ROLE OF THE OUTER MEMBRANE OF PSEUDOMONAS AERUGINOSA IN ANTIBIOTIC RESISTANCE

by

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ABSTRACT

It was demonstrated that induction of a major outer protein, H1, was associated with increased resistance to chelators of divalent cations such as EDTA and to the cationic antibiotics polymyxins and aminoglycosides. Outer membrane protein H1 was the major cellular protein in cells grown in Mg$^{2+}$-deficient medium (0.02 mM Mg$^{2+}$) and in mutants selected for resistance to polymyxin. Increase in protein H1 was associated with decrease in cell envelope Mg$^{2+}$. Induction of protein H1 was prevented by supplementation of Mg$^{2+}$-deficient medium with 0.5 mM Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ or Sr$^{2+}$, but not by Zn$^{2+}$, Ba$^{2+}$, or Sn$^{2+}$. Cells grown in Ca$^{2+}$, Mn$^{2+}$ or Zn$^{2+}$ showed enhanced levels of these cations as main major cell envelope associated cation. Only cells grown in the presence of those cations which failed to prevent H1 induction were resistant to chelators, polymyxin B and gentamicin. Protein H1 overproducing cells also demonstrated altered streptomycin uptake.

It was further demonstrated that aminoglycosides could interact with the outer membrane so as to make it more permeable to other substances. Mg$^{2+}$ inhibited aminoglycoside-mediated permeabilization. Both aminoglycosides and polymyxin B could be shown to displace a small amount of Mg$^{2+}$ from the cell envelope.
A mutant severely deficient in outer membrane protein F was isolated. Permeability of this strain was studied by measuring hydrolysis of a chromogenic beta-lactam by periplasmic beta-lactamase. It was found that outer membrane permeability of *P. aeruginosa* was low compared to *E. coli* and that loss of protein F caused a further decrease. The results suggest that only a small proportion of protein F molecules form functional channels in wild type cells so that the hydrophilic pathway of uptake across the outer membrane is relatively inefficient. Cationic antibiotics such as aminoglycosides and polymyxins may use an alternate pathway of "self promoted" permeation. It is proposed that EDTA, polymyxin and aminoglycosides act by attacking a critical divalent cation binding site on the lipopolysaccharide. Protein H1 is proposed to act by replacing divalent cations at this site, preventing the action of these agents.
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<td>A&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Absorbance at 600 nm.</td>
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<td>BM2</td>
<td>Basal medium number 2, a phosphate buffered minimal medium (Gilleland et al., 1974).</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate.</td>
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<td>EGTA</td>
<td>Ethyleneglycol-bis(beta-ethylether)N,N'tetraacetate.</td>
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<td>LPS</td>
<td>Lipopolysaccharide.</td>
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<td>MIC</td>
<td>Minimal inhibitory concentration.</td>
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<tr>
<td>NTG</td>
<td>N-methyl-N'-nitro-nitrosoguanidine.</td>
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<td>PP2</td>
<td>Proteose peptone number 2 medium.</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate.</td>
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I am grateful for the support of a Frank Wesbrook scholarship during most of my studies.
INTRODUCTION

*Pseudomonas aeruginosa* is a gram-negative bacterium found widely distributed in the environment. During the last 20-25 years *P. aeruginosa* has acquired notoriety as a major opportunistic pathogen. One of the primary reasons for its emergence in this role is its resistance to commonly used antibiotics.

1. **Medical importance of Pseudomonas aeruginosa.** The introduction of efficient anti-staphylococcal agents has played a major role in altering patterns of hospital-acquired infections. Consequently, gram-negative bacteria have replaced staphylococci as the major source of nosocomial infections. *P. aeruginosa* in particular is well known for its resistance to antiseptics, and is often difficult to eradicate. Extensive use of broad spectrum antibiotics has also favoured the selection of *P. aeruginosa*, so that it is now a major potential hazard in the hospital environment.

