up to maltoheptaose) which exceed the exclusion limits of the major E. coli porins (Luckey and Nikaido, 1980a; Nakae and Ishii, 1980). Mutants deficient in LamB protein exhibit a 1000-fold increase in the Km for maltose transport (from 1.0 uM to 1 mM) (Szmelcman et al., 1976) and are severely defective in transporting maltotriose (Szmelcman et al., 1976), while maltotetraose and higher molecular weight maltodextrins are not accumulated at all (Ferenci, 1980). The affinity of the channel for maltose and maltodextrins increases with increasing chain length of the dextrin (Km for maltose=14 mM; Km for maltodecanose=75 uM) (Ferenci et al., 1980) and has been attributed to binding sites in/near the channel (Ferenci et al., 1980; Luckey and Nikaido, 1980b).

The LamB protein is a component of a maltose operon in E. coli which includes, in addition to inner membrane transport proteins and cytoplasmic catabolic enzymes, a periplasmic high-affinity maltose binding protein (Dietzel et al., 1978). The binding protein was demonstrated to bind maltose and maltodextrins in the micromolar range (Wandersmann et al., 1979) in agreement with the observed kinetics of transport (Ks = 1.0 uM) (Szmelcman et al., 1976). Furthermore, a physical association between the
binding protein and the LamB porin was observed in vitro (Bavoil and Nikaido, 1981) confirming electron microscopic data which showed that the maltose-binding protein associated with the periplasmic face of LamB-containing outer membranes (Boos and Staehlin, 1981). Such an association was suggested to be necessary for the efficient transport of maltose and maltodextrins across the outer membrane in vivo (Wandersman et al., 1979; Luckey and Nikaido, 1983).

In addition to its role in maltose and maltodextrin uptake, the LamB channel functions as a general diffusion channel as well. In vitro studies have confirmed the ability of a number of amino acids and unrelated sugars (Nakae, 1979; Luckey and Nikaido, 1980a) as well as ions (Boehler-Kohler et al., 1979) to permeate the channel. LamB may also be capable of replacing the major porins in vivo in revertants of porin-deficient mutants (Von Meyenburg and Nikaido, 1977).

An analogous protein, designated protein D1, has been identified in the outer membrane of P. aeruginosa cells growing in glucose-containing media (Hancock and Carey, 1979). Co-regulated with a binding protein-dependent high-affinity glucose transport system (Midley and Dawes, 1973; Stinson et al., 1977), this 46,000 molecular weight protein forms channels in liposomes which are selectively permeable to glucose (Hancock and Carey, 1980).
b. The PhoE protein. Inducible in *E. coli* under conditions of phosphate-limitation (Overbeeke and Lugtenberg, 1980), porin protein PhoE was first identified in revertants of porin-deficient mutants (Henning *et al*., 1977; Van Alphen *et al*., 1978; Pugsley and Schnaitman, 1978; Chai and Foulds, 1979). The purified protein has been demonstrated to form large (1 nm dia) (Benz *et al*., 1985), weakly anion-selective (Benz *et al*., 1984) channels in reconstituted bilayer membranes. Examination of the transport properties of mutants expressing PhoE (previously protein e) as the sole porin revealed that a wide range of nutrients (sugars, amino acids, nucleosides, ions) could permeate the channel in vivo (Lugtenberg *et al*., 1978; Van Alphen *et al*., 1978), consistent with the formation of a general diffusion channel. The protein is immunologically cross-reactive with the major porin proteins OmpF and OmpC (Overbeeke *et al*., 1980), exhibiting 70% amino acid homology with OmpF (Tommassen *et al*., 1982) and 61% amino acid homology with OmpC (Mizuno *et al*., 1983). In addition, the cloned *phoE* and *ompF* genes have been demonstrated to hybridize in regions along their entire lengths (Tommassen *et al*., 1982).

Despite these similarities with the major porins, the PhoE channel exhibits properties consistent with a presumed role in phosphate acquisition. A component of the phosphate (*pho*) regulon in *E. coli* (Tommassen and Lugtenberg, 1982) (see section 4) the protein forms a channel which is more efficient in the uptake of anionic and phosphorylated
compounds than either OmpF or OmpC (Overbeeke and Lugtenberg, 1982). Overbeeke and Lugtenberg (1982) also demonstrated that a mutant deficient in PhoE grew more slowly than wild type in the presence of polyphosphate (P15) as the sole phosphate source. Furthermore, Korteland et al (1982) have demonstrated that a PhoE-deficient mutant exhibited a 10-fold increase in the Km for phosphate transport compared with strains expressing a PhoE channel. Unfortunately, this result was obtained in a porin-deficient background, rather than a background wild type for the major porins. Therefore, it was not possible to conclude whether the increase in Km for phosphate in the PhoE-deficient strain reflected a specific role for protein PhoE in phosphate transport, or whether any porin would have reversed the poor phosphate transport of the porin-deficient strain.

PhoE proteins have been identified in other Enterobacteriaceae, including Salmonella typhimurium (Bauer et al., 1985) and Enterobacter cloacae (Verhoef et al., 1984). These proteins form anion-selective channels consistent with their presumed roles in phosphate acquisition. A 36 kDa outer membrane protein inducible by phosphate-limitation has also been identified in Klebsiella aerogenes (Sterkenburg et al., 1984) although it was not assayed for porin function.

Although iron is an abundant metal in nature it occurs under aerobic conditions (at pH 7) as ferric hydroxide with low
water solubility (equilibrium concentration of $10^{-12}$ uM) (Braun and Hantke, 1982). Cells of *E. coli*, for example, require an iron concentration of approximately 0.1 uM for growth and to gain sufficient iron they must produce iron chelators (e.g. ferrichrome; enterochelin) concomittant with transport systems for these chelates (Hantke and Braun, 1975; Wayne and Neilands, 1975). A number of high molecular weight outer membrane proteins have been identified which are co-regulated with these chelators under iron-limiting conditions (Braun *et al.*, 1976; Hancock and Braun, 1976; McIntosh and Earhart, 1977). Two of these, the products of the *fhuA* (*tonA*) (Hantke and Braun, 1975) and *fepA* (*feuB*, *cbr*) (Pugsley and Reeves, 1976; McIntosh and Earhart, 1977; Wookey and Rosenberg, 1978) genes, function in the uptake of ferric-ferrichrome and ferric-enterochelin, respectively. This was supported by data which demonstrated directly the ability of the respective iron chelates to bind to their receptor proteins in the outer membrane (Braun and Hantke, 1977; Ichihara and Mizushima, 1978) and by the inactivation of specific transport systems in mutants deficient in the corresponding outer membrane proteins (Pugsley and Reeves, 1976; Wookey and Rosenberg, 1978). Similar proteins have been identified in other gram-negative bacteria, including *Salmonella typhimurium* (Ernst *et al.*, 1978), *Neisseria gonorrhoeae* (Norqvist *et al.*, 1978), *Vibrio cholerae* (Sciortino and Finkelstein, 1983), *Klebsiella aerogenes* (Williams *et al.*, 1984) and *Pseudomonas aeruginosa*
(Brown et al., 1984).

d. Others. The transport of vitamin B12 (cyanocobalamin) by \textit{E. coli} is a biphasic process involving an energy-independent rapid binding phase followed by an energy-dependent phase (DiGirolamo and Bradbeer, 1971). The initial vitamin B12 binding sites are firmly embedded in the outer membrane (DiGirolamo \textit{et al.}, 1971; White \textit{et al.}, 1973) and have been identified as the protein products of the \texttt{btuB} (bfe) gene (White \textit{et al.}, 1973; DiMasi \textit{et al.}, 1973; Kadner and Liggins, 1973). A minor outer membrane protein, the \texttt{btuB} gene product has not been shown to exhibit any porin function, although, together with LPS and the OmpF protein, it has been identified as a constituent of the colicin A receptor (Chai \textit{et al.}, 1982). The proximity of the vitamin B12 receptor and major porin protein OmpF \textit{in vivo} may be significant in terms of the mechanism by which vitamin B12 actually crosses the outer membrane.

The transport of nucleosides by \textit{E. coli} cells reportedly involves an outer membrane protein, the \texttt{tsx} gene product, which forms an especially efficient channel for nucleosides (Hantke, 1976). Although nucleosides are capable of permeating the outer membrane via the OmpF and PhoE channels (van Alphen \textit{et al}, 1978), their diffusion through these channels is expected to be slow due to the large size of nucleosides. Furthermore, the exceptionally high \(V_{\text{max}}\) of the nucleoside active transport system (Koch, 1971) probably necessitates a specific channel in the outer membrane.
3. **Bacterial phosphate transport — with specific reference to E. coli.** Inorganic phosphate transport has been characterized in a number of bacterial systems, including *Staphylococcus aureus* (Mitchell, 1954), *Bacillus cereus* (Rosenberg et al., 1969), *Micrococcus lysodeikticus* (Friedberg, 1977), *Streptococcus faecalis* (Harold and Baarda, 1966), *E. coli* (Medveczky and Rosenberg, 1971) and *P. aeruginosa* (LaCoste et al., 1981). In each case, the transport is concentrative, energy-dependent and inhibitable to varying degrees by arsenate, a phosphate analogue. In addition, the rate of and capacity for phosphate uptake appears, in many cases, to increase at low concentrations of phosphate suggesting that the transport systems involved are inducible.

The transport of inorganic phosphate by *E. coli* has been characterized in detail and two major systems of uptake, the PST and PIT systems, have been identified (Willsky et al., 1973). The PST system, which operates at approximately 20% of capacity in the presence of high levels of phosphate (Rosenberg et al., 1977), is completely derepressed under phosphate-limiting (< 1.0 mM) conditions (Rosenberg et al., 1977). A high-affinity system (Km = 0.16 to 0.43 μM) (Rosenberg et al., 1977; Willsky and Malamy, 1980) the PST phosphate transport system is responsible for the bulk of phosphate transport under limiting conditions. The PIT system, which operates constitutively, is of low affinity (Km = 25 to 38 μM) (Rosenberg et al., 1977; Willsky
and Malamy, 1980), and is the major transport system for phosphate under phosphate-sufficient conditions.

The PST system is comprised of the products of at least 5 genes (pst, phoU, phoV, phoS, phoT) (Levitz et al., 1984), one of which, the product of the phoS gene, functions as a periplasmic phosphate-binding protein (Gerdes and Rosenberg, 1974). The remaining gene products have not been identified, although they are probably localized in the cytoplasmic membrane (Tommassen and Lugtenberg, 1982). The phosphate-binding protein is inducible under conditions of phosphate-limitation (Yagil et al., 1976) and binds phosphate with high-affinity (Kd=0.8 uM) (Medveczky and Rosenberg, 1970), accounting for the inducibility and low Km of the PST transport system. Osmotic shock and spheroplast formation, both of which result in the release of the binding protein from whole cells, have been demonstrated to inactivate the PST system (Gerdes et al., 1977). Typical of binding protein-dependent transport systems in general, phosphate uptake via the PST system utilizes phosphate bond energy, in the form of ATP or a related metabolite, as the energy source (Rosenberg et al., 1979). The recent demonstration that the ornithine-arginine binding protein of E. coli is phosphorylated - dephosphorylated during substrate transport (Celis, 1984) may be a clue as to the role ATP (or a related metabolite) plays in binding protein-dependent transport. Although arsenate was capable of inhibiting phosphate uptake via the PST system (Ki = 0.39 uM) (Willsky and Malamy, 1980)
it was not transported by this system and cells expressing only the PST phosphate transport system were capable of growth in the presence of arsenate.

The low-affinity PIT phosphate transport system involves the product of a single known gene, pit (Bennet and Malamy, 1970; Willsky et al., 1973; Sprague et al., 1975), which is probably an inner membrane protein. Inorganic phosphate uptake via this system is not sensitive to spheroplast formation (Rosenberg et al., 1977), consistent with the absence of a binding protein associated with it. Characteristic of shock-resistant transport systems, phosphate transport via the PIT system is coupled to the proton motive force (Rosenberg et al., 1979). PIT-mediated phosphate transport is inhibited by arsenate which appears to be transported equally well by this system (Willsky and Malamy, 1980). Cells expressing only the PIT system cannot grow in an arsenate-containing medium in which they suffer an almost total depletion of intracellular ATP levels (Willsky and Malamy, 1980).

In addition to the two major inorganic phosphate uptake systems described above, three organophosphate transport systems have also been identified in E. coli with roles in hexose phosphate (the uhp operon) (Kornberg and Smith, 1969) and glycerol-3-phosphate (the gipT (Lin, 1976) and ugp (Schweizer et al., 1982) operons) transport. Two of these, involving the constituents of the glucose-6-phosphate-inducible uhp operon and the glycerol-3-phosphate-inducible
glpT operon appear to be pathways for the uptake of inorganic phosphate as well (Willsky and Malamy, 1974). The other involves the products of the ugpA and ugpB genes (Schweizer et al., 1982), which encode an inner membrane permease and a periplasmic glycerol-3-phosphate-binding protein, respectively (Tommassen and Lugtenberg, 1982). This system is derepressed upon phosphate-limitation (Argast and Boos, 1980) and forms part of a phosphate or pho regulon in E. coli (Schweizer et al., 1982; Tommassen and Lugtenberg, 1982) (see below).

Interestingly, P. aeruginosa also appears to possess two major transport systems for inorganic phosphate, of low and high-affinity, respectively (LaCoste et al., 1981), as well as an uptake system for glycerol-3-phosphate (Siegel and Phibbs, 1979). The transport of inorganic phosphate by P. aeruginosa is somewhat sensitive to osmotic shock, consistent with the involvement of a periplasmic binding protein. In addition, the two uptake systems appear to exhibit different energy requirements similar to the situation in E. coli.

4. The pho regulon of E. coli. Under conditions of phosphate-limitation, wild type cells of E. coli are derepressed for the synthesis of numerous proteins (Tommassen and Lugtenberg, 1982), and at least 18 phosphate-starvation-inducible genes have been described (Wanner et al., 1981). The roles of these gene products in the
scavenging of phosphate and phosphate-containing molecules from a dilute environment has, in many cases, been addressed (Tommassen and Lugtenberg, 1982). The phosphate-starvation-inducible proteins which have been identified include periplasmic binding proteins for phosphate (the phoS gene product) (Gerdes and Rosenberg, 1974) and glycerol-3-phosphate (the ugpB gene product) (Argast and Boos, 1980), a periplasmic alkaline phosphatase (the phoA gene product) (Torriani, 1960; Brickman and Beckwith, 1975), cytoplasmic membrane permeases for phosphate (the pst gene product) (Rosenberg et al., 1977) and glycerol-3-phosphate (the ugpA gene product) (Argast and Boos, 1980), an outer membrane pore-forming protein (the phoE gene product) (Overbeeke and Lugtenberg, 1980) and a cytoplasmic polyphosphatase (Yagil, 1975). Several presumably cytoplasmic regulatory molecules, including the products of the phoB (Makino et al., 1982; Tommassen and Lugtenberg, 1982), phoM (Ludtke et al., 1984) and phoR (Tommassen et al., 1982) genes have also been identified. Most if not all of these proteins are part of a single regulon, designated the pho regulon, which exhibits some similarity to the maltose regulon of E. coli which is also inducible for an outer membrane protein, a periplasmic binding protein, cytoplasmic membrane carrier proteins and cytoplasmic catabolic enzymes (Hengge and Boos, 1983). Phosphate-starvation-inducible proteins identified in P. aeruginosa include an extracellular phospholipase C (Stinson and Hayden, 1979) and an alkaline phosphatase which occurs
in both the periplasm and the extracellular medium (Hou et al., 1966; Cheng et al., 1979). A number of additional phosphate-repressible proteins have been identified in phospholipase C regulatory mutants of *Pseudomonas aeruginosa* (Gray et al., 1982) although their functional activities have not been elucidated.

The components of the *E. coli* pho regulon are under the control of a complex network of regulatory proteins which includes the products of three known genes - *phoB*, *phoM* and *phoR* (Tommassen and Lugtenberg, 1982). As a model for the regulation of pho regulon constituents, production of the *phoA* gene product (alkaline phosphatase) has been studied in detail (Echols and Garen, 1961; Brickman and Beckwith, 1975; Bracha and Yagil, 1973; Wanner and Latterell, 1980). Mutant studies and studies involving the cloned genes have indicated that the *phoB* gene product functions as a transcriptional activator of *phoA* (Bracha and Yagil, 1973; Brickman and Beckwith, 1975), while the *phoR* gene product acts as a repressor (high phosphate) (Echols et al., 1961) and activator (low phosphate) (Wanner and Latterell, 1980), the latter function being at least partially replaceable by the *phoM* gene product (Wanner and Latterell, 1980). Based on results of mutant studies it was also concluded that the *phoB* gene product was itself co-regulated with alkaline phosphatase and that *phoB* transcription was probably under the control of the *phoR* and *phoM* gene products (Tommassen et al., 1982). It remains to be seen whether phosphate acts
directly (as co-repressor) or indirectly in regulating this process.
METHODS

1. Media and growth conditions. The minimal medium used in this study contained 0.1 M N-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonate (Hepes) (pH 7.0), 0.5 mM MgSO$_4$, 7 mM (NH$_4$)$_2$SO$_4$, 20 mM potassium succinate or 0.4 % (wt/vol) glucose as the carbon source, 0.1 % (wt/vol) trace ion solution (as described by Hancock et al., 1981b) and either 0.2 mM potassium phosphate buffer (pH 7.0) for phosphate-deficient medium (with exceptions, see below) or 0.6-1.0 mM potassium phosphate buffer (pH 7.0) for phosphate sufficient medium. Amino acids were included, as required, at a final concentration of 1 mM. When the culture organism was Pseudomonas cepacia, Pseudomonas pseudomallei or Pseudomonas acidovorans the phosphate-deficient medium contained 0.1 mM phosphate. When the culture organism was Klebsiella pneumoniae, Enterobacter aerogenes or Serratia marcesens the phosphate-deficient medium contained 0.15 mM phosphate. Xanthomonas maltophilia (previously Pseudomonas maltophilia) cultures were supplemented with 1 mM methionine. L-broth [1 % (wt/vol) tryptone/ 0.5 % (wt/vol) yeast extract/ 0.05 % (wt/vol) NaCl] and proteose peptone No. 2 [1 % (wt/vol)] were used as the rich media throughout.

Liquid cultures were grown with vigorous aeration at 37°C unless otherwise indicated. Strains were routinely maintained on L-broth agar (L-agar) or phosphate-sufficient medium agar plates prepared by including 2 % (wt/vol)
Bactoagar (Difco) in L-broth and phosphate-sufficient minimal medium respectively.

Antibiotics were used in selective media at the following concentrations: tetracycline (Tc), 200 µg/ml; kanamycin (Kn), 300 µg/ml; carbenicillin (Cb), 1 mg/ml; mercuric chloride (HgCl₂), 15 µg/ml and trimethoprim (Tp), 1 mg/ml.

2. **Bacterial strains.** The bacterial strains and plasmids used in this study are listed in Table I. Plasmid pMT1000 was introduced into strain H103 by conjugation with PAO1594(pMT1000) on L-agar plates. Equal volumes of mid-log phase donor and recipient cells (grown in L-broth) were mixed and pelleted by centrifugation. The supernatant was decanted and the pellet resuspended gently in 0.05 ml of L-broth. The cells were spread over approximately one-third of the surface of an L-agar plate and incubated for 2 h at 30°C. The mating mixture was then resuspended in 1 ml of phosphate-sufficient medium, centrifuged and washed several times in the same medium. Transconjugants were selected at 30°C on phosphate-sufficient minimal medium containing 100 µg/ml Tc.

3. **Cell fractionation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Whole cell protein extracts were obtained as described by Nicas and Hancock (1980). Briefly, overnight cultures were centrifuged and
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa PAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H103</td>
<td>PAO1 wild type</td>
<td>Hancock &amp; Carey, 1979</td>
</tr>
<tr>
<td>H103(pMT1000)</td>
<td>contains plasmid pMT100</td>
<td>This study</td>
</tr>
<tr>
<td>H242</td>
<td>PAO1 <strong>thr-102</strong></td>
<td>S. Benson</td>
</tr>
<tr>
<td>H287</td>
<td>ATCC #19305</td>
<td></td>
</tr>
<tr>
<td>H553</td>
<td>Tn501 insertion mutant of H103 non-derepressible for alkaline phosphatase, phospholipase C, phosphate-binding protein and protein P</td>
<td>This study</td>
</tr>
<tr>
<td>H556</td>
<td>Tn501 insertion mutant of H103 requiring arginine</td>
<td>This study</td>
</tr>
<tr>
<td>H576</td>
<td>Tn501 insertion mutant of H103 non-derepressible for protein P</td>
<td>This study</td>
</tr>
<tr>
<td>H585</td>
<td>Phosphate-binding protein deficient mutant of H242 selected as constitutive for alkaline phosphatase</td>
<td>This study</td>
</tr>
<tr>
<td>H586</td>
<td>Alkaline phosphatase constitutive mutant of H242 producing a defective phosphate-binding protein</td>
<td>This study</td>
</tr>
<tr>
<td>H587</td>
<td>Mutant strain of H242 constitutive for alkaline phosphatase, phospholipase C, phosphate-binding protein and protein P.</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1594</td>
<td><strong>met-28  ilv-202  rmo-53</strong> str-1</td>
<td>M. Tsuda, Tokyo</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>P. aeruginosa PAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1594(pMT1000)</td>
<td>contains plasmid pMT1000</td>
<td>Tsuda et al, 1984</td>
</tr>
<tr>
<td><strong>Pseudomonadaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. putida</td>
<td>ATCC # 12633T</td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>ATCC # 949</td>
<td></td>
</tr>
<tr>
<td>P. chlororaphis</td>
<td>ATCC # 9446T</td>
<td></td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>ATCC # 13985T</td>
<td></td>
</tr>
<tr>
<td>P. cepacia</td>
<td>ATCC # 25609T</td>
<td></td>
</tr>
<tr>
<td>P. pseudomallei</td>
<td>ATCC # 23343T</td>
<td></td>
</tr>
<tr>
<td>P. acidovorans</td>
<td>strain 29</td>
<td>Warren, 1979</td>
</tr>
<tr>
<td><strong>P. stutzeri</strong></td>
<td>ATCC # 17588T</td>
<td></td>
</tr>
<tr>
<td><strong>P. syringae</strong></td>
<td>ATCC # 19310T</td>
<td></td>
</tr>
<tr>
<td><strong>P. solanacearum</strong></td>
<td>ATCC # 11696T</td>
<td></td>
</tr>
<tr>
<td><strong>P. maltophilia</strong></td>
<td>ATCC # 13637T</td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacteriaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>strain HMS174</td>
<td>R.A.J. Warren, U.B.C.</td>
</tr>
<tr>
<td></td>
<td>OmpF+ OmpC+</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>strain JF700</td>
<td>Foulds and Chai, 1978</td>
</tr>
<tr>
<td></td>
<td>OmpF- OmpC+</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> K12</td>
<td>strain JF694</td>
<td>Foulds and Chai, 1979</td>
</tr>
<tr>
<td></td>
<td>OmpF- OmpC- PhoE&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
Table I. - continued

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella typhimurium</td>
<td>strain SL1906</td>
<td>Stocker et al, 1979</td>
</tr>
<tr>
<td></td>
<td>OmpC⁺ OmpD⁻ OmpF⁺</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC # 13883T</td>
<td></td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>ATCC # 13048T</td>
<td></td>
</tr>
<tr>
<td>Serratia marcesens</td>
<td>ATCC # 13880T</td>
<td></td>
</tr>
</tbody>
</table>

a ATCC, American Type Culture Collection; only the relevant phenotypes are indicated; T, type strain; PhoEc, constitutive for PhoE
the cells resuspended in 2% (wt/vol) sodium dodecyl sulfate (SDS)/20 mM Tris-HCl (pH 8.0). After treatment at 100°C for 10 min, residual cells were removed by centrifugation at 27,000 x g for 10 min and the resulting supernatant sonicated (1 min, setting 5, Biosonik sonicator, Bronwill Scientific, Rochester, NY) to shear DNA and reduce viscosity.