*P. aeruginosa* does not cause disease in healthy, uninjured individuals. However, in individuals with impaired defense mechanisms, it is one of the most frequently isolated pathogens. As modern medicine improves the survival rate for patients with severe burns, neoplastic disease and cystic fibrosis, an increasing incidence of *Pseudomonas* infections has been noted (Levine *et al.*, 1974; Pennington *et al.* 1975; Reynolds *et al.*, 1975). Treatment with immunosuppressive and
cytotoxic drugs and corticosteroids, as well as many hospital techniques (e.g., catheterization) which can result in introduction of organisms into susceptible tissues have contributed to the growing prevalence of infections by gram-negative bacteria, especially \textit{P. aeruginosa}. \textit{P. aeruginosa} is a predominant cause of burn-wound infections. For example, some studies have found that it colonizes up to 60\% of patients by 5 days after injury (Pruitt \textit{et al.}, 1979). The frequency of \textit{P. aeruginosa} is also extremely high among cancer patients, especially in patients with acute leukemia or other diseases resulting in neutropenia (Rodriguez and Bodey, 1979). Fatality rates of such infections are reported to be as high as 50-80\% (Rodriguez and Bodey, 1979). \textit{P. aeruginosa} is a major pathogen in patients with cystic fibrosis, where it is the predominant bacterium associated with terminal pulmonary illness. It is also an important cause of hospital-acquired pneumonias, hospital-acquired urinary tract infections, and one of the major causes of infection of patients undergoing invasive procedures.

2. \textbf{Antibiotic resistance of \textit{Pseudomonas aeruginosa}.} \textit{P. aeruginosa} has been characteristically regarded as resistant to antimicrobial agents, although introduction of new antimicrobial drugs specifically developed for anti-pseudomonal activity has somewhat changed this situation (Bryan, 1979). The small number of antibiotics which show high \textit{in vitro} activity against \textit{P. aeruginosa} include some aminoglycoside antibiotics
such as gentamicin, tobramycin, amikacin and their recently developed analogues such as netilmicin and sisomycin, polymyxin antibiotics such as polymyxin B and colistin, and a variety of semi-synthetic beta-lactam antibiotics, including carbenicillin, its thienyl analogue ticarcillin, and some new broad spectrum cephalosporins such as cefotaxime and moxalactam.

For all anti-pseudomonal antibiotics currently in use, effectiveness is limited by the narrow margin between safe and effective doses (Young, 1979; Bryan, 1979). This is particularly true for aminoglycosides, which are ototoxic and can cause renal damage, (Wersall et al., 1969; Falco et al., 1969) and for polymyxins, which are generally considered to be too toxic for parenteral use (Bryan, 1979). Furthermore, there has generally been a poor correlation between the in vitro susceptibility to antimicrobial agents and in vivo therapeutic efficacy (Davis, 1974; Flick and Cuff, 1976). One possible cause for this is the occurrence of adaptive (non-mutational) resistance in vivo, as has been demonstrated for a variety of antibiotics in vitro (Brown and Melling, 1969; Gilleland et al., 1974; Pechey and James, 1974). One of the properties of P. aeruginosa which is becoming clear from recent studies is that the cell envelope of P. aeruginosa is highly variable depending on the growth conditions to which the organism is subjected (Brown and Melling, 1969; Costerton et al., 1979; Hancock and Carey, 1981; Hancock et al., 1982). In agreement with this, it now appears that the outer membranes of cells grown in vivo are somewhat different from those grown in vitro.
(P.A. Sokol, D.E. Woods, C.D. Cox, and B.H. Iglewski, manuscript in preparation; K. Poole and R.E.W. Hancock, unpublished data). Some of these changes may well affect antibiotic efficacy (Costerton et al., 1979).

An additional cause of the poor correlation between in vivo and in vitro effectiveness of antibiotics is the antagonism of antibiotic activity by Ca$^{2+}$ and Mg$^{2+}$, which occurs with polymyxin and aminoglycoside antibiotics (Newton, 1954; Zimelis and Jackson, 1973). In the case of aminoglycosides, divalent cation antagonism of antibiotic action is of much greater significance in P. aeruginosa than in other gram-negative bacteria (Madeiros et al., 1971). Differences of up to 32-fold have been observed in measurements of minimal inhibitory concentrations of gentamicin in Mueller Hinton medium with varying levels of Ca$^{2+}$ and Mg$^{2+}$ (Reller et al., 1974). It has been suggested by several workers that the site of Mg$^{2+}$ and Ca$^{2+}$ antagonism is the cell envelope (Newton, 1954; Zimelis and Jackson, 1973; Brown, 1975). The study reported here offers evidence that the site of competition is the outer membrane.

3. Mechanisms of antibiotic resistance. Resistance of P. aeruginosa to antimicrobial agents takes two general forms (Bryan, 1979). One of these is resistance to agents effective against most other gram-negative bacteria, generally termed "intrinsic" resistance. The second form is emerging resistance to the more recently introduced anti-pseudomonal agents such as gentamicin and carbenicillin. In many cases the latter form of
resistance may be attributed to inactivating enzymes usually specified by R factors (Bryan, 1979).

Intrinsic resistance does not, however, require the presence of R factors, and is present in nearly all strains of P. aeruginosa. Antibiotic inactivating enzymes appear to have only a minor role in intrinsic resistance. For example chromosomally-specified, inducible cephalosporinase is reported to be present in all strains of P. aeruginosa (Bryan, 1979; Sabath et al., 1965), which may in part account for resistance to some beta-lactam antibiotics. Other chromosomally-specified inactivating enzymes have not been found (Bryan et al., 1975). Resistance at the level of the targets of antibiotic activity also does not appear to account for intrinsic resistance. For example, Bryan et al. (1975) have shown that protein synthesis by cell-free systems derived from P. aeruginosa is fully sensitive to inhibition by streptomycin, whereas whole cells are quite resistant. Similarly, Mirleman and Nuchamowitz (1979) have shown that the enzymes which synthesize peptidoglycan in P. aeruginosa are at least as sensitive to benzylpenicillin as those of E. coli, whereas whole cells are much more resistant. As neither antibiotic inactivation nor altered targets of antibiotic action would seem to account for the high antibiotic resistance of P. aeruginosa, a possibility to be considered is that antibiotics are unable to reach their site of activity. Bryan and colleagues have shown that defective uptake of aminoglycosides (Bryan, 1979) and tetracycline (Tseng and Bryan,
1974) correlates with resistance to these drugs. In addition, enhanced permeability of the outer membrane has been shown to correlate with increased susceptibility to 27 different agents in the antibiotic supersusceptible *P. aeruginosa* mutant Z61 (Angus et al., 1982). Thus perhaps the most likely explanation of intrinsic resistance is that *P. aeruginosa* is relatively impermeable to antibiotics (Brown, 1975; Bryan, 1979).

4. The cell envelope and permeability. The cell envelope of *P. aeruginosa*, like that of other gram-negative bacteria has been shown by electron microscopy to consist of three layers. These are the inner, or cytoplasmic membrane, the peptidoglycan, and the outer membrane. In some instances, especially infections of children with cystic fibrosis, a capsule of mucoid material may also be present. The space between the inner and outer membrane, the periplasm, is the location of a variety of degradative enzymes, including those which inactivate antibiotics (Benveniste and Davies, 1973; Bryan, 1979). The inner membrane is the site of specific, energy-requiring, transport systems, as well as components involved in energy generation and most of the enzyme systems involved in synthesis of the external wall layers, peptidoglycan and the outer membrane. The inner membrane constitutes a highly selective permeability barrier (Costerton et al., 1974). Both the inner and outer face of the cytoplasmic membrane are thought to be hydrophobic (Machtiger and Fox, 1973), and it has been demonstrated that
this membrane does not constitute an effective barrier against hydrophobic substances (Teuber and Miller, 1977). In contrast, the inner membrane is relatively impermeable to hydrophilic substances. Such substances cross the inner membrane by means of substrate specific transport systems. Thus hydrophilic antibiotics are unable to enter the cytoplasm unless they are able to mimic a natural substrate and use its transport system, or disrupt the membrane sufficiently to allow permeation.