The preparation of cell envelopes was based on the method of Nicas and Hancock (1980). Cells from overnight or logarithmic phase cultures were centrifuged, resuspended in 15 mM Tris-HCl (pH 8.0) containing 10 μg/ml pancreatic deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO) and broken in a French pressure cell (Aminco) at 11,500-13,000 psi. Unbroken cells were removed by centrifugation (1,000 x g for 10 min) and the resulting supernatant centrifuged at 160,000 x g for 1 h. The cell envelope pellet was resuspended in deionized water.

Outer membranes were prepared using the two-step gradient method described by Hancock and Carey (1979). Cell envelopes prepared in 15 mM Tris-HCl (pH 8.0)/20% (wt/vol) sucrose were layered onto a sucrose step gradient of 60% (wt/vol) (top layer) and 70% (wt/vol) (bottom layer) and centrifuged overnight at 183,000 x g in a Beckman SW41 Ti rotor (Beckman Instruments Inc., Palo Alto, CA). A single outer membrane band was obtained at the interface of the two sucrose solutions.

Triton X-100-Tris, Triton X-100-Tris-EDTA and Triton X-100-Tris-lysozyme extraction of outer membranes was exactly
as described by Hancock et al. (1981a).

SDS-polyacrylamide gel electrophoresis was performed as described by Hancock and Carey (1979) using a 12 % (wt/vol) acrylamide running gel.

Two-dimensional (unheated x heated) SDS-polyacrylamide gel electrophoresis was based on a method described previously (Russel, 1976). Samples solubilized at room temperature were electrophoresed on an SDS-polyacrylamide slab gel and the lanes excised (1st dimension). The gel strips were then placed in screw capped tubes containing 2 % (wt/vol) SDS/20 mM Tris-HCl (pH 6.8) and heated at 88°C for 10 min. The heated gel strips were laid horizontally across the top of a second SDS-polyacrylamide slab gel, sealed in place with 0.8 % agarose (Biorad, Richmond, CA) and electrophoresed again (2nd dimension). Where indicated, urea was included in the second dimension slab gels at a final concentration of 6M.

4. Purification of protein P. The solubilization in Triton X-100-EDTA of outer membranes from phosphate-deficient cells and chromatography on a DEAE-Sephacel column was exactly as described previously for protein D1 purification (Hancock and Carey, 1980). Protein P-containing fractions (exhibiting some contamination with protein F) were pooled and concentrated 5-fold by dialysis against 20 % (wt/vol) polyethyleneglycol 20,000 (Sigma Chemical Co.). To this pooled concentrate a 4-fold excess of SDS over Triton X-100
(i.e. 2 % (wt/vol) SDS) was added and the solution was made 3 mM for sodium azide. This solution was added to a Sepharose 4B column (46 x 2 cm) pre-equilibrated with 0.1 % (wt/vol) SDS/ 5 mM Tris-HCl (pH 8.0)/ 3 mM sodium azide (column buffer) and eluted with column buffer. Three-milliliter fractions were collected at 12 ml/h and tested for absorbance at 280 nm and for protein composition on SDS-polyacrylamide gels. Protein P, slightly contaminated with protein F, eluted just after the void volume, whereas the Triton X-100 eluted in subsequent fractions. The protein P-containing fractions were again pooled, concentrated and reapplied to the Sepharose 4B column described above. The resultant protein P peak was homogeneous as determined by SDS-polyacrylamide gel electrophoresis.

5. Acetylation of protein P. Protein P was acetylated using acetic anhydride as described by Tokunaga et al (1981). Protein P (500 ug in 1.2 ml of 0.1 % (wt/vol) SDS/10 mM Tris-HCl (pH 8.0)/ 3 mM sodium azide) was diluted in 50 mM sodium phosphate buffer (pH 6.8)/ 0.1 % (wt/vol) SDS to a final volume of 2 ml. The reaction was started by the addition of 2 ul of acetic anhydride, which was subsequently added at 10 min intervals over a period of 1 h. The pH of the solution was monitored with a microprobe and maintained at approximately 7 with aliquots of 5 M NaOH. The solution was allowed to sit for an additional hour before it was dialysed against one liter of 35 mM sodium phosphate buffer.
(pH 6.8)/ 0.1 % (wt/vol) SDS for 4 h. The dialysis buffer was changed and dialysis continued overnight in the same buffer.

6. **Immunological methods.** Antigen specificity and titre of the various antisera was determined by the enzyme-linked immunosorbent assay (ELISA) as described by Mutharia and Hancock (1983) using 20 ug/ml final concentration of antigen in the wells. The Western immunoblot procedure, involving the electrophoretic transfer of SDS-polyacrylamide gel electrophoretograms to nitrocellulose and subsequent immunostaining, has been described previously (Mutharia and Hancock, 1983). In cases where a peroxidase conjugate was used as the second antibody, azide was omitted from all buffers and the blots developed using the peroxidase substrate described below.

7. **Preparation of protein P-phosphatidyl choline vesicles.** Phosphatidyl choline (0.5 umol in CHCl₃) was dried under N₂ and dessicated for 30 min at room temperature. Protein P (200 ug) was added to the dried lipid and vortexed for 30 sec. Deionized water was added to the protein-lipid solution to make a final volume of 1 ml. The lipid was scraped from the sides of the tube with a spatula and the solution vortexed a further 30 sec. Following sonication (4 pulses of 15 sec each at setting 50, Biosonik sonicator, Bronwill) the vesicle solution was cooled on ice and stored
at -20°C.

8. Preparation of protein affinity columns.
   a. Protein F-sepharose. Protein F was partially purified according to the procedure of Yoshimura et al. (1983), omitting the column chromatography step. The resultant preparation, which had only minor contamination with protein H2, was approximately 90% pure as judged by SDS-polyacrylamide gel electrophoresis. The partially purified protein was passed across a Biogel P-10 (Biorad) column (15x1.5 cm) equilibrated with 0.1 M NaHCO₃ (pH 8.3)/0.5 M NaCl/0.1% (wt/vol) SDS. Peak fractions (measured at an absorbance of 280 nm) were pooled and the protein (approximately 3.5 mg) was cross-linked to CNBr-activated Sepharose 4B beads (approximately 0.5 g dry weight) as recommended by the manufacturer (Pharmacia, Upsalla, Sweden). The column was stored at 4°C in phosphate-buffered saline (pH 7.4) (PBS) (Mutharia and Hancock 1983) containing 0.1% (vol/vol) Triton X-100. The final column volume was approximately 1 ml. Prior to use, the column was washed exhaustively with PBS to remove excess detergent.

   b. Protein P-affi-gel 10. Purified protein P was passed across a Biogel P-10 (Biorad) column equilibrated with 0.1 M acetic acid/sodium acetate buffer, pH 5.0/0.1% (wt/vol) SDS. Peak fractions, measured at an absorbance of 280 nm, were pooled and the protein (1.5 mg) cross-linked to approximately 0.65 ml of Affi-gel 10 (Biorad) for 2 h at
room temperature as recommended by the manufacturer. The column was stored at 4°C in PBS containing 0.1 % (wt/vol) Triton X-100. Prior to use, the column was washed exhaustively with PBS to remove excess detergent.

c. **Phosphate-binding protein-Sepharose.** Purified phosphate-binding protein was passed across a Biogel P-10 (Biorad) column (at 20 % of column volume) equilibrated with 0.1 M NaHCO₃/ 0.5 M NaCl (pH 8.0) (coupling buffer) and the protein-containing fractions were pooled (final volume of 5 ml at an absorbance at 280 nm of 0.38) and coupled to CNBr-activated Sepharose 4B (Pharmacia) exactly as described for protein F. The final column volume was approximately 1 ml. The column was stored in 20 mM Tris-HCl (pH 8.0) at 4°C.

9. **Preparation of protein P-specific antisera.**

a. **Trimer-specific.** Antibodies to protein P trimers were raised in New Zealand White rabbits using the following immunization schedule. Protein P (50 ug) was injected subcutaneously at weekly intervals over a three week period. Following this, the rabbits were rested, without injection, for three weeks. This cycle of three weeks of weekly immunization followed by three weeks without injection was repeated twice more, before a final subcutaneous injection of protein P (50 ug) was given. For the first two injections, protein P (diluted in PBS) was mixed 1:1 with Freund's Incomplete Adjuvant (Difco, Detroit, MI, USA), otherwise it was injected in PBS alone. Two weeks after the
final injection, blood was collected and the serum obtained after centrifugation of clotted blood.

The resultant antiserum contained antibodies to lipopolysaccharide (LPS) and porin protein F as well as to protein P. Thus in order to generate a protein P trimer-specific antiserum it was necessary to remove these contaminating activities. The antiserum was first adsorbed against whole cells of \textit{P. aeruginosa} PAO1 strain H103 as follows. Cells from a 10 ml overnight culture in L-broth were harvested by centrifugation in a table top centrifuge and washed twice with Hank's Balanced Saline Solution (Gibco, Burlington, Ont, Can). The cell pellet was resuspended directly into 1.0 ml of the antiserum, placed in a 1.5 ml polypropylene centrifuge tube (Evergreen Scientific, Los Angeles, CA, USA) and incubated for 45 min at room temperature in an end-over-end shaker. The cells were then pelleted and the antiserum-containing supernatant adsorbed a second time against a fresh batch of washed cells. Whole cell adsorption effectively removed all antibodies directed against smooth LPS as measured by ELISA and confirmed by Western immunoblots. There was, however, no decrease in antibody titre to protein F (or protein P).

The adsorbed antiserum (600 ul) was then incubated for 45 min at room temperature on a protein F-Sepharose affinity column (2.5x0.7 cm). At the completion of the incubation period, the column was washed with 4 ml of PBS and the unbound antibodies collected in 400 ul fractions. Fractions

33
containing antibodies to protein P, as determined by ELISA, were pooled to yield a protein F-adsorbed antiserum.

Adsorption of the antiserum on the protein F-Sepharose column facilitated removal of 99% of the antibody activity to protein F, with no decrease in antibody titre to protein P. The protein F-adsorbed antiserum, however, could only poorly distinguish between phosphate-limited cells producing protein P (e.g. strain H103) and phosphate-limited cells defective in protein P production (e.g. strain H553). Therefore, the protein F-adsorbed antiserum was subsequently adsorbed twice against phosphate-limited _P. aeruginosa_ strain H553 cells as described above for strain H103. The resultant antiserum was protein P trimer-specific (see Chapter One).

b. **Monomer specific.** Antibodies to protein P monomers were raised as follows. Purified protein P was heated at 88°C for 15 min to promote the heat denaturation of trimers to form monomers (see Chapter One). After cooling, the protein (20 µg), suspended in 0.1 ml PBS (Mutharia and Hancock, 1983), was injected interperitoneally into Balb/c mice (Department of Microbiology breeding colony, University of British Columbia, Vancouver, Canada). The injection was repeated on days 14, 28, 35, 42, 45 and 50. The blood was collected 7 days after the final injection and the serum obtained after centrifugation of clotted blood. Antibodies to LPS, as measured by ELISA using purified LPS as the antigen, were removed by adsorbtion against whole cells of
P. aeruginosa PAO1 strain H103 (see above).

c. **Antiserum to protein P in phosphatidyl choline vesicles.** Antiserum to protein P vesicles was raised in Balb/c mice as follows. Protein P vesicles (20 ug protein P) were preincubated at 37°C in the presence of monoclonal antibodies MA5-8 (LPS core-specific) (Hancock et al., 1983a) and MA1-8 (LPS O antigen-specific) (Hancock et al., 1983a) for 30 min prior to subcutaneous injection. The monoclonals were previously titrated against protein P vesicles in the ELISA to determine the amounts of the 2 antisera required to block all LPS molecules present in a given amount of protein P-containing vesicles. The injections were repeated on days 14,21,28,35,42,56,70,84, and 98. One week after the final injection, the blood was collected and the antiserum obtained after centrifugation of clotted blood.

10. **Isolation of a protein P-deficient mutant.**

a. **Tn501 insertion mutagenesis.** P. aeruginosa H103(pMT1000) was cultured overnight in L-broth at 30°C in the presence of HgCl₂. Dilutions were plated onto L-agar plates containing HgCl₂ and incubated at 42°C. Colonies growing up after 24 h (insertion mutants) were picked onto grids on fresh L-agar plates containing HgCl₂ and incubated once again at 42°C. After 24 h, these plates were then replica plated onto L-agar plates containing HgCl₂ and onto phosphate-deficient minimal medium plates, followed by
incubation at 42°C. The replicas on rich medium were retained as a master set from which desired mutants, once identified, could be rescued. The replicas grown on the phosphate-deficient minimal medium plates were screened for protein P-deficient mutants.

b. Selection of a protein P-deficient mutant using a protein P trimer-specific antiserum. Bacterial clones resistant to HgCl$_2$ at 42°C and replica plated onto phosphate-deficient minimal medium plates were transferred by contact onto nitrocellulose filter discs (Schleicher and Schuell Inc., Keene, NH, USA, type BA85, 82 mm). The nitrocellulose replicas were subsequently screened, by a modification of the procedure of Helfman et al. (1983), for the absence of protein P using the above-described protein P trimer-specific antiserum.

Blotted filters were placed in individual Petri dishes in 10 ml of 50 mM Tris-HCl (pH 7.4)/ 150 mM NaCl/ 5 mM MgCl$_2$ containing 3 % (wt/vol) bovine serum albumin and shaken gently for 1 h at 37°C. The filters were then washed twice at room temperature for 10 min in 10 ml of Tris-buffered saline (0.9 % NaCl/ 10 mM Tris-HCl (pH 7.4); Towbin et al. 1979) with shaking, followed by three 5 min washes in 10 ml PBS.

Protein P trimer-specific antiserum (with an ELISA titre of 2000, indicating that antibodies to protein P were detectable at a 1/2000 dilution of the antiserum) was diluted 1:249 in PBS containing 3 % (wt/vol) bovine serum albumin (10 ml) and then incubated on the filters overnight.
at room temperature with shaking. The filters were subsequently washed three times for 10 min at room temperature in 10 ml PBS. Affinity purified goat anti-rabbit IgG (H+L)-peroxidase conjugated antibody (Cappel Laboratories, West Chester, PA, USA) diluted 1:999 in 10 ml PBS containing 3 % (wt/vol) bovine serum albumin was then incubated on the filters at 37°C, again with shaking. After 2 h of incubation, the filters were washed twice for 10 min each in 10 ml of PBS at room temperature, followed by three washes of 10 min each in 10 ml of Tris-buffered saline. Peroxidase substrate (30 mg chloro-4-naphthol (Sigma) in 10 ml of methanol/ 50 ml of Tris-buffered saline/ 0.02 ml H₂O₂ (30 % (vol/vol)) was then added (10 ml/filter) and the filters incubated at 37°C until colour developed. Those colonies failing to develop colour were identified and picked from the master plates and screened for the absence of protein P in SDS-polyacrylamide gels of phosphate-limited cell envelopes.

11. Phosphate transport assays. Overnight cultures of P. aeruginosa grown in phosphate-deficient medium were harvested by filtration and washed with three volumes of minimal Hepes-buffered medium without phosphate. Washed cells were resuspended by vortexing in the same phosphate-less medium at a final absorbance at 600 nm of 0.2-0.3 and stored on ice until needed. Cells could be stored on ice for up to 3 h without any change in cell density or any
signs of cell damage (measured as release of periplasmic alkaline phosphatase into the medium). Prior to assaying phosphate accumulation, cells were shaken at 37°C for 5-25 min. To assay phosphate uptake, 1 ml samples of prewarmed cells were added to 10 ml culture tubes containing radioactively labelled orthophosphate. The cells were vortexed to ensure adequate aeration and 200 ul aliquots were removed at various times and filtered on nitrocellulose membrane filter cups (0.45 um dia, Amicon Corp) in an Amicon vacuum manifold. Filtered cells were washed twice with 1.5 ml of minimal Hepes-buffered medium containing 1 mM unlabelled phosphate. The filters were then removed and counted in 3 ml of PCS scintillation cocktail (Amersham). In some cases it was necessary to dilute cells in prewarmed phosphate-less minimal Hepes-buffered medium at the time of assay (1 ml final volume) due to excessively rapid transport rates of undiluted cell cultures.

12. Enzyme assays. Alkaline phosphatase was measured using para-nitrophenyl phosphate (pNPP) as the chromogenic substrate at a final concentration of 1 mg/ml in 0.1 M Tris-HCl (pH 8.5). The assay was read at an absorbance of 410 nm. Beta-lactamase was measured using, as the substrate, the chromogenic beta-lactam nitrocefin at a final concentration of 0.06 mg/ml in 50 mM sodium phosphate buffer (pH 7). The assay was read at an absorbance of 540 nm. Phospholipase C activity was measured using para-nitrophenyl phosphorylcholine
(NPPC) as the chromogenic substrate at a final concentration of 120 mg/ml in 60 % (vol/vol) glycerol/ 0.25 M Tris-HCl (pH 7.4). The assay was read at an absorbance of 410 nm.

13. **Nitrocefin permeability assay.** Outer membrane permeability was determined using a modification of the method of Angus et al. (1982). Intact cell beta-lactamase activity was measured on growing cultures by first taking aliquots of cells and dividing them in two. One fraction was taken up into a syringe and slowly squeezed through a millipore filter of 0.22 µm pore size to obtain a culture supernatant while the other fraction was left unfiltered. Equal volumes of each fraction were transferred to separate cuvettes. The cuvette containing the culture supernatant was placed in the reference beam of a Perkin-Elmer (Norwalk, CT, USA) Lambda 3 dual beam spectrophotometer. The other cuvette, containing intact cells and supernatant, was placed in the sample beam. Nitrocefin, a chromogenic beta-lactam, was added to each cuvette (final concentration of 0.06 mg/ml) and the differential rate of conversion of nitrocefin to nitrocefoic acid was recorded at an absorbance of 540 nm using a coupled Perkin-Elmer model 561 chart recorder. The recorded beta-lactamase activity was a direct measure of intact cell activity. Because beta-lactamase has been shown to be periplasmic, the activity of intact cells at a given concentration is limited by the diffusion of the beta-lactam, in this case nitrocefin, across the outer membrane.
rather than by the amount of enzyme. From theory (Zimmermann and Rosselet, 1977), the steady state rate of hydrolysis of beta-lactam in intact cells ($V_{\text{int}}$) equals the rate of beta-lactam diffusion across the outer membrane ($V_D$) and hence provides a measure of outer membrane permeability. Permeability parameters ($C$) were calculated using the formula $V_{\text{int}} = V_D = C(S_{\text{out}} - S_{\text{in}})$ according to Zimmermann and Rosselet (1977), where $C$ = permeability parameter; $S_{\text{out}}$ = concentration of substrate (nitrocefin) outside the cell and $S_{\text{in}}$ = concentration of substrate inside the cell (i.e. in the periplasm) (which is $\ll S_{\text{out}}$, and thus negligible).

14. Osmotic shock and purification of the phosphate-binding protein. *P. aeruginosa* PAO1 strain H103 was grown in phosphate-deficient medium to induce the synthesis of the periplasmic 34K protein. Induced cells were harvested by centrifugation (10,000 $x$ g for 10 min) and subjected to two rounds of the Tris-HCl/ MgCl$_2$/ cold shock procedure described by Hoshino and Kageyama (1980). The shocked cells were removed by centrifugation (10,000 $x$ g for 10 min) and the supernatant concentrated approximately 50-fold via Amicon pressure filtration using a PM10 microfilter (Amicon Corp, Danvers, MA, USA). Remaining whole cells and debris were removed by centrifugation in a clinical table top centrifuge. The concentrated shock fluids were then desalted by passage over a Biogel P-10 (Biorad) column equilibrated with 20 mM Tris-HCl (pH 7.4). The eluted
protein peak was applied to a DEAE-Sephacel (Pharmacia) column also equilibrated with 20 mM Tris-HCl (pH 7.4). The binding protein did not bind to DEAE-Sephacel at this pH and was collected in the flow through fraction. Binding protein containing fractions were pooled and applied to a CM-Sepharose (Pharmacia) column equilibrated with 20 mM sodium acetate-acetic acid buffer (pH 5.0). At this pH the binding protein bound to the column and was eluted with a NaCl gradient of 0.1 to 0.4 M. The binding protein eluted at between 0.1 and 0.2 M NaCl as a single peak of homogeneous protein as determined by SDS-polyacrylamide gel electrophoresis. $^{32}$P-orthophosphate-binding activity was monitored at all stages of the purification.