The peptidoglycan of *P. aeruginosa* does not appear to differ chemically from that of enteric organisms (Meadow, 1975), although it does not appear to be covalently linked to an outer membrane protein analogous to the major lipoprotein of *E. coli* (Hancock et al., 1981). However, it seems unlikely that the peptidoglycan makes a major contribution to the low antibiotic permeability of *P. aeruginosa*.

As the targets of antibiotic activity are either within the cytoplasm (e.g., the ribosomes) or within the cell envelope itself (e.g., the penicillin binding proteins exposed on the outer surface of the inner membrane), the common barrier which must be traversed is the outer membrane. There is a growing body of evidence that much of the intrinsic resistance of *P. aeruginosa* may be accounted for on the basis of the permeability properties of its outer membrane. The outer membrane of gram-negative bacteria has a major role as a permeability barrier. Nikaido (Nikaido and Nakai, 1979) has described two general pathways for diffusion of small molecules across the
outer membrane, one for hydrophobic compounds and one for hydrophilic compounds. The hydrophobic pathway is apparently unimportant in the outer membrane of organisms such as *E. coli*, *Salmonella* and *P. aeruginosa*, which synthesize complete lipopolysaccharides although its existence was demonstrated in deep rough organisms. The hydrophilic pathway is mediated by specific integral membrane proteins which form trans membrane channels or pores. Such molecules are generally termed "porins" (Nikaido and Nakai, 1979).

5. **Properties of the outer membrane of gram-negative bacteria.**

The outer membrane of enteric bacteria has been extensively studied (for reviews see Nikaido and Nakae, 1979; DiRienzo et al., 1980; Osborn and Wu, 1980) and considerable insight has been achieved with regard to both outer membrane structure and the relationship of structure to the function of the outer membrane as a permeability barrier. The major components of the outer membrane are protein, phospholipid and lipopolysaccharide (LPS). Like other membranes, the outer membrane appears as a bilayer in the electron microscope. Studies of the outer membrane of *E. coli* and *Salmonella* (reviewed by Nikaido and Nakae, 1979) have shown that the outer membrane is unusual, however, in the extreme assymetry of distribution of membrane components: virtually all the phospholipids are located on the inner face (except, possibly in certain mutants) while virtually all the LPS is on the outer surface. In contrast, the proteins of
outer membrane are present in both layers, and are, in some cases, membrane spanning (Enderman et al., 1978).

a. Lipopolysaccharide. The general structure of LPS is similar in all gram-negative bacteria and has been reviewed by Ørskov et al., 1977. LPS is an amphipathic molecule with a hydrophobic portion, lipid A, believed to be embedded in the membrane and a hydrophilic polysaccharide portion, which extends out from the cell surface. The distal portion of the polysaccharide, the O antigen, often consists of oligosaccharide repeating units, and shows wide variability even within a single species. This property, which can be simply screened by using antisera, allows fine serological typing of strains. The proximal portion, the R-core, shows less variability with given species, and often contains a unique eight carbon sugar (2-keto-3-deoxyoctonate), and a heptose. Phosphate and ethanolamine phosphate are also present in this region, and studies with fluorescent probes have indicated binding sites for divalent cations in this portion of the LPS (Schindler et al., 1978). The properties of the LPS of enteric bacteria and P. aeruginosa result in a membrane which is highly impermeable to hydrophobic compounds, in contrast to the permeability of phospholipid bilayers to such compounds (Nikaido and Nakae, 1979). This would account for the resistance of these organisms to hydrophobic antibiotics such as actinomycin D, erythromycin and rifampicin. It should be noted that the high level of resistance of P. aeruginosa to hydrophobic antibiotics (Bryan, 1979)
strongly suggests that no significant hydrophobic uptake pathway exists in this organism either.

b. **Protein.** The proteins of outer membranes are largely responsible for the permeability of the outer membrane to hydrophilic compounds. A number of outer membrane proteins appear to function in the transport of specific compounds across the outer membrane. These include proteins required for the uptake of iron chelates (Hancock et al., 1976), nucleosides (Hanke, 1976), vitamin B12 (Dimasi et al., 1973), and maltodextrins and maltose (Szmeleman and Hufnung, 1976). Such proteins are especially important in the uptake of substrates present at very low concentrations (Nikaido and Nakae, 1979). The major contribution to outer membrane permeability, however, comes from proteins called porins, which form general transmembrane diffusion channels. Such proteins, which generally have an apparent molecular weight of 32,000-42,000, are present in all gram-negative bacteria so far examined. They are present in high copy number, membrane spanning, and are generally closely associated with peptidoglycan (DiRienzo et al., 1978). The pore-forming function of these proteins has been established in reconstitution studies where porin is incorporated into phospholipid or phospholipid-LPS vesicles (Nakae, 1976a and 1976b) or into black lipid bilayers (Benz et al., 1978). Experiments by Nikaido and coworkers (Nikaido and Nakae, 1979) have established that porin channels appear to have a fairly constant diameter, thus limiting the size of molecules able to cross the
outer membrane, which consequently acts as a molecular sieve. Exclusion limits of *E. coli* and *Salmonella* porins have been measured as 550-650 daltons (Decad and Nikaido, 1976) while that of *P. aeruginosa* is larger, 3000-9000 daltons (Hancock and Nikaido, 1978). The function of porins in controlling permeability has been confirmed in studies of mutants deficient in these proteins (von Meyenburg and Nikaido, 1977; Lutkenhaus, 1977; Bavoil *et al.*, 1977). More than one porin species has been found in several bacteria (Osborn and Wu, 1980), and alternate porins which can be induced by specific growth conditions (von Meyenburg and Nikaido, 1977; Tommassen and Lugtenberg, 1970; Hancock and Carey, 1980; Hancock *et al.*, 1982) or prophage infection (Schnaitman, 1974) are also known. It is, however, unclear whether such porins are truly capable of mediating generalized permeability in the same way that the "major" porins do. The contribution of proteins other than porins and specific transport proteins is not known, but it would appear likely that their role in determining permeability is for the most part indirect.

Porin alterations in *E. coli* have been shown to affect the uptake of some beta-lactams, chloramphenicol, and tetracycline (Nikaido *et al.*, 1977; Van Alphen *et al.*, 1978; Chopra and Eccles, 1978; Foulds, 1976) and it would appear that these proteins are largely responsible for the permeability of hydrophilic antibiotics to the outer membrane. Only a single isolated example of an antibiotic which uses a specific outer
membrane transport protein to cross the outer membrane has been found: albomycin, which uses the Ton A ferrichrome transporting proteins (Braun et al., 1976) — although it is important to note that albomycin is a ferrichrome analogue.

6. The outer membrane of P. aeruginosa and its role in antibiotic permeability. Although the outer membrane of P. aeruginosa would appear to be similar to that of E. coli and Salmonella in its overall design, there appear to be significant differences which may well contribute to the different antibiotic susceptibility of P. aeruginosa.