15. **Filter assay of phosphate binding.** Periplasmic extracts (shock fluids) and purified phosphate-binding protein were screened for their ability to bind phosphate utilizing a nitrocellulose filter binding assay based on the methodology described by Lever (1972) for the histidine-binding protein. Briefly, protein extracts were added to Eppendorf tubes in a final volume of 250 ul containing 1 uM $^{32}$P-orthophosphate (specific activity = 1 mCi/umol phosphate, Amersham) and 10 mM Tris-HCl (pH 8.0). After 5 min at 23°C 100 ul aliquots were removed and filtered on nitrocellulose membrane filters (Millipore Corp., Bedford, MA, USA, type HA, 0.45 um pore size). After washing once with 600 ul of 10 mM LiCl, the filters were removed and
counted in 5 ml of PCS aqueous scintillation cocktail (Amersham). To determine the specificity of the phosphate binding, various inhibitors, as indicated, were included in the reaction mixture.

16. Equilibrium dialysis. To determine the Kd for orthophosphate binding to the phosphate-binding protein, the equilibrium dialysis technique was employed. Dialysis bags (Spectrapor, 6.4 mm dia, Spectrum Medical Industries Inc., Los Angeles, CA, USA) were filled with 7 ug of purified phosphate-binding protein in a final volume of 300 ul. The binding protein solutions were dialysed against 40 ml of radioactively labelled orthophosphate in 50 ml conical tubes. The concentration of phosphate ranged from 0.1 uM to 5.0 uM. After dialysis for 24 h at 4°C, 25 ul aliquots (in duplicate) were removed from the dialysis bags and from the solutions surrounding the bags and counted separately in 3 ml of PCS aqueous scintillation cocktail (Amersham).

17. Isolation of mutants lacking the phosphate-binding protein. Diethyl sulfate mutagenesis of P. aeruginosa PA01 strain H242 was carried out as described by T. Nicas (Ph.D. Thesis, U.B.C., Vancouver, Can, 1983) with modifications. An overnight culture (0.1 ml) was resuspended in 5 ml of a saturated solution of diethyl sulfate in 50 mM sodium Hepes buffer (pH 7.0) for 30 min at 25°C. Cells were then diluted 1:49 into proteose peptone No. 2 broth and allowed to grow
overnight at 37°C. After overnight growth, mutagenized cells were harvested by centrifugation (10,000 x g for 10 min) and washed three times in phosphate-less minimal Hepes-buffered medium. The washed cells were resuspended in the same medium and dilutions were plated onto phosphate-sufficient medium agar plates (1.0 mM phosphate) containing 20 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (XP) (Bachem). It was necessary to dissolve the XP in dimethylsulfoxide prior to its addition to plates but the final concentration of dimethylsulfoxide was < 1 % (vol/vol). This medium identified alkaline phosphatase constitutive mutants as blue-green colonies, the colour resulting from the hydrolysis of XP by alkaline phosphatase. Wild-type cells, which were repressed for alkaline phosphatase production in this medium, were non-pigmented. (Alkaline phosphatase constitutivity is a phenotype of phosphate-binding protein-deficient mutants in E. coli (Brickman and Beckwith, 1975)). After overnight growth at 37°C blue-green pigmented colonies were picked and cultured overnight in phosphate-deficient medium (to induce the phosphate-binding protein). Shock fluids and whole cell extracts were obtained and screened using SDS-polyacrylamide gel electrophoresis for the absence of the phosphate-binding protein under inducing conditions.
18. **Construction of a rabbit anti-protein P immunoadsorbant column.** Protein P trimer-specific antibodies were purified free of serum proteins by incubating the protein P trimer-specific rabbit antiserum (200 ul) on the above protein P-affi-gel affinity column (1.8x0.7 cm). After incubation at room temperature for 45 min the column was washed with 3 ml PBS followed by 3 ml PBS + 0.25 M NaCl, to wash off unbound and non-specifically bound material, respectively. Bound antibodies were eluted with 3 ml 0.1 M glycine-HCl (pH 2.5) and fractions (300 ul) collected in tubes containing enough solid Tris base (Schwartz-Mann, Cambridge, MA) to increase the pH of the fractions to between 7 and 9. The fractions containing antibody to protein P, as measured by ELISA (Mutharia and Hancock, 1983), were pooled and dialysed against one litre of 0.1 M borate buffer (pH 9.0) (0.1 M boric acid (pH 9.0)/0.1 M KCl) for 24 h at 4°C, with one buffer change. The dialysed antibodies (2.3 ml at an absorbance at 280 nm of 0.22) were then cross-linked to 0.5 gm CNBr-activated Sepharose 4B (Pharmacia) in 0.1 M borate buffer (pH 9.0) for 16 h at 4°C as recommended by the manufacturer. The final column volume was approximately 1.0 ml. The column was stored at 4°C in PBS.

19. **Electrophoretic elution of protein P from SDS-polyacrylamide gels.** Phosphate-limited outer membranes of *P. aeruginosa* PAO1 strain H103 were solubilized in Triton X-100-Tris-EDTA (pH 8.0) to release protein P. Protein P-
containing fractions, solubilized at 23°C in a
solubilization mix without 2-mercaptoethanol (Hancock and
Carey, 1979); were loaded onto SDS-polyacrylamide gels (1.5
mm thickness) and electrophoresed to separate protein
components. A portion of the gel was stained with Coomassie
blue to locate the appropriate trimeric band of protein P
and the corresponding region of the gel was cut from the
unstained-unfixed portion of the gel. The porin-containing
gel was crushed with a glass rod to increase its surface
area and then placed in dialysis bags in the presence of PBS
(Mutharia and Hancock, 1983) and 0.1 % (wt/vol) SDS. The
proteins in the preparations were electroeluted at 50V for 2
h at 4°C in a Biorad transblotting cell. The buffer used
for electroelution was the same as that used for Western
immunoblots.

20. **Purification of phosphate starvation-induced outer
membrane proteins of the fluorescent Pseudomonads.** Two
methods were used for the purification of phosphate
starvation-induced outer membrane proteins. The first was
an affinity chromatography method utilizing an
immunoabsorbant column. Triton X-100 insoluble cell
envelopes prepared from 100 ml stationary phase cultures of
phosphate-limited cells were solubilized in 1 ml 2 %
(wt/vol) Triton X-100/20 mM Tris-HCl, pH 8.0/10 mM EDTA
containing 1 mg/ml lysozyme at 37 C for 30 min. The Triton
X-100-EDTA-lysozyme soluble fractions (300 ul) were
subsequently incubated on the anti-protein P immunoadsorbant column at room temperature. After 45 min incubation, the column was washed successively with 3 ml of 2 % TX-100/20 mM Tris-HCl, pH 8.0 and 3 ml of 2 % (wt/vol) Triton X-100/20 mM Tris-HCl, pH 8.0/0.5 M NaCl, to remove unbound and non-specifically bound material respectively. Material bound specifically to the column was eluted with 3 ml of 1 % (wt/vol) Triton X-100/0.1 M glycine-HCl (pH 2.5), and fractions collected in tubes containing solid Tris base as described above. Peak fractions, as determined by SDS-polyacrylamide gel electrophoresis, were pooled.

The second method utilized electroelution from SDS-polyacrylamide gels. Phosphate-limited cell envelopes were solubilized in Triton X-100/Tris-HCl (pH 8.0)/EDTA after lysozyme treatment to release phosphate-starvation-inducible proteins. Solubilized proteins (300 ul) were incubated at 23°C in a solubilization mix without 2-mercaptoethanol, loaded onto individual SDS-polyacrylamide gels (1.5 mm thickness) and electrophoresed to separate protein components. The phosphate-starvation-induced protein oligomers were then excised from the gels and electroeluted as described above. The eluted proteins (5 ml final volume) were concentrated 10-fold against solid polyethylene glycol (20,000 molecular weight, Sigma Chemical Co., St. Louis, MO, USA) before being dialysed at room temperature for 16 h against one litre of 0.1 % (wt/vol) Triton X-100/20 mM Tris-HCl (pH 8.0) with one buffer change.
21. **Black lipid bilayer experiments.** The methods used for black lipid bilayer experiments have been described in detail (Benz et al., 1978; Benz and Hancock, 1981). The apparatus consisted of a Teflon chamber with two compartments separated by a small hole (0.1 mm for single channel experiments; 2 mm for macroscopic conductance experiments). A membrane was formed across the hole by painting on a solution of 1.5% (wt/vol) oxidized cholesterol in n-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. In single channel conductance experiments, conductance through the pores was measured after application of a given voltage, using a pair of Ag/AgCl electrodes inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope and recorded on a strip chart recorder.

The procedure for macroscopic conductance inhibition experiments has been described (Hancock and Benz, submitted). Briefly, the Ag/AgCl electrodes were replaced with Calomel electrodes and the current through the pores was monitored with a Keithley 610B electrometer. After addition of purified protein P to the solution bathing the lipid bilayer membrane the increase in conductance (measured as current increase) was followed for 15-25 min or until the rate of increase had slowed considerably. At this time membrane conductance had increased 2-4 orders of magnitude.
The bathing solutions in both compartments were stirred gently (approximately 60 revolutions per minute) with a magnetic stir bar and aliquots of phosphate added to both compartments. Sufficient time (30-90 sec) was allowed for the new current level to be established before addition of subsequent aliquots.

22. **Modified ELISA procedure for demonstrating an association between protein P and the phosphate-binding protein.**

   a. **Preparation of protein P.** A solution of purified protein P (approximately 1 mg in 0.5 % (wt/vol) SDS) was made 2 % (vol/vol) for Triton X-100 (final volume of 500 ul) and passed across a Sepharose 6B (Pharmacia) column (20x1.5 cm) equilibrated with 0.1 % (vol/vol) Triton X-100/10 mM Tris-HCl (pH 7.4)/3 mM azide (column buffer). The column was eluted with column buffer and 600 ul fractions collected at 10 ml/h. Protein-containing fractions (as determined by absorbance at 280 nm) were pooled and concentrated 5-fold against 20 % (wt/vol) polyethylene glycol 20,000 (Sigma) (final volume 1 ml). The concentrated protein was passed a second time across the Sepharose 6B column described above and protein-containing fractions (600 ul) again pooled and concentrated to yield a purified protein P solution in Triton X-100. In some experiments, protein P was used as an enriched (but not pure) preparation obtained from the NaCl gradient-eluted, protein P-containing fractions from the
DEAE column described in section 3. These preparations were dialysed against 10 mM Tris-HCl (pH 7.4) for 6-8 h prior to being used in the ELISA.

b. Modified ELISA procedure. Phosphate-binding protein, purified as described above and diluted in carbonate/bicarbonate buffer (Ruitenberg et al., 1974), was used to coat the bottom of the wells of polyvinyl-chloride microtitre plates (Falcon 3912 Microtest III, Becton Dickinson Labware, Oxnard, CA) exactly as described for conventional ELISA (Mutharia and Hancock, 1983). After blocking unbound sites in the wells with fetal calf serum (FCS) [1 % (vol/vol) in 10 mM Tris-HCl (pH 7.4)] for 45 min, protein P, was added to the phosphate-binding protein-containing wells diluted in a solution of 10 mM Tris-HCl (pH 7.4) containing 0.5-1 % (vol/vol) Triton X-100 and 1 % (vol/vol) FCS. After incubation for 2 h at 37°C, the wells were washed 4 times with 1 % Triton X-100/10 mM Tris-HCl (pH 7.4) and the protein P trimer-specific antiserum (diluted 1:999 in Triton-Tris (pH 7.4) + 1 % FCS) was incubated on the wells for 2 h at 37°C. The plates were again washed 4 times with Triton-Tris (pH 7.4) and subsequently incubated for 2 h at 37°C in the presence of a goat-anti-rabbit IgG (H+L)-alkaline phosphatase conjugated antibody (Cappell) diluted 1:999 in Triton-Tris (pH 7.4)/1 % FCS. After washing 4 times with Triton-Tris (pH 7.4), paranitrophenyl phosphate (pNPP) was added to the wells at 1 mg/ml final concentration in 10 % (vol/vol)
diethanolamine buffer (pH 9.8) (Ruitenbergen et al., 1974) and incubated at 37°C or 23°C until colour developed. The colour reaction was assayed by measuring absorbance at 405 nm using a Titretek Multiscan ELISA reader (Flow Labs, McLean, VA). All incubations were at 37°C.


a. Phosphate-binding protein-Sepharose 4B affinity column. Triton-Tris-EDTA soluble phosphate-deficient outer membrane (enriched for protein P) (300 ul) was incubated on the phosphate-binding protein Sepharose 4B column at 4°C, 23°C or 37°C. After 1 h incubation, the column was washed with 3 ml of 0.1 % (vol/vol) Triton X-100/20 mM Tris-HCl (pH 8.0)/10 mM EDTA (pH 8.0), followed by 3 ml of the same solution containing 1 M NaCl. The eluted fractions (400 ul) were collected, dialysed overnight in 20 mM Tris-HCl (pH 8.0) and concentrated 5-fold against 20 % (wt/vol) polyethylene glycol 20,000 before being run on SDS-polyacrylamide gels.

b. Protein P-Affigel-10 affinity column. Phosphate-binding protein-containing crude shockates, obtained by Tris-MgCl₂-cold shock treatment of phosphate-limited cells, were desalted by passage across a Biogel P-10 (Biorad) column equilibrated with 20 mM Tris-HCl (pH 8.0) and incubated (300 ul) on a protein P-Affigel-10 column equilibrated with 0.1 % (vol/vol) Triton X-100 at 4°C, 23°C
or 37°C. After 1 h, the column was washed with 3 ml 20 mM Tris-HCl (pH 8.0) + Triton X-100, followed by 3 ml 20 mM Tris-HCl (pH 8.0)/1 M NaCl + Triton X-100. Eluted fractions (400 µl) were dialysed overnight in 20 mM Tris-HCl (pH 8.0), concentrated 5-fold against polyethylene glycol as described above and run on SDS-polyacrylamide gels.

24. Isolation of regulatory mutants of alkaline phosphatase and phospholipase C. *P. aeruginosa* was mutagenized using diethylsulphate or Tn501 insertion into the chromosome (see above) and mutagenized cells were plated onto phosphate-sufficient minimal plates (for selection of constitutive mutants) or phosphate-deficient minimal plates (for selection of non-inducible mutants) containing XP (Bachem). As described above, this compound yields a blue-green colour when hydrolyzed by alkaline phosphatase. Alkaline phosphatase constitutive mutants were identified as blue-green colonies on phosphate-sufficient plates and mutants non-inducible for alkaline phosphatase were identified as non-pigmented colonies on phosphate-deficient plates. To distinguish regulatory mutants from mutants in the alkaline phosphatase gene, mutant colonies were tested for their phospholipase C phenotypes. Thus, mutagenized colonies were transferred by contact onto Whatman 3M paper previously soaked in NPPC (a chromogenic substrate for phospholipase C (see above)). Colonies demonstrating phospholipase C activity turned rapidly yellow on the filter paper.
Regulatory mutants were identified as those colonies constitutive or non-inducible for both enzyme activities.

25. **Other assays.** Protein determinations were made by either the method of Schacterle and Pollack (1973), using bovine serum albumin as the standard, or the method of Warburg *et al.* (1941), using absorbance at 260 and 280 nm. 2-keto-3-deoxy-octulosonic acid (KDO) was measured using the method of Osborn (1963). The silver staining procedures for protein (*Wray et al.*, 1981) and LPS (*Tsai and Frasch, 1982*) have been described.
1. **Induction of protein P by phosphate limitation.** Growth of *P. aeruginosa* PAO1 strain H103 in a phosphate-deficient medium (0.2 mM phosphate) was characterized by an initial logarithmic rate of growth indistinguishable from that observed for phosphate sufficient (0.6 mM phosphate) cells (60 min doubling time) (Fig. 1). The onset of phosphate-limitation in the phosphate-deficient cell culture was detectable as a marked decline in growth rate (> 2.5 h doubling time) which contrasted with the phosphate-sufficient cell culture which continued to grow with a doubling time of ca. 60 min (Fig. 1). At concentrations of phosphate below 0.2 mM the total growth yield was dependent upon the concentration of phosphate in the medium (Fig. 2) indicating that phosphate was indeed limiting for growth at these concentrations. Examination of the outer membranes prepared from *P. aeruginosa* cells harvested 4 h after the onset of phosphate limitation revealed the presence of a novel protein (Fig. 3, lane 2), hereafter referred to as protein P, not present in cells grown in a phosphate-sufficient medium (Fig. 3, lane 1). A major protein of ca. 22,000 molecular weight present in the outer membranes of cells grown in phosphate-sufficient medium (Fig. 1, lane 1) was consistently absent from the outer
Figure 1. Growth of *P. aeruginosa* in a phosphate deficient medium. Overnight cultures of *P. aeruginosa* PA01 strain H103 grown in phosphate-sufficient medium were harvested, washed twice with phosphate-deficient medium and resuspended in phosphate-deficient (X--X) or phosphate-sufficient (O--O) medium at an absorbance at 600 nm of 0.20 ($A_{600}$). Growth was followed by the time dependent increase in $A_{600}$. 
Figure 2. Growth yield of *Pseudomonas aeruginosa* as a function of the concentration of phosphate in a defined minimal medium. An overnight culture of *P. aeruginosa* H103 grown in phosphate-sufficient medium was harvested, washed twice in phosphate-free Hepes-buffered minimal medium and resuspended in the original volume of the same phosphate-free medium. Aliquots (0.1 ml) were added to flasks containing Hepes-buffered minimal medium and varying concentrations of phosphate and allowed to grow overnight at 37°C. The growth yield was determined from the culture density (measured as $A_{600}$) obtained after overnight incubation.
Phosphate Concentration (μM)

Growth Yield (A600)

0  40  80  120  160  200
Figure 3. SDS-polyacrylamide gel electrophoretogram of purified protein P and of outer membranes and shock fluids of phosphate-sufficient and phosphate-deficient cells of *P. aeruginosa* H103. Lane 1, outer membrane of phosphate-sufficient H103; lane 2, outer membrane of phosphate-deficient H103; lane 3, purified protein P solubilized at 75°C; lane 4, purified protein P solubilized at 55°C; lane 5, purified protein P solubilized at 25°C; lane 6, unconcentrated shock fluid of phosphate-sufficient H103; lane 7, unconcentrated shock fluid of phosphate-deficient H103. Because of the dilute nature of the samples in lanes 6 and 7 only major proteins were detected. Samples were solubilized at 88°C prior to electrophoresis unless otherwise indicated. P, protein P monomer; P*, protein P oligomer (trimer; Angus et al., 1983); F, protein F.
membranes of all phosphate-limited P. aeruginosa strains examined, including a protein P-deficient mutant (H576) (Fig. 9). Additional minor alterations in the outer membrane protein banding patterns of phosphate-sufficient and phosphate-deficient grown cells were consistently observed, and may reflect differences in growth stage since phosphate-limited cells were routinely harvested several hours after the onset of limitation when the growth rate was substantially lower than that observed for phosphate-sufficient cells (Fig. 1).

By previously published criteria (Hancock et al., 1981) protein P was not a peptidoglycan-associated protein although its inability to be solubilized in 2% SDS/20 mM Tris-HCl (pH 8.0) suggested that it was at least weakly associated with the peptidoglycan. Using the procedure outlined in Methods, a highly purified preparation of this protein was obtained (Fig. 3, lane 3). The apparent molecular weight of the purified protein in SDS-polyacrylamide gels after solubilization in SDS at 75°C was 48,000 (48K), corresponding exactly to the apparent molecular weight in outer membranes. Upon solubilization at temperatures < 60°C, however, the protein ran at an apparently higher molecular weight (Fig. 3, lanes 4 and 5) suggesting that the native form of the protein was an oligomer. The oligomeric nature of protein P was confirmed by cross-linking data which indicated that the native protein was, in fact, a trimer (Angus et al. 1983).
The formation by protein P of SDS-stable oligomers in polyacrylamide gels is consistent with properties of known enteric porin proteins (Lugtenberg and van Alphen, 1983), but in contrast to the previously described *P. aeruginosa* porin proteins F (Hancock and Carey, 1979) and D1 (Hancock and Carey, 1980) which did not demonstrate oligomer formation in SDS-polyacrylamide gels.

2. **Co-regulation with alkaline phosphatase, phospholipase C and a 34K periplasmic protein.** When phosphate became limiting for growth as indicated by a decline in growth rate (Fig. 4A) detectable levels of the enzymes alkaline phosphatase and phospholipase C were produced by wild-type *P. aeruginosa* cells, and the levels increased with time (Fig. 4, panels B and C). A protein of molecular weight 34K was also observed as the major protein in the periplasm (releasable by Tris-MgCl$_2$-cold shock) of cells grown in phosphate-deficient (Fig. 3, lane 7) but not phosphate-sufficient (Fig. 3, lane 6) media. In addition to their supernatant activities (Fig. 4B), alkaline phosphatase and phospholipase C exhibited cell-associated activity which was localized to the periplasm (Fig. 4C).

The above data indicated that protein P was co-regulated with the enzymes alkaline phosphatase and phospholipase C and the 34K periplasmic protein. To obtain support for this genetically, mutants non-inducible (H553) or constitutive (H587) for alkaline phosphatase were
Figure 4. Induction by phosphate-limitation and localization of alkaline phosphatase and phospholipase C of \textit{P. aeruginosa} H103. A) Growth in phosphate-deficient medium. B) Supernatant activities and C) Periplasmic activities of alkaline phosphatase (X--X) and phospholipase C (○---○). Logarithmic-phase cells in phosphate-sufficient medium (1 mM Pi) were washed and resuspended in phosphate-deficient medium (0.2 mM Pi) at time zero. Cells were harvested at various times during growth. Supernatants were obtained after removal of cells by centrifugation and periplasmic extracts were obtained using the Tris-MgCl₂ cold shock procedure of Hoshino and Kageyama (1980). Enzyme assays were carried out as described in Methods. The measurements in panels B and C were representative data of 5 separate experiments.
isolated (see legend to Fig. 5). Mutant strain H553 was similarly non-inducible for phospholipase C, and examination of the protein complement of this mutant revealed that it was also non-inducible for protein P (Fig. 5, lane C; c.f. wild-type, lane D) and the 34K periplasmic protein (Fig. 5, lane E, c.f. wild-type, lane F). Likewise, the alkaline phosphatase constitutive mutant strain H587 was additionally constitutive for phospholipase C (measured by NPPC hydrolysis) as well as protein P and the 34K periplasmic protein (Fig. 5, lane A; c.f. wild-type, lane B), albeit at levels below that obtained in the fully derepressed wild-type (lanes D and F). These data support the existence of a phosphate regulon in *P. aeruginosa* analogous to the *pho* regulon in *E. coli* (Tommassen and Lugtenberg, 1982).

3. **Outer membrane permeability.** Alkaline phosphatase is characteristically a periplasmic marker in gram-negative bacteria and some alkaline phosphatase and phospholipase C activity was always detectable in the periplasm of phosphate-limited *P. aeruginosa* cells (Fig. 4C). However, a portion of the alkaline phosphatase and the majority of the phospholipase C activity of phosphate-limited cells was released into the culture supernatant (see below). The extracellular release of these enzymes by phosphate-limited cells could be explained by a breakdown in the outer membrane permeability barrier, releasing them from a periplasmic location. Conversely, a mechanism of specific
Figure 5. SDS-polyacrylamide gel electrophoretogram of whole cell protein extracts and cell envelope and soluble (non-membrane) fractions of alkaline phosphatase regulatory mutants. Mutants of P. aeruginosa H103 constitutive (H587) and non-inducible (H585) for alkaline phosphatase were isolated following diethylsulphate mutagenesis as described in Methods. Lane A, whole cell protein extract of phosphate-sufficient H587; lane B, whole cell protein extract of phosphate-sufficient H103; lane C, cell envelope of phosphate-deficient H553; lane D, cell envelope of phosphate-deficient H103; lane E, soluble fraction of phosphate-deficient H553; lane F, soluble fraction of phosphate-deficient H103. All samples were solubilized at 88°C for 10 min prior to electrophoresis. P, protein P; 34K, 34,000 molecular weight periplasmic protein.
secretion across the outer membrane may be responsible for their release, in the absence of any gross permeability changes. To test the specificity of alkaline phosphatase and phospholipase C release, the distribution of two other proteins normally localized within the periplasm, the constitutive RP1-encoded beta-lactamase and the 34K protein, were examined at the time of enzyme induction and secretion. The results in Fig. 6 demonstrated that beta-lactamase remained almost wholly periplasmic (as Tris-MgCl₂-releasable enzyme) during growth on phosphate-deficient medium, with only 6% of the total activity present in the supernatant (extracellular medium) 2 h after the onset of phosphate limitation. The 34K protein, although present as the major protein in the periplasm upon induction (Fig. 3), was undetectable in the supernatant as determined by SDS-polyacrylamide gel electrophoresis of 50-fold concentrated supernatants. In contrast, up to 58% of the total alkaline phosphatase activity and 87% of the total phospholipase C activity were found in the supernatant 2h post-induction (Fig. 4B). These results confirmed that the release of alkaline phosphatase and phospholipase C by whole phosphate-limited cells was indeed specific and not explainable by a general increase in outer membrane leakiness. Furthermore, LPS (measured as KDO) or major outer membrane proteins were not detected in 50-fold concentrated supernatants, supporting the absence of membrane breakdown during release.
The periplasmic location of beta-lactamase (Hancock et al., 1981) and the demonstration by Angus et al. (1982) that nitrocefin is taken up by the hydrophilic (porin) pathway, provided a means by which outer membrane permeability could be measured directly, as a function of nitrocefin uptake and hydrolysis. Furthermore, treatment of cells with EDTA, an agent known to break down the outer membrane thus increasing permeability (Hague and Russel, 1974), is associated with a 10-fold increase in nitrocefin hydrolysis (Hancock et al., 1981). From the results of nitrocefin permeability assays, permeability coefficients (C) were calculated (see Methods) as a function of growth in phosphate-deficient medium. No increase in outer membrane permeability was detected concomittant with enzyme release (Fig. 6B). In fact, the only alteration in outer membrane permeability which was detected over the 2.5 h of the experiment was a general 2.8-fold decrease in permeability (Fig. 6).

Given the low permeability of the P. aeruginosa outer membrane and the lack of an increase in permeability during phosphate-limited growth, it seemed reasonable to hypothesize that protein P may function as a phosphate porin, mediating the uptake of phosphate from a dilute environment.
Figure 6. Outer membrane permeability during growth on phosphate-deficient medium. Panel A shows growth after transfer to phosphate-deficient medium at time zero as described in the legend to Figure 4. Panel B shows beta-lactamase activity in the supernatant (●—●) and periplasm (●—●) and the outer membrane permeability coefficient C (X—X) (expressed in ml/min/mg whole cell protein) calculated as described in Methods.
4. **LPS-free protein P forms channels in planar lipid bilayer membranes.** During the course of this study, protein P was demonstrated to form small (0.6 nm diameter), water-filled channels in lipid bilayer membranes which were specific for anions (Hancock *et al.*, 1982). The basis of this specificity was shown to be lysine residues in or near the channel (Hancock *et al.*, 1983), which also formed a binding site for phosphate (H Hancock and Benz, submitted). Data in the literature suggests that the ability of porins to form channels is dependent upon an association with LPS (Schindler and Rosenbusch, 1978). Furthermore, it has been proposed that LPS may function to modulate porin activity (Kropinski *et al.*, 1982). To determine if any of the properties hitherto attributed to protein P were related to its association with LPS (LPS is invariably detected in purified preparations of protein P (see below)) the protein was purified free of LPS by electroeluting the protein trimer out of SDS-polyacrylamide gels as described in Methods. Protein P isolated by electroelution lacked detectable LPS (<2-3.8x10^-2 mol/mol protein) as measured by ELISA using LPS-specific monoclonal antibodies (Table II) and as observed by silver staining for LPS in polyacrylamide gels (Fig. 7). In contrast, the conventionally purified protein contained significant levels of LPS (1-1.7 mol/mol protein) (Table II; Fig. 7). LPS-free protein P was still capable of forming channels in lipid bilayer membranes with a mean single channel conductance in 1 M KCl (234 pS)
Figure 7. SDS-polyacrylamide gel electrophoretogram of LPS associated with protein P. Conventionally purified (lane 1) and electroeluted (lane 2) protein P were electrophoresed on SDS-polyacrylamide gels after solubilization at 23°C for 10 min and stained for LPS using the procedure of Tsai and Frasch (1982). The area of densest stain in lane 1 occurs at the tracking dye front.
<table>
<thead>
<tr>
<th>Assay Method</th>
<th>Conventionally Purified</th>
<th>Electroeluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE(^a)</td>
<td>1.7</td>
<td>&lt;2x10(^{-2})</td>
</tr>
<tr>
<td>ELISA(^b)</td>
<td>1.1</td>
<td>&lt;3.8x10(^{-2})</td>
</tr>
</tbody>
</table>

\(^a\) Conventionally purified and electroeluted protein P were electrophoresed on SDS-polyacrylamide gels and stained for LPS using the method of Tsai and Frasch (1982). Contaminating LPS was estimated by comparing silver stained electrophoretograms of dilutions of the protein P preparations with silver stained electrophoretograms of dilutions of pure LPS of known concentration.

\(^b\) Protein P preparations were serially diluted and used to coat the bottoms of microtitre wells. Monoclonal antibodies specific for \(P.\ aeruginosa\) LPS were then used to detect the presence of LPS at each dilution. Based on the detection limits of the antibodies employed (approximately 50 ng LPS), derived from ELISA analysis of serially diluted pure LPS preparations of known concentration, the LPS levels could be estimated from the highest dilution which still gave a positive LPS response.
Table III. Functional properties of conventionally purified and electroeluted protein P in planar lipid bilayer membranes

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>Average single channel conductance in 1 M KCl (pS)</th>
<th>Number of events</th>
<th>Selectivity(^a) (Pc/Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>235</td>
<td>317</td>
<td>&lt;0.01(^b)</td>
</tr>
<tr>
<td>Electroeluted</td>
<td>234</td>
<td>224</td>
<td>0.005</td>
</tr>
</tbody>
</table>

\(^a\) Permeability ratio of K\(^+\) to Cl\(^-\) derived from the Goldman-Hodgkin-Katz equation as described by Hancock et al. (1982)

\(^b\) Taken from Benz et al. (1985)
almost indistinguishable from that obtained for the conventionally purified, LPS-contaminated protein (235 pS) (Table III). Furthermore, the LPS-free protein remained anion-specific (Pc/Pa = 0.005) and single channel conductance through LPS-free protein P channels was observed to saturate at high concentrations of KCl, consistent with a binding site in the channel for anions. These properties were in agreement with the published properties of the conventionally purified protein (Hancock et al., 1982; Table III). LPS association was therefore not required for channel formation by protein P and was not responsible for the functional properties of this protein in vitro.

5. Isolation of a protein P-deficient mutant.

a. Preparation of a protein P trimer-specific antiserum. A polyclonal rabbit antiserum raised against purified protein P trimers (see Methods) reacted specifically with the native trimer form of the protein (Fig. 8A, lane 3), exhibiting no reaction with heat-dissociated monomers (Fig. 8A, lane 4). The smearing pattern evident in the reaction of the antibody with electrophoresed protein P trimers suggested some heterogeneity. This may be due to an association of the trimer form of the protein with LPS or due to aggregation of the trimers. Nevertheless, all of the material in the smear reacting with the antibody was protein P as confirmed by the ability to convert this
Figure 8. Immunoblots of electrophoretically separated P. aeruginosa H103 cell envelopes and purified protein P, and whole cells. A) Cell envelopes from phosphate-sufficient cells (lane 1), phosphate-limited cells (lane 2) and purified protein P (lanes 3 and 4) were separated on SDS-polyacrylamide gels after solubilization at 23°C (lanes 1-3) or 88°C (lane 4) for 10 min. After electrophoretic transfer to nitrocellulose, the blots were interacted with the protein P-specific polyclonal antiserum and subsequently immunostained using a peroxidase-conjugated goat-anti-rabbit IgG antibody and a histochemical stain for peroxidase (see Methods). B) A colony immunoblot showing the interaction of the protein P-specific polyclonal antiserum with phosphate-limited Tn501 insertion mutants of P. aeruginosa PAO1 strain H103. The protein P-deficient mutant, strain H576, is indicated by the arrowhead.
material to protein P monomers by heating (see Chapter Three, Fig. 19A).

The specificity of the polyclonal antiserum to protein P was demonstrated by the ability of the antiserum to react with a component present in envelopes from phosphate-starved cells (Fig. 8A, lane 2) which was absent in envelopes from phosphate-sufficient cells (Fig. 8A, lane 1). The reaction profile was very similar to that seen with purified protein P trimers (Fig. 8A, lane 3).

b. Tn501 mutagenesis of P. aeruginosa. In our search for a suitable vehicle for use in the transposon insertion mutagenesis of P. aeruginosa PAO1 (H103), a number of vectors were tested (see Table IV). One class, which included plasmids pME9 and pME319, were temperature sensitive for maintenance due to mutation, so that selection for transposon-encoded resistance at the non-permissive temperature (42°C) resulted in insertions into the PAO chromosome. Recovery of the transposable element at the non-permissive temperature was usually (> 98 %) associated with recovery of all plasmid antibiotic resistance markers as well, indicating that the entire plasmid had probably inserted.

The second class of vectors tested included plasmids pUW942 (::Tn501), pUW964 (::Tn5), pAS8Rep-1 (::Tn7) and pKP100 (::Tn5-132). These plasmids were hybrids comprising the broad host range transfer functions of the Inc P-1
a Abbreviations: Cb\textsuperscript{r}, carbenicillin resistant; Kn\textsuperscript{r}, kanamycin resistant; Tp\textsuperscript{r}, trimethoprim resistant; Tc\textsuperscript{r}, tetracycline resistant; Hg\textsuperscript{r}, mercury resistant; ts, temperature sensitive. Genotype symbols are according to Bachmann (1983). Only the relevant phenotypes are indicated.

b A derivative of Tn5 carrying a Tc\textsuperscript{r} determinant in place of the Kn\textsuperscript{r} determinant (Berg and Berg, 1983).

c The temperature sensitive phenotype is due to a mutation in trf\textit{A} (Rella \textit{et al}, 1985).

d A derivative of Tn5 carrying the Tp\textsuperscript{r} determinant of plasmid R751 (Rella \textit{et al}, 1985).
Table IV. Plasmids tested for utility in transposon insertion mutagenesis of *P. aeruginosa*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptiona</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS8Rep-1</td>
<td>RP4-ColE1 hybrid/rep(RP4)::Tn7</td>
<td>Sato <em>et al.</em>, 1981</td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;s&lt;/sup&gt; Tp&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pUW942</td>
<td>pAS8Rep-1::Tn501</td>
<td>Weiss and Falkow, 1983</td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;s&lt;/sup&gt; Tp&lt;sup&gt;r&lt;/sup&gt; Hg&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pKP100</td>
<td>putative pAS8Rep-1::Tn5-132&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt; Tp&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pRK2013</td>
<td>RK2-ColE1 hybrid Tra&lt;sup&gt;+&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Figurski &amp; Helinski, 1979</td>
</tr>
<tr>
<td>pUW964</td>
<td>pRK2013(Kan::Tn7)::Tn5</td>
<td>Weiss <em>et al.</em>, 1983</td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;s&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;s&lt;/sup&gt; Tp&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pME319</td>
<td>RP1&lt;sup&gt;ts&lt;/sup&gt; [Rep A&lt;sup&gt;ts&lt;/sup&gt; Rep B&lt;sup&gt;ts&lt;/sup&gt;] (carries Tn&lt;sub&gt;J&lt;/sub&gt;)</td>
<td>Haas <em>et al.</em>, 1981</td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pME305</td>
<td>RP1&lt;sup&gt;ts&lt;/sup&gt; with a 12 kb deletion&lt;sup&gt;c&lt;/sup&gt; in Kn&lt;sup&gt;r&lt;/sup&gt;, primase and IS21 (Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;s&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>Rella <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>pME9</td>
<td>pME305::Tn5-751</td>
<td>Rella <em>et al.</em>, 1985</td>
</tr>
<tr>
<td></td>
<td>(Tra&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;r&lt;/sup&gt; Kn&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt; Tp&lt;sup&gt;r&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>pMO190</td>
<td>a temperature-sensitive mutant of R68 (=RP4)</td>
<td>Tsuda <em>et al.</em>, 1984</td>
</tr>
<tr>
<td>pMT1000</td>
<td>pMO190::Tn501</td>
<td>Tsuda <em>et al.</em>, 1984</td>
</tr>
</tbody>
</table>

77
plasmids (eg. RP1) and the narrow host range replication functions of the ColE1-like plasmids. Thus, these vectors could be transferred from *E. coli* to *P. aeruginosa* but were unable to replicate in this recipient (Bagdasarian *et al*., 1979). Selection for transposon-encoded resistances in *P. aeruginosa* PAO strains mated with *E. coli* strains harbouring these plasmids revealed colonies with insertions in the chromosome. Almost without exception, insertion events associated with these vectors involved insertion of the whole plasmid, as indicated by the recovery of all plasmid markers. The plasmid markers were stably maintained in *P. aeruginosa* and were not readily transferrable to a second host strain indicating that they were, indeed, present as inserts in the chromosome.

Although curing of transposon-mediated whole plasmid inserts (co-integrates), to leave a single copy of the transposon in the mutated gene, has been documented (Harayama *et al*., 1981; Tsuda *et al*., 1984), the consistent isolation of whole plasmid inserts made the vectors outlined above unsuitable for our needs. The isolation of a protein P-deficient mutant, which involved a negative selection, required the screening of thousands of potential mutants. It was thus desirable to have a system whereby mutants generated would represent resolved, single transposon inserts, without the requirement for additional manipulations to obtain the desired insertional mutation.
Plasmid pMT1000, recently described by Tsuda et al. (1984), is a temperature-sensitive R68 plasmid carrying a Tn501 element. Insertion mutants in P. aeruginosa can be readily selected on HgCl₂-containing plates at 42°C. After raising the incubation temperature of P. aeruginosa PA01 strain H103 (pMT1000) to the restrictive temperature (42°C), colonies resistant to HgCl₂ were isolated at a frequency of > 1x10⁻³/viable cell. Of these, approximately 30% (3x10⁻⁴/viable cell) apparently represented whole plasmid inserts in that they were resistant to Cb, Tc and Kn, as well as to HgCl₂, and this proportion decreased to < 15% after a single passage on HgCl₂-containing plates. The remainder of the mercury resistant colonies were sensitive to Cb, Tc and Kn. This, together with the high frequency of isolation suggested that they were Tn501 insertion mutants.

Examination of colonies growing on HgCl₂-containing plates at 42°C revealed the existence of two colony morphologies which could be correlated to the type of insertion event which had occurred in these clones in the rescue of the Tn501 element. Colonies containing whole plasmid inserts (Hg⁰, Tc⁰, Kn⁰, Cb⁰ at 42°C) were typically flat, translucent and irregularly shaped. Colonies containing a resolved Tn501 insertion (Hg⁰, Tc⁰, Kn⁰, Cb⁰ at 42°C) were opaque, dome-shaped and generally circular, typical of wild type.

The isolation, in the majority of cases, of resolved Tn501 inserts in P. aeruginosa PA01 strain H103 meant that
the Tn501 insertion mutagenesis was significantly simpler than published procedures (Harayama et al., 1981; Tsuda et al., 1984) requiring no curing of plasmid sequences. In addition, the mutagenic capability of plasmid pMT1000 (Tsuda et al., 1984) was confirmed in P. aeruginosa PA01 strain H103 by the isolation of auxotrophs (frequency=2x10⁻³/Hg⁺ colony), mutants deficient in pigment production (6x10⁻⁴/Hg⁺ colony), and a number of pho regulon mutants, including phosphate-binding protein-deficient mutants (2x10⁻⁴/Hg⁺ colony), alkaline phosphatase constitutive mutants (1x10⁻³/Hg⁺ colony) and alkaline phophatase-deficient mutants (3.3x10⁻⁴/Hg⁺ colony).

c. Isolation of a Tn501-induced protein P-deficient mutant. In order to confirm a role for protein P in phosphate transport in P. aeruginosa, it was necessary to isolate a mutant deficient in protein P. Plasmid pMT1000-mediated Tn501 insertion mutants, isolated as resistant to HgCl₂ at 42°C, were transferred from phosphate-deficient minimal medium plates to nitrocellulose by contact and screened for the absence of protein P using a protein P-specific antiserum. Of 3,200 mercury resistant colonies screened, only one failed to react strongly with the protein P-specific antiserum in the colony blot assay (see Fig. 8B). SDS-polyacrylamide gel electrophoresis of cell envelopes of this mutant (designated strain H576), grown under phosphate-deficient conditions, confirmed the absence of detectable
Figure 9. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from a protein P-deficient mutant of *P. aeruginosa* and its wild type parent. Lane 1, purified protein P. The outer membranes were prepared from: lane 2, phosphate-sufficient H103 (wild type) cells; lane 3, phosphate-deficient H103 cells; lane 4, phosphate-sufficient H576 (mutant) cells; lane 5, phosphate-deficient H576 cells. All preparations were solubilized at 88°C prior to electrophoresis such that protein P (P) ran as the monomer.
protein P (see Fig. 9, lane 5). In contrast, the parent strain H103 grown under the same conditions produced large quantities of protein P (Fig. 9, lane 3). Western immunoblots of electrophoretically-separated cell envelope and whole cell proteins confirmed the absence of detectable protein P in phosphate-limited mutant cells using both a protein P trimer-specific and monomer-specific antiserum. The mutant, like its parent, was normally derepressible for alkaline phosphatase and phospholipase C under conditions of phosphate deficiency. In addition, the presence of the phosphate-binding protein in shock fluids and whole cell extracts of the mutant was confirmed using SDS-polyacrylamide gel electrophoresis and Western immunoblotting with a phosphate-binding protein specific antiserum (data not shown). These results supported the specific loss of protein P in this mutant, and distinguished this strain from a class of mutants isolated previously which were pleiotropically deficient in the phosphate-regulated components of \textit{P. aeruginosa}, including protein P (see Fig. 5).

6. \textbf{Phosphate transport}. Phosphate transport in wild-type \textit{P. aeruginosa} is characterized by the presence of two major systems of uptake, of low and high-affinity, respectively (LaCoste \textit{et al.}, 1981). When stationary phase, phosphate-starved cells of \textit{P. aeruginosa} were pre-incubated, with aeration, at 37°C for only 5 min prior to transport assays,
it was possible to examine high-affinity phosphate transport alone, since it was found necessary to incubate cells for longer periods (15-25 min) at 37°C for the low-affinity uptake system to become operative. Thus it was possible to precisely examine what role, if any, protein P played in high-affinity phosphate transport by comparing phosphate uptake in the protein P-deficient mutant with that of its parent H103.

Compared with the wild type parent strain, the protein P-deficient mutant was significantly defective in phosphate transport, exhibiting a Km for high-affinity transport roughly 10 times greater than that of the parent (Table V). No effect on the Vmax of the system was seen, however, as a result of the loss of protein P in the mutant (Table V). This confirmed the involvement of protein P in high-affinity phosphate transport in *P. aeruginosa*.

It was not possible to accurately determine the kinetic parameters of low-affinity phosphate transport in the mutant owing to the simultaneous operation of a high-affinity uptake component (see Chapter Two, section 4). However, the rates of phosphate transport measured in the mutant (H576) and wild-type (H103) were comparable at higher concentrations of phosphate (> 25 uM) suggesting that low-affinity phosphate uptake was not affected by the protein P-deficiency of the mutant.
Table V. Kinetics of high-affinity phosphate transport in a protein P-deficient mutant strain and its wild-type parent

<table>
<thead>
<tr>
<th>Strain</th>
<th>Km (uM)</th>
<th>Vmax (umol/min/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103</td>
<td>0.39 ± 0.07</td>
<td>5.34 ± 0.59</td>
</tr>
<tr>
<td>H576</td>
<td>3.60 ± 0.64</td>
<td>5.56 ± 0.66</td>
</tr>
</tbody>
</table>

Initial rates of phosphate transport at various concentrations of phosphate were plotted as an Eadie-Hofstee plot, from which kinetic parameters were derived by least squares analysis. The results are the mean values ± standard deviations of four experiments.
7. **Growth in low phosphate medium.** It was of interest to determine if the transport differences attributable to a lack of protein P were significant in terms of the growth capabilities of the cell under phosphate-limiting conditions (under which conditions protein P is normally derepressed). Thus wild-type strain H103 and mutant strain H576 cells were grown in phosphate-deficient medium for 14-16 hrs to deplete internal phosphate pools and thus make growth dependent on transported phosphate. These cells were then placed in a Hepes-buffered minimal medium containing 50 uM phosphate. Typically, a lag period of 30-45 min was observed followed by logarithmic growth for 2-4 h at a very reduced rate, after which the cells stopped growing. Determination of the rate of growth during this period revealed that the protein P-deficient mutant grew more slowly than its wild type parent strain H103 (Table VI).