The proteins of the outer membrane of P. aeruginosa have been examined by several laboratories (Stinnett and Eagon, 1973; Mizuno and Kageyama, 1978; Hancock and Nikaido, 1978; Hancock and Carey, 1979). Six to eight major outer membrane proteins have been found. Porin activity has been demonstrated for protein F (Hancock et al., 1980). This molecule differs from porins of enteric bacteria in that it is unusually unstable to sodium dodecyl sulphate, and has two intra-chain disulfide bridges (Hancock and Carey, 1979) Two inducible porins have also been demonstrated. These are protein D1, a protein induced during growth on glucose as sole carbon source (Hancock and Carey, 1980), and protein P, an anion-selective pore with a relatively low exclusion limit, which appears during growth on phosphate-deficient medium (Hancock et al., 1981). A lipoprotein, protein I, analogous to the Braun
lipoprotein of *E. coli* (Braun, 1975), has been demonstrated in *P. aeruginosa*, and, although this protein is peptidoglycan associated, it may well differ from *E. coli* protein in that 30% of the *E. coli* protein is covalently attached to the peptidoglycan whereas the covalent association of the *P. aeruginosa* protein I is disputed (Mizuno and Kageyama, 1979; Hancock et al., 1981). In any case, the amino acid compositions of these two lipoproteins differ substantially. A second lipoprotein has also been demonstrated (Mizuno, 1979). In sodium dodecyl sulphate gel electrophoresis, this protein (H2) is separated from a protein of similar molecular weight (H1) only under conditions described by Hancock and Carey (1979). The function of H2, and of the other major outer proteins D2, E, and G, is still unknown.

Although it has been established that the size of individual porin channels is larger in *P. aeruginosa* than in enteric bacteria (Benz and Hancock, 1981; Hancock and Nikaido, 1978), there is evidence accumulating that the number of functional pores and consequently the total area of pore available for diffusion is much lower, so that total permeability via the hydrophilic pathway is relatively low. Low outer membrane permeability has been shown in *in vivo* studies of wild type *P. aeruginosa* (Angus et al., 1982). These workers showed that an antibiotic super susceptible mutant of *P. aeruginosa*, Z61, selected as antibiotic susceptible (Zimmerman and Rosselet, 1979) showed greatly enhanced outer membrane permeability, thus
providing evidence that low outer membrane permeability is indeed a major determinant in intrinsic resistance to antibiotics. In Z61, increased permeability and antibiotic supersusceptibility were associated with an LPS alteration (Kropinski et al., 1982). Low in vivo porin activity of wild type cells correlates well with the low pore forming activity of protein F which has been found in in vitro studies (Benz and Hancock, 1981). These observations, however, did raise the question as to whether the actual porin was protein F, or some minor contaminant copurified with protein F. This study reports the isolation of a mutant severely deficient in protein F. Results of permeability studies with this mutant confirm the pore-forming function of protein F and indicate that less than 1% of protein F molecules form functional channels across the outer membrane.

Another unusual property of the LPS of P. aeruginosa is its unusually high phosphate content (Drewry et al., 1971). This phosphate is associated with the core region, and is, in part, as triphosphate (Wilkinson, 1981). The cell envelope of P. aeruginosa also has very high levels of divalent cations (Brown and Wood, 1972), which may well be associated with these phosphate groups since phosphate carries a net negative charge at neutral pH. While P. aeruginosa is unusually resistant to antibiotics, it is highly susceptible to chelators of divalent cations such as EDTA (Cox and Eagon, 1968), and to polymyxin antibiotics. For example, although treatment of E. coli with
EDTA is insufficient to allow osmotic lysis unless lysozyme is present (Leive, 1965), EDTA treatment of *P. aeruginosa* results in osmotically fragile cells. Tris(hydroxymethyl)aminomethane (Tris) maximizes this EDTA effect (Eagon and Asbell, 1966). Treatment with EDTA results in the release of LPS-protein complexes with low (less than 10%) phospholipid content (Rogers *et al.*, 1969; Stinnett and Eagon, 1975). These complexes can be visualized by electron microscopy of freeze fractured cells and appear to be distinct aggregates in the plane of the membrane (Stinnett and Eagon, 1975). It has been concluded from these and other studies (Roberts *et al.*, 1970; Kenward *et al.*, 1979; Boggis *et al.*, 1979) that divalent cations play a critical role in maintaining the stability of the outer membrane.