To eliminate possible growth differences attributable to the presence of a Tn501 element in the chromosome of the protein P-deficient mutant, an arginine auxotroph, strain H556, obtained by Tn501 insertion mutagenesis, was used as the protein P-derepressible control. Again, the mutant lacking protein P exhibited a slower rate of growth than the strain producing wild type levels of protein P (Table VI). The 18-35% increase in doubling time characterized by the protein P mutant strain stresses the importance of protein P channels in the outer membrane of *P. aeruginosa* cells growing in a phosphate-limited environment.
Table VI. Growth of a protein P-deficient mutant and strains wild type for protein P in a phosphate-limited medium

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H103</td>
<td>12.82 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>H576</td>
<td>15.87 ± 0.60</td>
</tr>
<tr>
<td>2</td>
<td>H103</td>
<td>9.98 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>H576</td>
<td>15.43 ± 2.57</td>
</tr>
<tr>
<td>3</td>
<td>H556</td>
<td>13.55 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>H576</td>
<td>19.60 ± 1.62</td>
</tr>
<tr>
<td>4</td>
<td>H556</td>
<td>11.27 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>H576</td>
<td>13.67 ± 0.33</td>
</tr>
</tbody>
</table>

a
Overnight cultures, grown in phosphate-deficient medium, were resuspended in triplicate in Hepes-buffered minimal medium containing 50 uM phosphate at A_600 = 0.20 and growth measured by the increase in A_600.

b
H103, wild type PA01; H556, arginine requiring Tn501 insertion mutant; H576, Tn501 insertion mutant deficient in protein P.

c
Doubling times represent the reciprocal of growth rate, u, calculated from a plot of ln A_600 vs. time (min) using least squares analysis. Results are expressed as the mean doubling times ± standard deviations for three cultures (see above). Variations from experiment to experiment in growth rates obtained for a given strain reflect technical difficulties in obtaining precisely the same degree of phosphate limitation every time. For a given experiment, however, the degree of limitation was the same for each strain.
8. Summary. When wild-type cells of *Pseudomonas aeruginosa* PAO1 were grown in a medium containing 0.2 mM or less inorganic phosphate (phosphate-deficient medium) a new major outer membrane protein, P, was induced. The protein was purified and demonstrated to form SDS-resistant oligomers in polyacrylamide gels, a property shared by most known porins. The enzymes alkaline phosphatase and phospholipase C, as well as a major periplasmic protein of 34K were co-induced in a phosphate-deficient medium at the onset of phosphate-limitation identifiable by a marked decrease in growth rate. Mutants constitutive or non-inducible for alkaline phosphatase and phospholipase C were isolated and demonstrated to be similarly constitutive and non-inducible, respectively, for protein P and the 34K periplasmic protein consistent with the existence of a phosphate regulon in *P. aeruginosa*. The phosphate-starvation-inducible enzymes alkaline phosphatase and phospholipase C were secreted into the growth medium upon induction although enzyme release was shown not to be associated with a breakdown in the outer membrane or an increase in outer membrane permeability. As such, an increase in outer membrane permeability, which could conceivably increase the rate of phosphate movement across the outer membrane, is not the means by which *P. aeruginosa* adapts to a phosphate-limited environment. Protein P has been demonstrated to form small, anion-specific channels and the protein purified free of LPS exhibited unaltered channel-forming properties.
in planar bilayer membranes. In order to demonstrate a role for protein P channels in phosphate transport in P. aeruginosa, a transposon insertion mutant deficient in protein P was sought. A number of transposon delivery systems were tested which yielded, for the most part, whole plasmid inserts. Plasmid pMT1000 (Tsuda et al., 1984), a temperature-sensitive R68 plasmid carrying the transposon Tn501, was successfully employed in the isolation of a Tn501 insertion mutant lacking protein P under normally inducing conditions. To identify the mutant deficient in protein P, a protein P-specific polyclonal antiserum was used. This mutant, strain H576, was deficient in high-affinity phosphate transport, exhibiting a Km for uptake (3.60 uM phosphate) almost ten times greater than that of the wild-type strain (0.39 uM phosphate). There was, however, no change in the Vmax for high-affinity transport as a result of the loss of protein P in this mutant. The protein P-deficiency of the mutant correlated with a growth defect in a phosphate-limited medium, resulting in an 18-35 % decrease in growth rate compared with the wild-type.
CHAPTER TWO

Role of a periplasmic phosphate-binding protein in phosphate transport in Pseudomonas aeruginosa

1. Purification and properties of the periplasmic phosphate-binding protein. Phosphate limitation of P. aeruginosa PA01 strain H103 cells resulted in the induction of a major protein of molecular weight 34,000 (Fig. 10). Present as the major protein in the periplasm (releasable by cold osmotic shock) (Fig. 11, lane A) it was readily purified, (Fig. 11, lane B), using the procedure outline in Methods. A Scatchard plot of the data obtained from equilibrium dialysis binding studies (Fig. 12) revealed that the purified protein bound one molecule of phosphate per molecule of protein \( n = 0.91 \) from the Scatchard plot) with a \( K_d \) of \( 0.34 \pm 0.05 \) \( \mu M \) (mean of three \( K_d \) determinations \( \pm \) standard deviation). The specificity of the phosphate-binding protein was tested using a number of potential inhibitors of phosphate binding (Table VII). The organic phosphates glucose-6-phosphate, glycerol-3-phosphate and adenosine-5'-monophosphate did not compete with orthophosphate for binding, even at 1000-fold excess over orthophosphate (Table VII). In contrast, polymers of phosphate from \( P_2 \) (pyrophosphate) to \( P_{15} \), as well as arsenate, inhibited the binding of orthophosphate to the binding protein (Table VII).
Figure 10. Induction of the 34K periplasmic protein by phosphate limitation. Cells grown under phosphate-sufficient conditions were harvested, washed in phosphate-free minimal Hepes-buffered medium and resuspended in phosphate-deficient minimal Hepes-buffered medium at an absorbance at 600 nm of 0.20. Cells were shaken at 37°C, and at 15 min intervals cell samples were removed and whole cell protein extracted and run on SDS-polyacrylamide gels (lanes A-M). All samples were solubilized at 88°C for 10 min prior to electrophoresis. P, protein P.
Figure 11. SDS-polyacrylamide gel electrophoretogram of purified phosphate-binding protein and whole cell protein extracts of alkaline phosphatase constitutive mutants of P. aeruginosa H242. Lane A, 50-fold concentrated shock fluid of phosphate-limited strain H242 cells; lane B, purified phosphate-binding protein; lane C, whole cell protein extract of phosphate-limited strain H585; lane D, whole cell protein extract of phosphate-limited strain H586; lane E, whole cell protein extract of phosphate-limited strain H587; lanes F and G, whole cell protein extracts of phosphate-sufficient and phosphate-deficient H242, respectively. All samples were solubilized at 88°C prior to electrophoresis. P, protein P; PBP, phosphate-binding protein.
Figure 12. Scatchard plot of phosphate-binding activity. Equilibrium dialysis binding assays were performed with 7 ug of binding protein and varying amounts of phosphate. V represents nmol phosphate bound per nmol phosphate-binding protein. L represents the concentration of phosphate.
### Table VII. Substrate specificity of the phosphate-binding protein

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition(^a) (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mM</td>
<td>1.0 mM</td>
<td></td>
</tr>
<tr>
<td>Arsenate</td>
<td>17</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate (P2)</td>
<td>40</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Tripolyphosphate (P3)</td>
<td>50</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Trimetaphosphate (Cyclic P3)</td>
<td>65</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Polyphosphate (P5)</td>
<td>62</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Polyphosphate (P15)</td>
<td>41</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Orthophosphate (P1)</td>
<td>95</td>
<td>&gt;99</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphate</td>
<td>0</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Adenosine-5'-monophosphate</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Representative data from three determinations; N.D., not determined
2. **Isolation of mutants lacking the phosphate-binding protein.** Mutants lacking the phosphate-binding protein of *E. coli* (designated phoS) are constitutive for alkaline phosphatase (Willsky *et al.*, 1973). Therefore, to obtain mutants lacking the phosphate-binding protein in *P. aeruginosa*, alkaline phosphatase constitutive mutants were selected using the procedure of Brickman and Beckwith (1975). Of nine alkaline phosphatase constitutive mutants obtained, four lacked the phosphate-binding protein on SDS-polyacrylamide gels (e.g. strain H585: Fig. 11, lane C). The remainder were constitutive for all measured phosphate-regulated constituents in addition to alkaline phosphatase, including protein P, phospholipase C and the phosphate-binding protein, typical of the regulatory mutants described in Chapter One (e.g. H587 Fig. 11, lane E).

The absence of the phosphate-binding protein in periplasmic (and whole cell extracts) of phosphate-limited cells of mutant strain H585 (Fig. 11, lane C) and in extracts of the uninduced (i.e. phosphate-sufficient) wild-type parent strain H242 (Fig. 11, lane F) was correlated with an inability of these extracts to bind $^{32}$P-orthophosphate (Table VIII). In contrast, periplasmic extracts of the induced (phosphate-limited) parent strain (H242) (Fig. 11, lane G) and the alkaline phosphatase constitutive mutants retaining the phosphate-binding protein (H587) (Fig. 11, lane E) demonstrated excellent binding of $^{32}$P-orthophosphate (Table VIII). Interestingly, extracts of
Table VIII. $^{32}$P-orthophosphate binding by periplasmic$^a$
extracts of wild type and mutant strains of *P. aeruginosa*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phosphate-binding$^b$ protein</th>
<th>$^{32}$P-orthophosphate$^c$ bound (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H585</td>
<td>-</td>
<td>2,370</td>
</tr>
<tr>
<td>H586</td>
<td>+</td>
<td>3,500</td>
</tr>
<tr>
<td>H587</td>
<td>+</td>
<td>18,100</td>
</tr>
<tr>
<td>H242 (Phosphate- sufficient)</td>
<td>-</td>
<td>2,404</td>
</tr>
<tr>
<td>H242 (Phosphate- deficient)</td>
<td>+</td>
<td>17,100</td>
</tr>
</tbody>
</table>

$^a$ Ten ml cultures were grown overnight under phosphate-deficient conditions (except as indicated) and shock fluids obtained as described in Methods. Aliquots (25 ul) were incubated in the presence of 0.5 mM orthophosphate (specific activity = 1 mCi/ml) in a final volume of 250 ul and phosphate-binding measured using the filter-binding assay of Lever (1972) as described in Methods.

$^b$ The presence (+) or absence (-) of the phosphate-binding protein in periplasmic extracts was determined by SDS-polyacrylamide gel electrophoresis (see Fig. 5).

$^c$ Representative data from two determinations
alkaline phosphatase-constitutive strain H586 apparently contain the phosphate-binding protein (Fig. 11, lane D) yet fail to bind $^{32}$P-orthophosphate (Table VIII). This mutant may well represent a structural gene mutation which does not alter expression of the binding protein but does affect activity.

3. **Phosphate transport.** The involvement of the periplasmic phosphate-binding protein in phosphate uptake *in vivo* was examined using a wild-type strain containing the phosphate-binding protein (strain H242) and a mutant lacking the binding protein (strain H585). The loss of the binding protein in H585 resulted in a marked deficiency in phosphate transport compared with the parental strain (H242) (Fig. 13). The rapid plateauing observed for the uptake curve of the parental strain indicated that the available phosphate was being depleted, precluding the determination of an accurate rate of transport at the concentration of phosphate used in the experiment depicted in Fig. 13. Indeed, it was necessary to dilute the wild-type parental cells 1:4 compared with mutant cells in order to obtain a comparable rate of phosphate uptake (Fig. 13). At lower concentrations of phosphate it was often necessary to dilute wild-type cells 1:19 in order to obtain linear rates of transport.
Figure 13. Phosphate uptake in *P. aeruginosa*. The procedure for transport assays is described in Methods. The concentration of phosphate was 2.5 uM. All cells were assayed at an absorbance at 600 nm of 0.30 except as indicated. Strain H242 (○—○); strain H585 (△—△); strain H242 diluted 1:4 (final $A_{600} = 0.06$) (○—○).
4. Kinetics of phosphate transport. In wild-type cells of *P. aeruginosa*, two major components of phosphate uptake were observable (Fig. 14B), confirming preliminary results (LaCoste *et al.*, 1981). The high-affinity component of uptake was characterized by an apparent Km of 0.46 ± 0.10 μM phosphate and a Vmax of 5.4 ± 0.2 nmol phosphate taken up/min/mg cell protein while the low-affinity component was characterized by an apparent Km of 12.0 ± 1.6 μM phosphate. The extrapolated Vmax value for the 'low-affinity' curve (16.0 ± 1.5 nmol/min/mg cell protein) actually represented the sum of both the high and low-affinity parameters. Given that the extrapolated Vmax of the high-affinity system in the wild-type was 5.4 nmol/min/mg cell protein, the actual Vmax of the low-affinity system could be estimated as approximately 11 nmol/min/mg cell protein. This was in good agreement with the value derived from the phosphate-binding protein-deficient mutant strain H585 containing only the low-affinity transport system. In the mutant H585, only a single phosphate uptake component with a Km of 19.3 ± 1.4 μM phosphate and a Vmax of 12.1 ± 0.5 nmol/min/mg cell protein was observable (Fig. 14A). (Kinetic constants represent the mean of at least three determinations ± standard deviation). Thus, the loss of the phosphate-binding protein by mutation in H585 correlated with the loss of high-affinity phosphate uptake.
Figure 14. Kinetics of phosphate uptake in P. aeruginosa. Data from uptake assays performed at various concentrations of phosphate was plotted as an Eadie-Hofstee plot with kinetic constants derived by least squares analysis. A) phosphate-binding protein-deficient mutant strain H585 containing only a single phosphate uptake system. B) wild-type strain H242 containing two phosphate uptake systems.
5. Growth in phosphate-deficient medium. In order to test whether the defect in high-affinity phosphate transport resulting from the phosphate binding protein-deficiency in H585 could be correlated with a growth defect, the growth of wild-type and mutant cells in phosphate-deficient medium was followed by measuring the time dependent increase in absorbance at 600 nm. Upon resuspension of phosphate-sufficient cells in phosphate-deficient medium (under which conditions the phosphate-binding protein would be induced in the wild-type), both strains were seen to grow logarithmically (Fig. 15) after a short lag (not shown). However, the phosphate-binding protein-deficient mutant H585 grew at a markedly slower rate (doubling time of 124 min) compared with the wild-type H242 (doubling time of 67 min) confirming the importance of the binding protein to P. aeruginosa cells growing in a limiting environment.

6. Physical association between outer membrane protein P and the periplasmic phosphate-binding protein. An association between maltose-binding protein and LamB porin protein of E. coli, demonstrated in vitro (Bavoil and Nikaido, 1981), has been suggested to be necessary in vivo for the efficient transport of maltose and maltodextrins across the E. coli outer membrane (Wandersmann et al., 1979). To determine if this was the case for protein P-phosphate-binding protein-mediated phosphate uptake in P. aeruginosa the phosphate-binding protein and protein P were
Figure 15. Growth of a phosphate-binding protein-deficient mutant and its wild-type parent in phosphate-limited medium. Overnight cultures of H242 (wild type parent strain) (O—O) and H585 (phosphate binding protein-deficient mutant) (X—X) grown in phosphate-sufficient medium were harvested, washed twice in phosphate-deficient medium and resuspended in phosphate-deficient medium at an absorbance at 600 nm of 0.20. Growth was followed by the time-dependent increase in A\textsubscript{600}. 
examined for their abilities to associate \textit{in vitro}. Using a modified ELISA procedure (see Methods) the phosphate-binding protein was immobilized on the bottom of the wells of mictotitre plates and examined for its ability to specifically retain protein P following incubation with protein P-containing extracts. The results in Table IX demonstrated that protein P and the phosphate-binding protein were apparently capable of associating \textit{in vitro}. At least 3 ug/well of phosphate-binding protein was required to demonstrate protein P retention in the wells. At this concentration of binding protein, protein P retention in the wells could be detected when as little as 20 ug/ml (approximately 0.14 uM) of protein P was added to the binding protein-containing wells (Table IX). At a concentration of 20 ug/ml (0.14 uM), protein P binding was 16 % above background and this increased to 33 % above background at 100 ug/ml (0.69 uM) and 42 % above background at 250 ug/ml (1.74 uM) (Table IX). In contrast, protein P binding was <10 % above background in the absence of the phosphate-binding protein (Table IX). Increasing the amount of phosphate-binding protein in the wells did not increase the amount of protein P which bound (at a given concentration) suggesting that levels of binding protein > 3 ug were saturating the wells. The observation that phosphate-binding protein-dependent protein P binding was detectable at uM concentrations of protein P (Table IX) implied that the Kd for protein P-binding protein

106
Table IX. In vitro association of the phosphate-binding protein and outer membrane protein P

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[Protein P]</th>
<th>A$_{405}$ $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ug/ml</td>
<td>uM$^b$</td>
</tr>
<tr>
<td>1$^d$</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.69</td>
</tr>
<tr>
<td>2$^e$</td>
<td>250</td>
<td>1.74</td>
</tr>
</tbody>
</table>

$^a$ Absorbance at 405 nm ± standard deviation of duplicate assays

$^b$ Assuming a molecular weight of 144,000 [deduced from the protein P monomer molecular weight of 48,000 and the demonstrated trimer form of the native protein (Angus et al, 1983)]

$^c$ Protein P binding to microtitre wells pre-coated with 3 ug of phosphate-binding protein (+PBP) or no phosphate-binding protein (-PBP) was detected using a protein P trimer-specific antiserum. Antibody binding, which was expected to be proportional to the amount of protein P present in the wells, was measured at A$_{405}$ following incubation with an alkaline phosphatase-conjugated second antibody and a chromogenic substrate, para-nitrophenyl phosphate (pNPP).

$^d$ After addition of the pNPP, colour development was allowed to proceed for 18 h at room temperature (in the dark) before measuring absorbance at 405 nm

$^e$ After addition of pNPP, colour development was allowed to proceed for 2 h at 37°C before measuring absorbance at 405 nm
association was in the uM range. This was consistent with data obtained for the association of maltose-binding protein and protein LamB (Kd = 0.15 uM) (Neuhaus et al., 1983).

One of the problems with the ELISA method used above was the very low levels of protein P which were in fact being retained in the wells (in one experiment overnight incubation was necessary to detect protein P retention). Since protein P retention was apparently dependent upon the presence of phosphate-binding protein in the wells (Table IX) this could be attributed to saturation of the microtitre wells by phosphate-binding protein at low levels (> 3 ug). This was compounded by an observed decrease in the affinity of the antiserum used to detect protein P binding in the presence of detergent (e.g. Triton X-100). As a result, it was difficult to accurately and consistently measure binding between protein P and the binding protein since background levels often equalled or exceeded levels due to specific binding. Thus, in order to obtain higher levels of binding it was deemed necessary to devise a method whereby larger amounts of the binding protein could be immobilized.

Bavoil et al. (1981) demonstrated an association between the maltose-binding protein and protein LamB by immobilizing large amounts (10 mg) of the binding protein on Sepharose beads and using affinity chromatography to demonstrate specific binding of the LamB protein. Using this methodology, a phosphate-binding protein Sepharose 4B column was constructed (see Methods) and examined for the
ability to specifically bind protein P. In a reciprocal experiment, protein P was immobilized on Sepharose beads and its ability to specifically bind the phosphate-binding protein was also examined. Unfortunately, these affinity columns failed to bind any proteins, including the relevant phosphate transport proteins. The cross-linking of these proteins to Sepharose may well distort the proteins sufficiently to prevent the adoption of the necessary binding conformations.

7. **Summary.** A binding protein for inorganic phosphate was purified to apparent homogeneity from the shock fluids of phosphate-limited *Pseudomonas aeruginosa*. The purified protein bound one molecule of phosphate per molecule of binding protein with a Kd of $0.34 \pm 0.05$ uM. Arsenate, pyrophosphate and inorganic polyphosphates up to 15 units long could inhibit the binding of phosphate to the binding protein, although organic phosphates such as glucose-6-phosphate, glycerol-3-phosphate and adenosine-5'-monophosphate could not. Mutants lacking the phosphate-binding protein were isolated and shown to be deficient in phosphate transport compared with wild-type cells. Two kinetically distinct systems for phosphate uptake could be observed in wild-type cells, with apparent Km values of $0.46 \pm 0.10$ uM (high affinity) and $12.0 \pm 1.6$ uM (low affinity). In contrast, only a single low-affinity transport system was observable in mutants lacking the phosphate-binding protein.
(Km apparent = 19.3 ± 1.4 uM phosphate), suggesting the involvement of the binding protein in the high-affinity phosphate uptake system of *P. aeruginosa*. Mutants deficient in the binding protein were also defective in their ability to grow in a phosphate-limiting medium consistent with the specific induction and requirement for the phosphate-binding protein-dependent high-affinity transport system under limiting conditions. An apparent association between the phosphate-binding protein and the phosphate-limitation-inducible outer membrane protein P was demonstrated *in vitro*.
CHAPTER THREE

Immunological cross-reactivity of phosphate-starvation-induced outer membrane proteins of the families Enterobacteriaceae and Pseudomonadaceae

1. Phosphate-starvation-induction of membrane proteins of the Pseudomonaceae and the Enterobacteriaceae. Under conditions of phosphate-limitation, P. aeruginosa is derepressed for the synthesis of protein P, a phosphate-selective (Hancock and Benz, manuscript submitted), channel-forming outer membrane protein. Growth of other Pseudomonads as well as members of the Enterobacteriaceae (Table X) in a phosphate-deficient medium resulted, in many of these strains, in the induction of novel membrane proteins (Fig. 16), many of which existed as the major cell envelope protein. The observation that these proteins were enriched in cation-aggregated membrane preparations demonstrated that they were probably outer membrane proteins. The phosphate starvation-induction of the PhoE outer membrane proteins of E. coli (Overbeeke and Lugtenberg, 1980) and S. typhimurium (Bauer et al., 1985) has been demonstrated previously. In experiments reported here, however, the PhoE protein of E. coli co-migrated with the OmpF protein of this strain (Fig. 16, lanes 17 and 18) making it necessary to use an ompF mutant strain (JF700) (Table 1) to demonstrate PhoE induction (Fig. 16, lanes 15 and 16). New membrane proteins
Enriched, soluble preparations of the phosphate starvation-induced proteins were solubilized at 23°C or 88°C prior to electrophoresis on SDS-polyacrylamide slab gels. Low molecular weight standards (Sigma Chemical Co, St. Louis, Mo.) were co-electrophoresed and a plot of log molecular weight vs Rf (measured as distance migrated in cm) for the standards was derived, from which molecular weights of the phosphate starvation-induced proteins were determined from their respective Rf values. Because the proteins solubilized at 23°C occurred as smeared bands (see text) the distance migrated (Rf) was determined for the midpoint of the area of densest stain. 48K e.g. signifies a molecular weight of 48,000

A, extractable from cell envelopes in 2 % (wt/vol) Triton X-100/20 mM Tris-HCl pH 8.0/0.5 M EDTA; B, extractable from cell envelopes in 2 % (wt/vol) Triton X-100/20 mM Tris-HCl pH 8.0 after 30 min incubation at 37°C in the presence of 1 mg/ml of lysozyme; C, extractable from cell envelopes in 2 % (wt/vol) SDS/0.5 M NaCl

Probable cross-reactive protein (in the oligomer form); see text for discussion

not determined

The 37K protein of P. chlororaphis was extractable from cell envelopes in 2 % (wt/vol) Triton X-100 alone and is probably an inner membrane protein

The 37K and 24K proteins co-purified, using all methods tested, such that the resultant oligomers at 23°C could not be distinguished, but appeared as a high molecular smear of approximately 115K
Table X. Properties of phosphate starvation-induced membrane proteins of the Enterobacteriaceae and the Pseudomonadaceae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Apparent Molecular Weight&lt;sup&gt;a&lt;/sup&gt; (in thousands) after solubilization at 88°C</th>
<th>23°C</th>
<th>Native oligomers</th>
<th>Solubility&lt;sup&gt;b&lt;/sup&gt; properties of native oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>48</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>50</td>
<td>102&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>22</td>
<td>N.D.&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>P. putida</td>
<td>45.5</td>
<td>97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>P. chlororaphis</td>
<td>49.5</td>
<td>110&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>N.D.</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>48</td>
<td>107&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>P. cepacia</td>
<td>37</td>
<td>115&lt;sup&gt;f&lt;/sup&gt;</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>115&lt;sup&gt;f&lt;/sup&gt;</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.5</td>
<td>20.5</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>P. pseudomallei</td>
<td>39</td>
<td>104&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>37</td>
<td>83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B,C</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>36</td>
<td>82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B,C</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>36.5</td>
<td>83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B,C</td>
<td></td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>36</td>
<td>85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B,C</td>
<td></td>
</tr>
<tr>
<td>S. marcesens</td>
<td>37</td>
<td>87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>
were not detected in cell envelopes of *P. maltophilia* (now *Xanthomonas maltophilia*), *P. acidovorans* and *P. solanacearum* strains grown in a phosphate-deficient medium, although these strains were derepressed for the synthesis of alkaline phosphatase in this medium (data not shown). *P. stutzeri*, which apparently failed to produce a new membrane protein when grown in phosphate-deficient medium, grew extremely poorly in this medium, and *P. syringae* did not grow at all, although both of these strains grew quite well in phosphate-sufficient medium.

While most strains expressed a single phosphate starvation-induced membrane protein band (Fig. 16), *P. fluorescens* and *P. chlororaphis* each apparently expressed two (Fig. 16, lanes 4 and 8, respectively), and *P. cepacia* apparently expressed three (Fig. 16, lane 12). With the exception of the 20.5K protein of *P. cepacia* (Fig. 16, lane 12) and the 22K protein of *P. fluorescens* (Fig. 16, lane 2), all of the phosphate starvation-induced membrane proteins were heat-modifiable (Table X). SDS-polyacrylamide gel electrophoretograms of soluble preparations of these proteins revealed that the native or unheated forms of the proteins ran as higher molecular weight oligomers which could be dissociated to monomers upon heating at temperatures greater than 60°C, a property shared by the majority of porins described to date (Lugtenberg and van Alphen, 1983). In addition, the monomer molecular weights ranged from 36K to 39K or from 46K to 50K (Table X),
Figure 16. SDS-polyacrylamide gel electrophoretogram of cell envelopes prepared from phosphate-deficient and phosphate-sufficient grown strains of the families Pseudomonadaceae and Enterobacteriaceae. Cell envelopes (lanes 1 to 14) and Triton X-100 insoluble cell envelopes (lanes 15-27) were prepared from: lane 1, phosphate-sufficient and lane 2, phosphate-deficient P. aeruginosa; lane 3, phosphate-sufficient and lane 4, phosphate-deficient P. fluorescens; lane 5, phosphate-sufficient and lane 6, phosphate-deficient P. putida; lane 7, phosphate-sufficient and lane 8, phosphate-deficient P. chlororaphis; lane 9, phosphate-sufficient and lane 10, phosphate-deficient P. aureofaciens; lane 11, phosphate-sufficient and lane 12, phosphate-deficient P. cepacia; lane 13, phosphate-sufficient and lane 14, phosphate-deficient P. pseudomallei; lane 15, phosphate-sufficient and lane 16, phosphate-deficient E. coli K-12 strain JF700; lane 17, L-broth grown E. coli K-12 strain HMS174; lane 18, L-broth grown E. coli K-12 strain JF694; lane 19, phosphate-sufficient and lane 20, phosphate-deficient S. typhimurium LT2; lane 21, phosphate-sufficient and lane 22, phosphate-deficient S. marcesens; lane 23, phosphate-sufficient and lane 24, phosphate-deficient K. pneumoniae; lane 25, phosphate-sufficient E. aerogenes; lanes 26 and 27, phosphate-deficient E. aerogenes. The arrows indicate the phosphate-regulated proteins. All samples were solubilized at 88°C prior to electrophoresis. The gel in lanes 15-27 contained urea at a final concentration of 6M.
characteristic of the major Enterobacterial porin proteins (Lugtenberg and van Alphen, 1983) and protein P of *P. aeruginosa* (see Chapter One), respectively.

Typically, porin protein P can be extracted in its native state from peptidoglycan-associated cell envelopes or outer membranes with Triton X-100 in the presence of EDTA (Chapter One), in contrast to the major porin protein F of *P. aeruginosa* and the major porins of *E. coli* and *S. typhimurium*, which are solubilized (in their native forms) in Triton X-100 only after lysozyme digestion of the peptidoglycan (Hancock *et al.*, 1981) or in the presence of SDS and high salt (> 0.4 M NaCl) (Nakamura and Mizushima, 1976; Tokunaga *et al.*, 1979; Yoshimura *et al.*, 1983). A number of the phosphate starvation-inducible proteins examined were soluble in Triton X-100-EDTA, including those of *P. putida*, *P. fluorescens*, *P. aureofaciens* and the 49.5K protein of *P. chlororaphis* (Table X). The 37K phosphate-starvation-induced protein of *P. chlororaphis* was extractable with Triton X-100 in the absence of EDTA. In contrast to the other proteins described here, this protein may well be from the cytoplasmic membrane. The remainder of the phosphate-starvation-induced membrane proteins were insoluble in Triton X-100-Tris-EDTA but could be solubilized in their oligomeric forms in SDS/0.5 M NaCl (Table X). Interestingly, lysozyme digestion of the peptidoglycan did not facilitate Triton X-100 solubilization of the phosphate starvation-induced membrane proteins of *P. cepacia*,
P. pseudomallei or S. marcesens, although such treatment did yield Triton-soluble phosphate-starvation-induced proteins in the cases of E. coli, S. typhimurium, K. pneumoniae and E. aerogenes (Table X).

2. Immunological cross-reactivity of phosphate starvation-induced outer membrane proteins.

   a. Cross-reactivity of protein oligomers in phosphate-limited cell envelopes. To test for immunological cross-reactivity of the phosphate starvation-induced membrane proteins of the various strains examined, SDS-polyacrylamide gel electrophoretograms of phosphate-limited cell envelopes were electrophoretically transferred to nitrocellulose and probed with a protein P trimer-specific polyclonal antiserum (for specificity of the antiserum see Fig., 17 lane 1; c.f. lane 2). This antiserum was demonstrated to specifically detect protein P trimers in cell envelopes of phosphate-limited P. aeruginosa cells (Fig. 17, lane 4). The protein P-specific antiserum was capable of reacting with a component present in the phosphate-limited cell envelopes of all strains which had produced a phosphate-starvation-induced membrane protein and with a component present in cell envelopes of E. coli K-12 strain JF694 which lacks the major porin proteins OmpF and OmpC but is constitutive for PhoE (Fig. 18). In each case smeared bands of high molecular weight reacted with the antiserum, suggesting that the native (unheated) oligomers
Figure 17. Interaction of protein P trimer-specific or monomer-specific antiserum with Western blots of purified protein P and *Pseudomonas aeruginosa* PAO1 strain H103 cell envelopes. Cell envelopes or purified proteins were electrophoretically transferred from SDS-polyacrylamide gel electrophoretograms to nitrocellulose and incubated with a protein P trimer-specific (lanes 1 to 5) or monomer-specific (lanes 6 to 10) antiserum. Antibody binding was detected using an alkaline phosphatase-conjugated goat-anti-rabbit IgG antibody (for the trimer-specific antiserum) or an alkaline phosphatase-conjugated goat-anti-mouse IgG antibody (for the monomer-specific antiserum) and a histochemical alkaline phosphatase substrate. Lanes 1 and 7, purified protein P solubilized at 23°C (trimer form); lanes 2 and 6, purified protein P solubilized at 88°C (monomer form); lane 3, cell envelope preparation of phosphate-sufficient *P. aeruginosa* solubilized at 23°C; lanes 4 and 10, cell envelope preparation of phosphate-deficient *P. aeruginosa* solubilized at 23°C; lanes 5 and 9, cell envelope preparation of phosphate-deficient *P. aeruginosa* solubilized at 88°C; lane 8, cell envelope preparation of phosphate-sufficient *P. aeruginosa* solubilized at 88°C. A small amount of monomer protein P can be seen in the trimer preparation in lane 7. The protein P oligomer (trimer) band observable in Coomassie-stained gels (e.g. Fig. 3, lanes 4 and 5) migrates at a position corresponding to the bottom of the smeared band in lane 1.
Figure 18. Interaction of protein P trimer-specific antiserum with Western blots of cell envelope preparations of different bacteria grown under phosphate-deficient or sufficient conditions. Lane 1, phosphate-deficient and lane 2, phosphate-sufficient *P. aeruginosa*; lane 3, phosphate-deficient and lane 4, phosphate-sufficient *P. fluorescens*; lane 5, phosphate-deficient and lane 6, phosphate-sufficient, *P. putida*; lane 7, phosphate-deficient and lane 8, phosphate-sufficient *P. chlororaphis*; lane 9, phosphate-deficient and lane 10, phosphate-sufficient *P. aureofaciens*; lane 11, phosphate-deficient and lane 12, phosphate-sufficient *P. cepacia*; lane 13, phosphate-deficient *P. cepacia*; lane 14, phosphate-deficient *P. cepacia* cell envelopes solubilized in 2% SDS/0.5 M NaCl to inactivate contaminating alkaline phosphatase; lane 15, phosphate-deficient and lane 16, phosphate-sufficient *P. pseudomallei*; lane 17, phosphate-deficient and lane 18, phosphate-sufficient *K. pneumoniae*; lane 19, phosphate-deficient and lane 20, phosphate-sufficient *E. aerogenes*; lane 21, phosphate-deficient and lane 22, phosphate-sufficient *S. marcesens*; lane 23, phosphate-deficient and lane 24, phosphate-sufficient *S. typhimurium*; lane 25, L-broth grown *E. coli* K-12 strain JF694; lane 26, L-broth grown *E. coli* K-12 strain HMS174; lane 27, phosphate-deficient and lane 28, phosphate-sufficient *E. coli* K-12 strain JF700. The cell envelopes were solubilized at 23°C prior to electrophoresis. The blots, with the exception of lane 11, were developed with the protein P-trimer-specific antiserum as described in the legend to Fig. 17. The blot in lane 13 was incubated directly with an alkaline phosphatase histochemical substrate to detect contaminating cell envelope bound alkaline phosphatase. Similar controls were negative for all other strains shown here.
of the phosphate starvation-induced proteins were reacting. In support of this, the non-heat-modified oligomeric forms of the phosphate starvation-induced membrane proteins were identifiable as high-molecular weight smeared bands in Coomassie stained SDS-polyacrylamide gel electrophoreterograms of enriched, soluble preparations of these proteins. Solubilization of cell envelopes at 88°C for 10 min, which converted oligomeric proteins to monomers (Table X), destroyed this reactivity. This was consistent with the inability of the antiserum to react with protein P monomers in heat treated, phosphate-limited cell envelopes (Fig. 17, lane 5) or purified in detergent (Fig. 17, lane 2). These data excluded the non-heat-modifiable 20.5K and 22K proteins of \textit{P. cepacia} and \textit{P. fluorescens}, respectively, as the cross-reactive species in these strains. In the case of \textit{P. cepacia}, a strong cross-reactivity originally seen (Fig. 18, lane 11) was demonstrated to be due, in part, to the presence of alkaline phosphatase associated with the cell envelope (Fig. 18, lane 13). Using a 2% SDS/0.5 M NaCl soluble preparation of a phosphate-limited \textit{P. cepacia} cell envelope, which contained the phosphate starvation-induced proteins but lacked alkaline phosphatase, a weak reactivity with the protein P trimer-specific antiserum was detected (Fig. 18, lane 14). No reactivity was observed with any cell envelopes derived from phosphate-sufficient cells (Fig. 18).
b. Identification of the cross-reactive proteins. To confirm that the cross-reactivity seen in native phosphate-limited cell envelopes was indeed due to the oligomeric forms of the phosphate-starvation-induced protein in each case, we attempted to convert the material present in the cross-reactive smeared bands to the appropriate monomeric proteins by heating. Thus, SDS-polyacrylamide gel electrophoretograms of native (unheated) phosphate-limited cell envelopes (first dimension) were heated at 88°C and electrophoresed on fresh SDS-polyacrylamide slab gels (second dimension). A typical result is shown in Fig. 19. Proteins which were not-heat modifiable typically (Russel, 1976) appeared on the diagonal of a 2-dimensional (unheated vs heated) SDS-polyacrylamide gel, since their molecular weights would remain unchanged in the second dimension after heating. Proteins which form native oligomers which dissociate in response to heating would typically appear at a position to the left of the diagonal at their appropriate monomer molecular weights. As expected, protein P occurred to the left of the diagonal (Fig. 19B) since it ran as a trimer in the first dimension (unheated) and a monomer in the second dimension (heated). Furthermore, the protein P monomers ran as a broad band in the second dimension, consistent with the apparent heterogeneity of protein P trimers in phosphate-limited cell envelopes (Fig. 19B) or purified in detergent (Fig. 19A).
Figure 19. Two-dimensional (unheated x heated) SDS-polyacrylamide gel electrophoretogram of purified protein P and cell envelopes prepared from phosphate-limited strains of the fluorescent Pseudomonads. A) purified protein P; B) P. aeruginosa; C) P. chlororaphis; D) P. pseudomallei; E) S. marcesens; and F) P. cepacia. SDS-polyacrylamide gel electrophoretograms of cell envelopes solubilized at 23°C for 10 min prior to electrophoresis were excised (1st dimension) were heated at 88°C for 10 min, laid across the top of a second SDS-polyacrylamide slab gel with (4E) or without (4A,B,C,D,F) urea, and electrophoresed in the second dimension as described in Materials and Methods. Western immunoblots of first dimension gels (e.g. Fig 3) are included above the 2-D gels to indicate the position of the cross-reacting material in native cell envelopes prior to heating in the second dimension. The phosphate-starvation-induced protein monomers are indicated by arrows.
For all strains expressing a heat-modifiable phosphate starvation-induced protein, it was possible to demonstrate the presence of phosphate-starvation-induced monomers to the left of the diagonal in the second dimension of a two-dimensional unheated vs heated SDS-polyacrylamide slab gel. In all cases these monomeric proteins ran as broad bands, the positions of which corresponded with the position of the cross-reactive smeared oligomer bands present in native phosphate-limited cell envelopes in the first dimension (Fig. 19). Since the phosphate starvation-induced proteins of P. putida, P. fluorescens and P. aureofaciens represented the only heat-modifiable membrane proteins in these strains, demonstrating broad bands which appeared to the left of the diagonal in the second dimension and which corresponded with the cross-reactivity seen in first dimension gels (e.g. Fig. 19B), they were readily confirmed as the cross-reactive species in these strains. Similarly, the constitutively produced PhoE protein of the porin-deficient E. coli strain JF694 existed as the lone heat-modifiable, oligomeric protein in cell envelopes of this strain (Fig. 16; Table X), occurring to the left of the diagonal and thus accounting for the cross-reactivity observed with cell envelopes of this strain. Of the two heat-modifiable phosphate starvation-induced membrane proteins produced by P. chlororaphis, only the 49.5K monomer protein ran as a broad band in the second dimension, whose position also corresponded with the cross-reactivity seen in first
dimension gels (Fig. 19C), consistent with its being the cross-reactive membrane protein in this strain. The remaining strains, which included *P. cepacia*, *P. pseudomallei*, *E. coli* K-12, *S. typhimurium*, *K. pneumoniae*, *E. aerogenes* and *S. marcesens*, all produced a number of constitutive membrane proteins which, like the various phosphate-starvation-induced membrane proteins, were heat-modifiable, appearing to the left of the diagonal in the second dimension of a two-dimensional unheated vs heated SDS-polyacrylamide gel (e.g. Figs. 19D and 19E). Furthermore, they occurred as broad bands of monomer molecular weight in a position which also corresponded with the cross-reactive smears observed in first dimension gels of native phosphate-limited cell envelopes (e.g. Figs. 19D and 19E). However, the expression of these constitutive proteins in the cell envelopes of phosphate sufficient cells, which had previously failed to react with the protein P-trimer specific antiserum (Fig. 17), excluded these proteins as the cross-reactive components in these strains. Thus the single heat-modifiable phosphate starvation-inducible protein present in each strain (except *P. cepacia*) must be responsible for the cross-reactivity observed with the protein P-specific antiserum. Nonetheless, this demonstrated that the smearing (heterogeneity) on SDS-polyacrylamide gels of native oligomers was not restricted to phosphate-starvation-induced membrane proteins. Rather, it may well be a property of oligomeric membrane proteins,
specifically porins.

The expression by P. cepacia of two heat-modifiable phosphate starvation-induced membrane proteins, both of which migrated as broad bands to the left of the diagonal in the second dimension, and in a position which corresponded with the cross-reactive smeared band seen in the first dimension (Fig. 19F), made it difficult to unambiguously identify the cross-reactive species. Furthermore, the proteins were invariably co-purified by all tested methods, making it impossible to individually examine their reactivities with the protein P-specific antiserum. Based on molecular weight, however, the likely candidate for the cross-reactive species was the 37K protein, whose monomer molecular weight was more typical of porins in general than that of the 24K protein.

c. Cross-reactivity of phosphate-starvation-induced monomers. Porin monomers, obtained by heat denaturation of porin trimers, have been shown to exhibit an alpha-helical structure (Nakamura and Mizushima, 1976) very distinct from the beta-structure of the native protein (Nakamura and Mizushima, 1976). Consistent with this, the monomer and trimer forms of individual porins have been demonstrated to be immunologically non-cross-reactive (Hofstra and Dankert, 1981). The demonstration, then, that the porin monomers of different species of the family Enterobacteriaceae could immunologically cross-react (Hofstra and Dankert, 1980;
Overbeeke and Lugtenberg, 1980), although implying that linear epitopes present in porin monomers had been conserved during porin evolution, did not demonstrate the existence of conserved epitopes in the native oligomers. In order to determine whether the cross-reactivity of phosphate starvation-induced membrane proteins could be attributed to conserved linear epitopes, the monomer and oligomer forms of the various phosphate-starvation-inducible membrane proteins were tested for their ability to react with an antiserum raised against heat-dissociated protein P monomers. The antiserum was demonstrated to react specifically with protein P monomers in heat denatured cell envelopes of phosphate-limited P. aeruginosa (Fig. 17, lane 9) or with purified protein P monomers (Fig. 17, lane 6), exhibiting no reactivity with the trimer form of the protein (Fig. 17, lanes 7 and 10) or with uninduced (phosphate-sufficient) cell envelopes (Fig. 17 lane 8). This antiserum failed to react with the phosphate-starvation-inducible membrane proteins, in monomer or oligomer form (data not shown), indicating that the phosphate-starvation-inducible membrane proteins do not cross-react immunologically with protein P monomers.

3. **Summary.** Bacteria from the families Enterobacteriaceae and Pseudomonadaceae were grown under phosphate-deficient (0.1 - 0.2 mM inorganic phosphate) conditions and examined for the production of novel membrane proteins. Twelve of
the seventeen strains examined expressed a phosphate-starvation-induced outer membrane protein which was heat-modifiable, in that after solubilization in SDS at low temperature the proteins ran on gels as diffuse bands of higher apparent molecular weight, presumably oligomer forms, which shifted to their apparent monomer forms after solubilization at high temperature. These proteins fell into two classes based on their monomer molecular weights and the detergent conditions required to release the proteins from the peptidoglycan. The first class, expressed by species of the *P. fluorescens* branch of the family *Pseudomonadaceae*, was similar to the phosphate-starvation-inducible channel-forming protein P of *P. aeruginosa*. The second class resembled the major Enterobacterial porin proteins and the phosphate-regulated PhoE protein of *E. coli*. Using a protein P trimer-specific polyclonal antiserum it was possible to demonstrate cross-reactivity of the oligomeric forms of both classes of these proteins on Western blots. However, this antiserum did not react with the monomeric forms of any of these proteins, including protein P monomers. Using a protein P monomer-specific antiserum, no reactivity was seen with any of the phosphate-starvation-inducible membrane proteins (in either oligomeric or monomeric form) with the exception of protein P monomers. These results suggest the presence of conserved antigenic determinants only in the native, functional proteins.
CHAPTER FOUR

Characterization of protein P-like porins from the fluorescent Pseudomonadaceae

1. Purification of the phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonads. In the previous chapter a number of bacterial strains were demonstrated to synthesize phosphate-starvation-inducible outer membrane proteins. Members of the fluorescent Pseudomonadaceae, including P. putida, P. fluorescens, P. aureofaciens and P. chlororaphis synthesized an oligomeric, heat-modifiable outer membrane protein which exhibited a number of properties in common with protein P of P. aeruginosa. Using the observed immunological cross-reactivity of these proteins with protein P, an attempt was made to purify these phosphate-regulated proteins by specific retention on an immunoadsorbent column constructed using the protein P trimer-specific antiserum described in Chapter one. Protein P, the original antigen, was readily purified and in reasonable quantities by this method (Fig. 20, lanes 1 and 2). It was also possible to isolate the other phosphate-starvation-inducible outer membrane proteins using this column, although the yields were substantially lower, requiring a sensitive silver staining procedure to detect the proteins in SDS-polyacrylamide gels (eg. Fig. 20, lanes 3 and 4). Apparently, the cross-reactive antibodies in the protein P trimer-specific
Figure 20. SDS-polyacrylamide gel electrophoretogram of purified phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonads. Phosphate-starvation-inducible proteins were purified from the outer membranes of lanes 1, 2, 11 and 12, P. aeruginosa (i.e. protein P); lanes 3 and 4, P. fluorescens; lanes 5 and 6, P. putida; lanes 7 and 8, P. aureofaciens; lanes 9 and 10, P. chlororaphis. The proteins in lanes 3, 4, 11 and 12 were purified via affinity chromatography using a rabbit anti-protein P immunoadsorbant column. The proteins in lanes 1, 2 and 5-10 were purified by electroelution from polyacrylamide gels. Samples were solubilized at 88°C (lanes 1, 3, 5, 7, 9, 11) or 23°C (lanes 2, 4, 6, 8, 10, 12) prior to electrophoresis. Lanes 3 and 4 were stained for protein using a sensitive silver staining procedure (Wray et al 1981). Artifact bands visible in these lanes are a product of the silver staining procedure. All other lanes were stained by Coomassie brilliant blue. The faint continuous band seen in the middle of the gel is an artifact and was observable in lanes were no protein was loaded.
antiserum represent only a minor or low-affinity component of this antiserum. The purified proteins occurred as higher molecular weight oligomers in SDS-polyacrylamide gels when solubilized at room temperature prior to electrophoresis (e.g. Fig. 20, lane 4), dissociating to lower molecular weight monomers when solubilized at 88°C (e.g. Fig. 20, lane 3). This was consistent with the observed properties of these proteins in phosphate-limited cell envelope preparations (see Chapter Three, Fig. 16) and with the properties of purified protein P (Fig. 20, lanes 1 and 2).

To improve yields, these proteins were also purified using a procedure for the electroelution of proteins out of SDS-polyacrylamide gels (Parr et al., 1986). Phosphate-starvation-induced protein-containing extracts prepared from each of the above strains were run on SDS-polyacrylamide gels, the relevant protein oligomer bands excised and the protein electroeluted from the gel. This method produced substantially increased yields of all proteins (Fig. 20, lanes 5-12) which were easily visible in Coomassie stained gels. Again, the purified proteins retained their oligomeric structure, as attested by their resistance to SDS denaturation (Fig. 20, lanes 6,8,10,12) unless heated at high temperature (Fig. 20, lanes 5,7,9,11).

2. Single channel experiments. When the purified phosphate-starvation-inducible outer membrane proteins were added in small quantities (5-10 ng/ml) to the aqueous
solutions bathing a black lipid bilayer membrane, membrane conductance was seen to increase in a stepwise fashion (e.g. Fig. 21), presumably due to the incorporation of individual protein oligomers into the membrane as suggested for other porins (Benz et al., 1978; Benz et al., 1979; Benz and Hancock, 1981). The observed single channel conductance increments were distributed about a mean (e.g. Fig. 22), although larger increments were also seen at 2 (Fig. 22), 3 and 4 (not shown) times the average single channel conductance. These probably represented multiple insertions of the protein oligomers into the bilayer membrane as has been observed for other porins, including protein P (Hancock et al., 1982). The average single channel conductances in 1 M KCl measured for a given protein purified by either affinity chromatography or electroelution were not significantly different (Table XI), confirming both the utility of the anti-protein P immunoadsorbent column in purifying functional cross-reactive molecules and the general applicability of the electroelution procedure in purifying functionally active porin proteins. In addition, the derived average single channel conductance values obtained for each of the phosphate-starvation-inducible proteins were not significantly different, falling between 233 and 252 pS (Table XI). These values were substantially less than those obtained for the E. coli porins, including the phosphate-starvation-inducible PhoE porin of this strain (approximately 2 nS) (Benz et al., 1985), and for the major
Figure 21. Strip chart recordings of stepwise increases in the conductance of a small (0.1 mm$^2$) oxidized cholesterol membrane (1.5% in n-decane) caused by the addition of 10 ng/ml of the phosphate-starvation-induced outer membrane protein from P. putida to the aqueous phase (1 M KCl, pH 6.0). The applied voltage was 50 mV and the temperature was 25°C.
Figure 22. Histogram of the conductance fluctuations observed with membranes of oxidized cholesterol (1.5 % in n-decane) in the presence of the phosphate-starvation-induced outer membrane protein of P. putida and 1 M KCl (pH 6.0) in the aqueous phase. The applied voltage was 50 mV and the temperature was 25°C. P(A) is the probability of a given conductance increment A - taken from recorder tracings such as that shown in Figure 21.
$\bar{\Lambda}_1 = 247 \text{ pS} \quad n = 152$

$\bar{\Lambda}_2 = 490 \text{ pS} \quad n = 7$
<table>
<thead>
<tr>
<th>Strain</th>
<th>Affinity Purified</th>
<th>Electroeluted</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single channel $^a$</td>
<td>n $^b$</td>
<td>Single channel (pS)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>239</td>
<td>317</td>
<td>234</td>
</tr>
<tr>
<td>P. putida</td>
<td>233</td>
<td>74</td>
<td>247</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>241</td>
<td>117</td>
<td>-</td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>237</td>
<td>54</td>
<td>252</td>
</tr>
<tr>
<td>P. chlororaphis</td>
<td>243</td>
<td>45</td>
<td>237</td>
</tr>
</tbody>
</table>

$^a$ Average value from n events

$^b$ Number of single channel events measured
porin protein F of *P. aeruginosa* (5 nS) (Benz and Hancock, 1981). They were, however, in excellent agreement with the observed single channel conductance of protein P in 1 M KCl (Table XI) suggesting that the phosphate-regulated porin proteins of the fluorescent Pseudomonads all form small channels typical of protein P and in contrast to the majority of porins described to date (Benz *et al.*, 1981), including other phosphate-starvation-inducible porins proteins (Benz *et al.*, 1981; Verhoef *et al.*, 1984; Bauer *et al.*, 1985).

3. **Ion-selectivity.** To examine the ion-selectivity of these channels, single channel conductance was measured in salts of varying cation or anion size. The anion-specific protein P channel has previously been demonstrated to yield average single channel conductances, the magnitudes of which were dependent exclusively upon the size of the anion (Benz *et al.*, 1983). Thus conductance through protein P channels was demonstrated to be inversely related to the size of the anion (Benz *et al.*, 1983), while remaining basically unaffected by changes in cation size of the salt bathing a protein P-containing lipid bilayer membrane (Hancock *et al.*, 1982). By comparing the average single channel conductance values obtained in K⁺Cl⁻ with, for example, Tris⁺Cl⁻ and K⁺Hepes⁻, in which cases the cation and anion sizes, respectively, are increased, it should be possible to gain some idea of the ion selectivity of each of
the phosphate-starvation-induced porin proteins. The results in Table XII suggested that in all cases the observed single channel conductance was dependent upon anion size only, such that increasing the anion size in the case of $K^+\text{Hepes}^-$ (anion dimensions of $1.4\times0.6\times0.5$ nm compared with a radius of 0.181 nm for $K^+\text{Cl}^-$) resulted in no detectable conductance increments, while increasing the cation size in the case of $\text{Tris}^+\text{Cl}^-$ (cation radius of 0.67 nm compared with 0.133 nm for $K^+\text{Cl}^-$) yielded a single channel conductance which was not discernably different from that observed in $K^+\text{Cl}^-$. These data were consistent with the formation of anion-selective, if not specific, channels by these proteins.

The anion-specificity of protein P has been shown to be due to the presence of an anion-binding site within the channel (Benz et al., 1983). Thus conductance through protein P channels saturates at high salt concentrations (Benz et al., 1983), in contrast to porin proteins which lack binding sites and typically reveal a linear dependence of single channel conductance on salt concentration (Benz and Hancock, 1981; Benz et al., 1984). To determine if the anion-selectivity of the phosphate-regulated porin proteins could be attributed to binding sites within their respective channels, single channel conductance was measured as a function of salt (KCl) concentration. In every case, single channel conductance was seen to saturate at high salt concentrations (e.g. Fig. 23) consistent with the presence
Table XII. Single channel conductance of phosphate-starvation-inducible porin proteins of the fluorescent *Pseudomonads* in salts of varying anion and cation size

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average single channel(^a) conductance (pS) in</th>
<th>(K^+\text{Cl}^-)(^b)</th>
<th>Tris(^+\text{Cl}^-)(^b)</th>
<th>K(^+)Hepes(^-)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td>158</td>
<td>141</td>
<td>&lt;25</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td></td>
<td>144</td>
<td>143</td>
<td>&lt;25</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td></td>
<td>156</td>
<td>149</td>
<td>&lt;25</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td></td>
<td>164</td>
<td>169</td>
<td>&lt;25</td>
</tr>
<tr>
<td><em>P. chlororaphis</em></td>
<td></td>
<td>166</td>
<td>167</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

\(^a\) Average of at least 60 single channel events

\(^b\) Salts were employed at a concentration of 0.5 M. Ion radii (in nm) are as follows: \(K^+\), 0.133; \(\text{Cl}^-\), 0.181; Tris\(^+\), 0.670. Hepes\(^-\), an ellipsoid molecule, has dimensions 1.4x0.6x0.5 nm.
of a binding site within these channels. By plotting the data as an Eadie-Hofstee plot (e.g. Fig. 23 inset), Kd values for Cl\(^-\) binding were readily derived (Table XIII). While some variability in the affinity of Cl\(^-\) for each of the channels was observed, there was only a 2-fold range in Kd values for Cl\(^-\) binding for all channels, including protein P, demonstrating that the relative affinities of each of the channels for Cl\(^-\) were similar to protein P.


Because the single channel conductance of protein P channels in phosphate is low (6-9 pS in 1 M H\(_2\)PO\(_4\)^-) (Hancock et al., 1982), approaching the resolution limits of the black lipid bilayer apparatus, the presence of a phosphate-binding site within protein P channels was supported by the ability of orthophosphate to inhibit Cl\(^-\) conductance through protein P channels. The derived I\(_{50}\) value for phosphate (defined as the concentration of phosphate which yielded 50 % inhibition of chloride conductance) indicated that orthophosphate had a 60-100 fold higher affinity for protein P channels than did Cl\(^-\) (Hancock and Benz, submitted) the anion previously exhibiting the highest affinity for protein P channels (Benz et al., submitted). In order to identify potential phosphate-binding sites in the phosphate-starvation-induced porin proteins of P. putida, P. fluorescens, P. aurefaciens and P. chlororaphis, this strategy was also applied. Thus, after formation of a lipid bilayer (indicated by the
Figure 23. Average single channel conductance of the phosphate-starvation-induced porin protein of P. aureofaciens as a function of the KCl concentration in the aqueous solution bathing an oxidized cholesterol (1.5 % in n-decane) membrane. The applied voltage was 50 mV and the temperature was 25°C. The aqueous phase contained approximately 10 ng/ml of porin protein at KCl concentrations of 300 mM and higher. For KCl concentrations below 300 mM, 100 ng/ml of protein had to be added to obtain a sufficient number of single channels. Inset. An Eadie-Hofstee plot of the data obtained from measurements of single channel conductance (V) as a function of the KCl concentration (S). Binding constants (Table XIII) were obtained from such a plot using least squares analysis.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Kd for Cl$^-$ (mM)</th>
<th>I$_{50}$ for phosphate (mM)$^b$ at</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>153</td>
<td>0.59 12.7</td>
</tr>
<tr>
<td>P. putida</td>
<td>192</td>
<td>1.08 -</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>220</td>
<td>- 9.7</td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>297</td>
<td>- 27.0</td>
</tr>
<tr>
<td>P. chlororaphis</td>
<td>204</td>
<td>2.40 -</td>
</tr>
</tbody>
</table>

The average single channel conductance from at least 75 recorded events was determined at each of 5 concentrations of KCl between 50 and 1000 mM. The data was plotted as an Eadie-Hofstee plot (Figure 23 inset) from which Kd values were obtained by least squares analysis.

Inhibition of macroscopic chloride conductance by phosphate was carried out as described in Methods. The % inhibition of initial conductance was measured for different concentrations of phosphate and the data plotted as an Eadie-Hofstee plot (see Fig. 24 inset) from which I$_{50}$ values were obtained by least squares analysis.
membrane's turning optically black) a small amount of protein was added to the aqueous solution bathing the lipid membrane and conductance followed until the rate of increase had slowed considerably (usually 15-25 min). At this time membrane conductance had usually increased 2-4 orders of magnitude and > 1000 channels were present in the membrane. Aliquots of phosphate were added sequentially and the new conductance level measured after each addition.

For each protein studied, phosphate addition was seen to decrease the level of conductance originally observed in the presence of KCl alone, and the magnitude of this decrease was directly related to the concentration added (e.g. Fig. 24). By plotting the data as % inhibition of chloride conductance as a function of % inhibition of chloride conductance/phosphate concentration (i.e an Eadie-Hofstee plot) (Fig. 24 inset) it was possible to derive an apparent $I_{50}$ value for phosphate inhibition of chloride conductance for each of the phosphate-regulated porins (Table XIII). These data were consistent with the presence of a phosphate-binding site within each of these channels. The apparent $I_{50}$ values varied with the concentration of KCl, ranging from 9.7 to 27 mM phosphate in 1 M KCl and from 0.59 to 2.5 mM phosphate in 40 mM KCl (at pH 7) (Table XIII). At a given concentration of KCl, however, the variation in $I_{50}$ values obtained for all of the channels did not exceed 4-fold, indicating that the relative affinities of these channels for phosphate were quite similar.
Figure 24. Phosphate inhibition of chloride (Cl⁻) flux through protein P channels. Protein P (100 ng/ml) was added to the aqueous solution (40 mM KCl/1 mM Tris-HCl, pH 7.0) bathing an oxidized cholesterol (1.5 % in n-decane) membrane and the membrane conductance allowed to increase until it had stabilized (usually at a level 2-4 orders of magnitude higher than the initial level). At this time aliquots of potassium phosphate buffer pH 7.0 were added to the aqueous phase on both sides of the membrane and the new conductance level recorded. The % decrease in conductance was calculated and plotted as a function of the aqueous phase phosphate concentration [Pi]. The applied voltage was 20 mV and the temperature was 25 C. Inset. An Eadie-Hofstee plot of the data derived from measurements of the % inhibition of chloride conductance as a function of phosphate concentration. I₅₀ values for phosphate inhibition of chloride conductance (Table XIII) were calculated using least squares analysis.
5. **Summary.** Phosphate-starvation induced oligomeric proteins from the outer membranes of *P. fluorescens*, *P. putida*, *P. aureofaciens* and *P. chlororaphis* were purified to homogeneity. The incorporation of purified proteins into planar lipid bilayer membranes resulted in stepwise increases in membrane conductance. Single channel conductance experiments demonstrated that these protein were all capable of forming small, protein P-like channels with an average single channel conductance in 1 M KCl of between 233 and 252 pS. The conductance properties were not altered when the proteins were purified free of LPS prior to reconstitution in lipid bilayer membranes. Single channel conductance measurements made in salts of varying cation or anion size indicated that the channels were uniformly anion-selective. The measurement of single channel conductance as a function of KCl concentration revealed that all channels saturated at high salt concentrations, consistent with the presence of a binding site in the channel. Apparent Kd values for Cl\textsuperscript{−} were calculated and shown to vary only two-fold (180 - 297 pS) amongst all channels, including protein P channels. Phosphate was capable of inhibiting chloride conductance through all channels, with apparent I\textsubscript{50} values of between 0.59 and 2.5 mM phosphate at 40 mM Cl\textsuperscript{−}, and between 9.7 and 27.0 mM phosphate at 1 M Cl\textsuperscript{−}. These data were consistent with the presence of a phosphate-binding site in the channels of these phosphate-regulated proteins. Furthermore, they indicated that these channels had at least
...a 20 to 80-fold higher affinity for phosphate over chloride.
DISCUSSION

The transport of inorganic phosphate in the gram-negative bacterium *Pseudomonas aeruginosa* involves translocation across two membranes. In this study only those constituents external to the cytoplasmic membrane were examined in detail, in an attempt to address the mechanism(s) by which phosphate overcomes the permeability barrier of the outer membrane under phosphate-limiting conditions.

1. A phosphate regulon in *Pseudomonas aeruginosa*. In response to phosphate-deficiency, wild type cells of *P. aeruginosa* were shown to be derepressed for the synthesis of the enzymes alkaline phosphatase and phospholipase C (Fig. 4), in addition to a periplasmic phosphate-binding protein and an outer membrane channel-forming protein, P (Figs. 3 and 10). The observation that collectively these species were similarly constitutively produced or non-inducible in mutants of *P. aeruginosa* (Fig. 5) suggested that these constituents were indeed co-regulated, forming a phosphate regulon analogous to the *pho* regulon of *Escherichia coli* (Tommassen and Lugtenberg, 1982). Additional, as yet uncharacterized, orthophosphate-regulated proteins have been identified in phospholipase C regulatory mutants of *P. aeruginosa* PAO (Gray et al., 1981, 1982) suggesting that a phosphate regulon in *P. aeruginosa* PAO may be significantly

154
more extensive than described here. The coordinate regulation of the constituent genes of operons or regulons usually reflects the roles of their gene products in a common process. The maltose regulon in *E. coli*, for example, involves constituents of the transport and catabolism of maltose and maltodextrins (Bedouelle, 1984), while many of the components of the *pho* regulon in *E. coli* function in the acquisition of inorganic phosphate (Tommassen and Lugtenberg, 1982).

Like *E. coli*, *P. aeruginosa* demonstrates two major uptake systems for inorganic phosphate, of low and high-affinity, respectively (LaCoste et al., 1981; Fig. 14B). The observation that the low-affinity system operates constitutively is consistent with a role in the transport of phosphate in a phosphate-rich medium. Its high capacity (Vmax = 12.1 nmol/min/mg cell protein) undoubtedly reflects the ready availability of phosphate in a rich medium, as well as the growth potential of the organism under these conditions. In a dilute environment, however, phosphate uptake via the low-affinity system will be limiting for growth (compare the rate of growth of wild-type *P. aeruginosa* with that of a mutant expressing only the low-affinity system (Fig. 13)). The derepression of a high-affinity system permits efficient transport of phosphate from a dilute environment. Thus, during phosphate limitation strains capable of expressing a high-affinity uptake system transport phosphate at significantly greater
rates than mutants deficient in this system (Fig. 12). The lower capacity of this system ($V_{\text{max}} = 5.4$ nmol/min/mg cell protein) compared with the low-affinity system undoubtedly reflects the decreased availability of phosphate and a consequently reduced growth potential in a phosphate-limited environment (Fig. 2).

Alkaline phosphatase (a phosphate monoesterase) may function to provide the inorganic orthophosphate (Pi) substrate for these transport systems by its action on phosphate-containing molecules. Its concerted action with phospholipase C, which releases the phosphoryl choline moiety from specific phospholipid molecules, probably functions to make phospholipids available as a phosphate source as well. The derepression of these enzymes in phosphate-limiting media suggests that in an orthophosphate-rich environment, larger phosphate-containing molecules may not normally be used as phosphate sources. However, upon depletion of the available orthophosphate supply, other sources are made available by the action of these hydrolytic enzymes. Similarly, the induction of phosphate-selective outer membrane porin protein P in a phosphate-limited environment is a response to the consequently low rate of diffusion of phosphate across the outer membrane which would otherwise be limiting for transport and growth (see below).

High-affinity transport in *P. aeruginosa* was demonstrated to obligately require a periplasmic phosphate-binding protein, such transport being absent in a phosphate-
binding protein-deficient mutant (Fig. 14A). The binding affinity for phosphate ($K_d = 0.34$ uM) was in good agreement with the observed kinetics of high-affinity phosphate transport ($K_m = 0.46$ uM). This is characteristic of binding protein-dependent transport systems where the kinetics appear to be dictated by the binding proteins which function as the rate determining step. The binding affinity and ability to be released by cold-osmotic shock are typical of binding proteins isolated from *P. aeruginosa* (Stinson *et al*., 1977; Hoshino and Kageyama, 1980; Eisenberg and Phibbs, 1982) and of binding proteins in general (Oxender and Quay, 1976). Binding protein-dependent transport systems are typically energized by phosphate-bond energy (e.g. ATP) (Berger and Heppel, 1974). Thus, an inhibitory effect of arsenate on high-affinity phosphate transport in *P. aeruginosa* (LaCoste *et al*., 1981) might be due to its affect on energization, although the competitive nature of the inhibition ($K_i = 0.24$ mM; LaCoste *et al*., 1981) suggests that it was acting at some component of the high-affinity transport system. The observation that arsenate was capable of inhibiting the binding of phosphate to the binding protein at concentrations comparable to the $K_i$ (Table VII) implied that the effect of arsenate was at the binding protein stage of transport.

It is noteworthy that, upon induction in phosphate-deficient medium, the enzymes alkaline phosphatase and phospholipase C were secreted from the cell (Fig. 4B).
Theories of extracellular protein release generally held that outer membrane breakdown was a means of releasing proteins from, for example, a periplasmic location. Obviously, if such a mechanism were responsible for the observed enzyme release, the concomittant increase in outer membrane permeability could have functioned to increase the rate of diffusion of phosphate across the outer membrane without the need for a specific channel. Nevertheless, enzyme secretion was specific, as demonstrated by the failure to observe any concomittant release of the periplasmic beta-lactamase and phosphate-binding protein, and the barrier properties of the outer membrane were maintained (Fig. 6). This implied that the synthesis of a phosphate-selective outer membrane porin protein was not superfluous. Interestingly, it has been suggested (Ingram et al., 1973; Bhatti and Ingram, 1981) that alkaline phosphatase release by \textit{P. aeruginosa} is associated with a change in outer membrane permeability in addition to LPS release. Unfortunately, their use of a Tris-buffered medium invalidated their results, given the observed ability of Tris to permeabilize and cause significant structural reorganization of the outer membrane (Irvin et al., 1981).

2. \textbf{Properties of outer membrane protein P.} Outer membrane protein P of \textit{P. aeruginosa} was demonstrated to form SDS-stable oligomers (trimers; Angus and Hancock, 1983) in polyacrylamide gels, a property shared by the majority of
porin proteins described to date. The monomer molecular weight of this protein, 48,000, was significantly greater than that of most Enterobacterial porins and porin protein F of *P. aeruginosa* (36-39,000) (Lugtenberg and van Alphen, 1983; Hancock and Carey, 1979). Attempts at determining the molecular weight of the native (trimer) form of the protein on SDS-polyacrylamide gels [from Ferguson plots of the electrophoretic mobility of the trimers as a function of the acrylamide concentration (Tokunaga *et al.*, 1979)] have failed because the native protein migrates anomalously in this gel system. The anomalous migration of porin oligomers in SDS-polyacrylamide gels has been described previously (Tokunaga *et al.*, 1979) and has been attributed to the high degree of beta-structure present in the native porins (Nikaido and Vaara, 1985), which results in the binding of less SDS (wt/wt) by these proteins (Rosenbusch, 1974).

With few exceptions (e.g. proteins F and D1 of *P. aeruginosa*; Hancock and Carey, 1979; Hancock and Carey, 1980), porins exist as undenatured trimers when extracted with SDS. The observation that protein P formed SDS-stable trimers, although typical of porins in general, is thus in contrast to the other porin proteins described in *P. aeruginosa*. However, protein P was distinguishable from the majority of porins, including protein F, in that it was readily solubilized in a non-denaturing detergent (Triton X-100) in the presence of EDTA. Most porin proteins are soluble in Triton only after digestion of the peptidoglycan
with lysozyme, or in SDS and high salt (> 0.4 M NaCl) (Lugtenberg and van Alphen, 1983). This has been interpreted as indicating a strong non-covalent attachment of these porins to the peptidoglycan. In addition, the channels formed by protein P in lipid bilayer membranes (0.6 nm dia.) were significantly smaller than those formed by protein F (2.2 nm) and the major Enterobacterial porins (1–1.4 nm) (Benz et al., 1985). Finally, while the majority of porins studied to date, including the phosphate-starvation-inducible PhoE porin protein of *E. coli*, exhibit only weak ion selectivity in planar lipid bilayer membranes (Benz et al., 1985), protein P channels are anion-specific (Hancock et al., 1982; Table III).

As confirmed in this study, (Table XIII), the anion-specificity of protein P channels can be attributed to the presence of a binding site for anions in the channel (Benz et al., 1983). Acetylation of available amino groups on protein P (see Methods), which did not affect the ability of protein P to form SDS-stable oligomers (trimers) in polyacrylamide gels (not shown), resulted in the loss of the anion-binding site and a concomittant loss of the anion-specificity of the channel (Hancock et al., 1983b). From these studies it was concluded that the anion-specificity of protein P channels was a function of epsilon-amino groups of lysine residues present on the protein (Hancock et al., 1983b). Similarly, the phosphate-regulated PhoE porin of *E. coli* has been demonstrated to possess acetylatable lysine
residues which were responsible for the observed, albeit weaker, anion-selectivity of this channel in black lipid bilayer membranes (Darveau et al., 1984).

The contamination of purified porin preparations with LPS is well documented (Nikaido and Vaara, 1985) and suggests a strong tendency for these molecules to associate. Indeed, an in vivo association of LPS with porins, suggested by the marked reduction in porin levels in the outer membranes of LPS-deficient mutants (e.g. Koplow and Goldfine, 1974), has been suggested to be necessary, not only for porin function (Schindler and Rosenbusch, 1978), but also for the modulation of porin activity (Kropinski et al., 1982). Although conventionally purified protein P preparations invariably contained significant levels of LPS (Fig. 7; Table II), such an association was demonstrated to be dispensable in the formation of anion-specific channels by this protein (Table III). Recently, Korteland and Lugtenberg (1984) have reported that E. coli mutants with heptose-less LPS produce PhoE porins which facilitate a 6- to 8-fold more efficient permeation of anionic solutes. In contrast to results presented here with protein P, these data suggest that LPS may indeed influence porin activity.

In addition to being co-regulated with components of a high-affinity phosphate uptake system in P. aeruginosas (see above), protein P channels have recently been demonstrated to possess a binding site for inorganic phosphate (Hancock and Benz, submitted; Table XIII). The observation that
protein P channels exhibited a 60 to 80-fold higher affinity for phosphate over chloride (Hancock and Benz, submitted; Table XIII) was consistent with the demonstrated role for protein P in phosphate uptake (Table V) (see below).

3. The outer membrane of *P. aeruginosa* as a permeability barrier to phosphate under phosphate-limiting conditions. The low intrinsic permeability of the *P. aeruginosa* outer membrane is well documented, supported by observations of the increased crypticity of periplasmic enzymes in *P. aeruginosa* compared with the analogous enzymes in, for example, *E. coli* (Yoshimura and Nikaido, 1982) as well as direct measurements of outer membrane permeability (Angus et al., 1982; Yoshimura and Nikaido, 1982; Nicas and Hancock, 1983). Thus, the apparent transport and growth Km values for given nutrient molecules are often higher in *P. aeruginosa* than in *E. coli*. The results of this study demonstrated that the outer membrane of *P. aeruginosa* indeed functions as a permeability barrier to phosphate molecules under phosphate-limiting conditions. This was supported by the observed increase in the apparent Km for high-affinity phosphate transport in the protein P-deficient mutant (Table V), explainable by a decrease (at a given external phosphate concentration) in the rate of diffusion of phosphate molecules across the protein P-deficient outer membrane compared with the wild type outer membrane. The fact that the Vmax of high-affinity uptake remained unaltered in the
mutant (Table V) further indicated that the diffusion of phosphate across the protein P-deficient outer membrane was only rate-limiting for transport at low concentrations of phosphate. At higher concentrations of phosphate (e.g. > 25 uM) the rate of phosphate transport was not detectably different in the mutant compared with the wild-type. Furthermore, a defect in the growth capabilities of the protein P-deficient mutant strain was only seen at a very low concentration of phosphate (50 uM) (Table VI), indicating that phosphate diffusion across the outer membrane was dependent upon protein P at low external phosphate concentrations only. At higher concentrations of phosphate, diffusion across the outer membrane through the major porin protein F of *P. aeruginosa* is apparently capable of satisfying the cellular requirements for this nutrient. In addition, the small size and the selectivity of the protein P channels would undoubtedly serve to maintain the low intrinsic permeability of this membrane to other constituents.

Whether the protein P channels are sufficient to facilitate the efficient movement of phosphate from a dilute environment across the outer membrane remains in question. The apparent ability of the phosphate-binding protein to associate *in vitro* with protein P may have physiological relevance *in vivo* concerning the mechanism by which phosphate diffusion across the outer membrane is facilitated in a dilute environment. Certainly the ability of the
periplasmic maltose-binding protein to associate with the coregulated LamB outer membrane porin protein has been documented (Bavoil and Nikaido, 1981). Such an association, demonstrated in vitro, has been suggested to be necessary in vivo for the translocation, across the outer membrane, of maltose, when present at low concentrations, and maltodextrins (Wandersman et al., 1979; Luckey and Nikaido, 1983). From these studies it was suggested that a physical association between a high-affinity binding protein and an outer membrane porin could function to bring a high-affinity binding site near the outer surface of the outer membrane. Such an association might also ensure rapid binding of substrate once it entered the periplasm, maintaining a concentration gradient across the outer membrane, even under dilute conditions, by reducing the levels of free substrate in the periplasm. In the case of the phosphate-binding protein and protein P of P. aeruginosa, such an association would certainly not be obligatory given the ability of the phosphate-binding protein-dependent high-affinity uptake system to operate in the absence of protein P. The increase in the Km for high-affinity phosphate transport observed for the protein P-deficient mutant strain may reflect higher free periplasmic concentrations of phosphate and a subsequently smaller concentration gradient to drive diffusion across the outer membrane owing to the inability of the phosphate-binding protein and protein P to associate at the outer membrane.
A further analogy with the maltose transport system of *E. coli* is seen in the ability of long phosphate polymers to bind to the phosphate-binding protein. If polyphosphates are capable of being transported intact by the high-affinity phosphate transport system *in vivo*, without prior hydrolysis by alkaline phosphatase or other phosphatases, their permeation of protein P channels will undoubtedly depend upon the proper linear orientation of the molecules at the channel mouth, since these molecules exceed the exclusion limit of protein P channels. Such orientation could be carried out by the phosphate-binding site of protein P and/or the high-affinity phosphate-binding site present on the phosphate-binding protein, in association with the outer membrane channel-forming protein. Similarly, the ability of the LamB protein to allow the diffusion of maltodextrins which exceed the apparent exclusion limit of the LamB pore, has been postulated to depend upon binding sites both in the channel and on the maltose-binding protein (Von Meyenburg and Nikaido, 1977; Ferenci and Boos, 1980).

Growth of *P. aeruginosa* on polyphosphates as the sole phosphate source has been demonstrated (Valette *et al.*, 1966). Confirmed in this study, it was further demonstrated that growth in media containing phosphate polymers of up to 5 units in length occurred without the derepression of alkaline phosphatase (data not shown). This suggested that polyphosphates could be transported intact, without prior hydrolysis to constituent orthophosphate molecules. As
such, phosphatase(s) must be present in the cytoplasm to convert these polymers to the usual currency of inorganic phosphate, orthophosphate. In this regard, a number of gram-negative organisms, including *E. coli*, have been demonstrated to possess cytoplasmic polyphosphatases (Yagil, 1975). Interestingly, growth of *P. aeruginosa* in a medium containing a phosphate polymer of 15 units as the sole phosphate source was, in fact, accompanied by the induction of detectable alkaline phosphatase in both the periplasm and in the external medium (data not shown). Whether this reflects an inability of polyphosphate P15 to be transported intact or a rate of uptake so slow as to mimic phosphate-deficiency is uncertain. It may be a moot point, however, since alkaline phosphatase hydrolysis of such large polyphosphates was apparently necessary for growth.

In contrast to *P. aeruginosa*, the observed higher intrinsic permeability of the *E. coli* outer membrane leads one to conclude that the major porins themselves may be capable of satisfying the phosphate requirements of the cell, without the need for a facilitated diffusion channel for phosphate. According to Fick's first law of diffusion, the rate of phosphate transport across the *E. coli* outer membrane via the constitutive porin pathway will be higher, at a given concentration of phosphate, than in *P. aeruginosa*. This makes the need of a phosphate channel in *E. coli* less obvious. Despite the fact that *E. coli* cells deprived of phosphate are derepressed for a channel-forming outer
membrane protein, PhoE (Overbeeke and Lugtenberg, 1980), a specific role in phosphate uptake has not been demonstrated and unlike protein P, PhoE does not bind phosphate (Benz et al., 1984). Thus, although the PhoE porin functions as a more efficient channel for phosphate than do the OmpF or OmpC porins (Korteland et al., 1982), it does not provide any advantage to cells growing in medium containing orthophosphate as the sole phosphate source (Overbeeke and Lugtenberg, 1982). Furthermore, a 10-fold increase in the Km for phosphate transport reported for a PhoE-deficient mutant compared with a PhoE+ strain (Korteland et al., 1982) was obtained in a background deficient in the major porins. Thus it was possible that the increase in Km of the PhoE mutant simply reflected the overall porin-deficiency of the PhoE mutant strain such that e.g. an OmpF+PhoE- strain might have transported phosphate as well as the above OmpF-PhoE+ strain. The E. coli PhoE channel does, however, provide a growth advantage to cells growing in a medium containing polyphosphate as the sole phosphate source (Overbeeke and Lugtenberg, 1982), and it is also an efficient channel for the uptake of organic phosphates. Possibly its physiological role is in the transport of larger phosphorylated molecules.

4. Protein P and protein PhoE as members of two distinct classes of phosphate-regulated porins. In addition to E. coli and P. aeruginosa, a number of other gram-negative
bacteria, including *S. typhimurium* (Bauer *et al.*, 1985) and *E. cloacae* (Verhoef *et al.*, 1984), have been demonstrated to synthesize novel outer membrane porin proteins in response to phosphate-limitation. These proteins form anion-selective channels in reconstituted lipid bilayer membranes (Bauer *et al.*, 1985; Verhoef *et al.*, 1984; Benz *et al.*, 1984) consistent with their presumed roles in phosphate acquisition. The results of this study extend the list of bacteria synthesizing phosphate-starvation-inducible outer membrane proteins. Existing as oligomers (probably trimers) in SDS, these proteins can be dissociated to monomers when subjected to temperatures above 60°C (Table X), a property characteristic of porins (Lugtenberg and van Alphen, 1983). Furthermore, based on the monomer molecular weight, peptidoglycan association and abundance of these proteins, they are likely to be porins.

Two classes of these proteins were distinguishable, based on monomer molecular weight (36 to 39K or 45.5 to 50K) and detergent requirements to remove the proteins from the peptidoglycan (SDS-high salt or Triton-Tris-EDTA). The former class, typified by protein PhoE, included the phosphate-starvation-inducible proteins of *P. cepacia*, *P. pseudomallei* and the Enterobacteriaceae. The latter class included the phosphate-starvation-inducible proteins of the *P. fluorescens* branch of the family Pseudomonadaceae and was exemplified by protein P. These two proteins were also distinguishable from a functional point of view. Thus,
while protein PhoE forms large (1 nm), weakly anion-selective channels (Benz et al., 1985) which lack binding sites for anions and for phosphate (Benz et al., 1984), protein P channels were small (0.6 nm) (Hancock et al., 1982), possessing binding sites for anions and phosphate (Benz et al., 1983; Hancock and Benz, submitted; Table XIII) consistent with the observed anion-specificity (Hancock et al., 1982) and phosphate-selectivity (Hancock and Benz, submitted; Table XIII) of this channel. Furthermore, the observed channel-forming properties of the phosphate-starvation-inducible outer membrane proteins of the fluorescent Pseudomonads were indeed consistent with the formation of protein P type porins by these proteins.

One can only speculate on the purpose of synthesizing one or the other of these phosphate-regulated porin proteins. It seems probable that the production of a small specific channel like protein P, in P. aeruginosa and the fluorescent Pseudomonads, reflects the need to maintain low outer membrane permeability. Because these fluorescent Pseudomonads occur naturally in the soil, where many antibiotic-producing microorganisms are found, it may be necessary for their survival that they maintain a barrier to antibiotics. Indeed, the outer membrane of P. aeruginosa has been implicated in the high intrinsic resistance of this organism to antibiotics (Yoshimura and Nikaido, 1982; Nicas and Hancock, 1983). In contrast, E. coli demonstrates significantly higher outer membrane permeability, implying
that an outer membrane of extremely low permeability is not essential for \textit{E. coli} cells. The production of a phosphate-starvation-inducible porin protein, PhoE, whose channel size is not significantly different from the major constitutive porins of \textit{E. coli} (Benz et al., 1985) would obviously function to maintain the same degree of permeability, especially since total porin levels in the outer membrane are regulated in a stringent fashion (Lugtenberg and van Alphen, 1983). Unlike the protein P channel of \textit{P. aeruginosa}, which is too small to act as a channel for larger phosphate-containing molecules [except perhaps linear polymers of inorganic phosphate (see above)], the \textit{E. coli} PhoE channel has been shown to allow the passage of large organic phosphate molecules (Overbeeke and Lugtenberg, 1982). Given the predicted high intrinsic permeability of the \textit{E. coli} outer membrane to inorganic phosphate, the transport of larger phosphorylated molecules may, in fact, be the most important function of the PhoE channel. Indeed, \textit{E. coli} is often isolated from sewage effluent where polyphosphates (and organic phosphates) will be in abundance, as a result of the action of anaerobic organisms which typically make and store large quantities of polyphosphate (Kulaev, 1975). A cytoplasmic polyphosphatase has been identified in \textit{E. coli} (Yagil, 1975), suggesting that polyphosphates are a potential source of phosphate in this organism. Because protein P channels are too small to act as channels for large phosphate-containing molecules,
the ability of *P. aeruginosa* to utilize large phosphorylated molecules under dilute conditions, where uptake across the outer membrane via the major porin(s) will be limiting for transport and growth, may be dependent upon prior hydrolysis of these molecules by alkaline phosphatase, to release orthophosphate. Interestingly, alkaline phosphatase has been demonstrated to be secreted from *P. aeruginosa* cells into the external medium (Cheng et al., 1970; Fig. 4B) where it could, by cleavage of organic phosphates, provide a source of inorganic phosphate for transport. *E. coli*, on the other hand, synthesizes a porin protein in phosphate-limiting media (PhoE) which is capable of transporting these larger organic phosphates, and this might explain why alkaline phosphatase is exclusively located in the periplasm of this bacterium (Malamy and Horecker, 1961).

The production of phosphate-starvation-induced porins by the fluorescent Pseudomonads, all of which form protein P-like channels, suggests an evolutionary/taxonomic relationship. This is borne out by several pieces of data. All of these strains produce a major, constitutive protein (porin) which does not form SDS-stable oligomers. This is in contrast to the *Enterobacteriaceae* and other Pseudomonads, including *P. cepacia* and *P. pseudomallei*, which produce major porin proteins which are stable to SDS denaturation at temperatures below 60°C (Lugtenberg and van Alphen, 1983). This "heat-unmodifiable" phenotype is typical of porin protein F of *P. aeruginosa* (Hancock and
In addition, these fluorescent Pseudomonads also produce a lipoprotein which cross-reacts immunologically with outer membrane protein H2 of P. aeruginosa PAO1 (Mutharia and Hancock, 1985). Furthermore, based on the results of DNA and rRNA hybridization studies, these strains were similarly classified in the same homolgy group and were clearly distinct from other species of the family Pseudomonadaceae (De Vos and De Ley, 1983). These data suggest that outer membrane protein profiles and immunological relationships may be important indicators of evolutionary links.

Because a number of distinct bacterial species have been demonstrated to produce a protein P-like porin, the opportunity exists to study the biogenesis of the protein and to identify functional domains. Given the similarities of the functional properties of these proteins in different strains, which were quite distinct from other phosphate-regulated porins, it should be possible to identify regions of the protein involved in, for example, anion/phosphate-selectivity as regions of close homology in the genes encoding these proteins. Such data will undoubtedly contribute to elucidating the topology of this protein in P. aeruginosa. Furthermore, by examining the expression of these foreign genes in P. aeruginosa PAO1 we can identify regions of the protein important in synthesis, secretion and assembly.
5. Conserved antigenic determinants in phosphate-starvation-inducible outer membrane (porin) proteins. The demonstration here that phosphate-starvation-induced membrane oligomers cross-reacted immunologically (Fig. 18), indicated that these proteins express conserved antigenic sites (regions of structural homology). The observed destruction of this cross-reactivity by the heat-dissociation of oligomers to monomers, which also destroys porin function, and the failure of phosphate starvation-induced monomers to cross-react with a protein P monomer-specific antiserum, suggested that the conserved regions of homology were maintained in the native, functional proteins only. Furthermore, the inability of the protein P trimer-specific antiserum to react with the major porin proteins in these strains (Fig. 18) indicated that the cross-reactivity was distinct from any homologies relating to porin structure in general. Such homologies do, in fact, exist as indicated by the observed immunological cross-reactivity of OmpF, OmpC and PhoE porins of \textit{E. coli} (Overbeeke \textit{et al.}, 1980) and of porin proteins F and P of \textit{P. aeruginosa} (K. Poole, unpublished result).

The cross-reactivity of phosphate starvation-induced oligomeric proteins may well relate to specific functional properties of these phosphate-regulated proteins. In this vein, a number of the phosphate starvation-induced membrane proteins have been demonstrated to form anion-selective channels (Hancock \textit{et al.}, 1982; Benz \textit{et al.}, 1984; Verhoef
et al., 1984; Bauer et al., 1985; Table XIII), in contrast with the major Enterobacterial porin proteins and the major porin protein F of *P. aeruginosa*, which are weakly cation-selective (Benz et al., 1985). The anion-selectivity of some of these proteins has been attributed to fixed positive charges, possibly epsilon-amino groups of lysine residues, in or near the mouth of the respective channels (Hancock et al., 1983b; Darveau et al., 1984). The demonstration of at least 14 exposed/accessible lysine residues in the protein P and PhoE channels (R.E.W. Hancock, unpublished result) is consistent with this idea. By comparison, porin protein OmpF contains 5 accessible lysine residues (R.E.W. Hancock, unpublished result). It is tempting to hypothesize that exposed lysine residues present in these, and perhaps other, phosphate-regulated porin proteins may be involved in the observed cross-reactivity of phosphate-starvation-induced membrane proteins, perhaps forming part of the conserved antigenic site(s). In support of this, protein PhoE channels are functionally indistinguishable from OmpF and OmpC channels upon acetylation of available lysine residues. The observed immunological cross-reactivity of these proteins (Overbeeke et al., 1980), the 60-70 % amino acid homology (Tommassen et al., 1982; Mizuno et al., 1983), and the ability of the *ompF* and *phoE* genes to hybridize in regions along their entire lengths (Tommassen et al., 1982) implies that the property of anion-selectivity as a function of lysine residues in the channel may well be the major
discriminating feature of PhoE compared with the other major porins in *E. coli*.

If indeed the cross-reactive determinant(s) are involved in the anion-selectivity of these proteins, then the question remains as to whether these phosphate-regulated porins evolved directly from the same ancestral phosphate porin gene, or whether they evolved from the various porin genes in these bacteria. Clearly, to answer this question it will be necessary to obtain sequence information for each of the porins concerned.
LITERATURE CITED


Publications


Publications cont'd