Polymyxins are amphipathic molecules consisting of a highly cationic peptide head and a hydrophobic tail (Storm *et al.*, 1977). Like EDTA, polymyxins appear to act directly on the outer membrane, and are known to bind with high affinity to LPS (Cooperstock, 1974; Schindler and Osborn, 1979). The action of polymyxins is inhibited by the presence of divalent cations (Newton, 1954). These observations have led to the suggestion that polymyxins and EDTA act at a common site on the outer membrane, a divalent cation binding site on the LPS which is required for outer membrane stability (Brown, 1975).

The study reported here provides experimental evidence supporting this suggestion. Furthermore, evidence is
presented that aminoglycoside antibiotics may also be active at this same site in \textit{P. aeruginosa}, and that disruption of this site by aminoglycosides and similar cationic substances may provide an alternate pathway across the outer membrane. This study also provides an explanation for the long-standing observation that the susceptibility of \textit{P. aeruginosa} to EDTA and polymyxin may be reversed by growth in Mg$^{2+}$-limited medium (Brown and Melling, 1969). It is demonstrated that this increase in resistance is associated with induction of a major outer membrane protein H1. It is suggested that H1 acts to replace divalent cations at a critical divalent cation binding site on the LPS, protecting this site from attack by EDTA and cationic antibiotics.
1. **Media and growth conditions.** Proteose peptone no. 2 (Difco, PP2) was used as a rich medium. The minimal medium used was Basal Medium No. 2 (BM2) described by Gilleland et al. (1974), containing 10 mM FeSO₄, and either 20 mM potassium succinate (BM2 succinate) or 0.4% (wt/vol) glucose (BM2 glucose). The usual level of MgSO₄ added was 0.5 mM (Mg²⁺ sufficient media). Mg²⁺ deficient media contained 0.02 mM MgSO₄. Other cations were added as chloride salts in the amounts specified in the text. Liquid cultures were grown with vigorous aeration at 37°C except where stated otherwise. All glassware used with defined media was cleaned by autoclaving with distilled water.

Nutrient broth (Difco) was used to grow cells for streptomycin uptake assays.

2. **Bacterial strains.** Sources and properties of the principal bacterial strains used in this study are listed in Table I.

*Pseudomonas aeruginosa* PA01 strain H103 was used as the wild type and reference strain throughout. The outer membrane of this strain has previously been well characterized (Hancock and Carey, 1979; Hancock et al., 1981).

Strains H185 and H181 were independently isolated from H103 by diethyl sulfate mutagenesis followed by selection on
### TABLE 1. Bacterial strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103</td>
<td><em>P. aeruginosa</em> PAO 1 wild type</td>
<td>A. Kropinski (Queen's University Kingston, Ont.)</td>
</tr>
<tr>
<td>H181</td>
<td>Polymyxin B resistant mutants of H103</td>
<td>This study</td>
</tr>
<tr>
<td>H185</td>
<td>Revertant of H181</td>
<td>This study</td>
</tr>
<tr>
<td>H207</td>
<td>Antibiotic super-susceptible <em>P. aeruginosa</em></td>
<td>W. Zimmerman (Ciba-Geigy, Basel Switzerland)</td>
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<td>H283</td>
<td>Protein F-deficient mutant of H103</td>
<td>This study</td>
</tr>
<tr>
<td>H284</td>
<td>Revertants of H283</td>
<td>This study</td>
</tr>
<tr>
<td>H321</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H324</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK43</td>
<td>LPS-deficient mutants of H103</td>
<td>A. Kropinski</td>
</tr>
<tr>
<td>AK1160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK1012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK1213</td>
<td>Non-piliated mutant of H103</td>
<td>A. Kropinski</td>
</tr>
<tr>
<td>AK1114</td>
<td>Non-piliated, non-flagellated mutant of H103</td>
<td>A. Kropinski</td>
</tr>
<tr>
<td>UB1636(RP1)</td>
<td><em>E. coli</em> K12 *trp his strA lac / amp&lt;sup&gt;r&lt;/sup&gt; tet&lt;sup&gt;r&lt;/sup&gt; neo&lt;sup&gt;r&lt;/sup&gt; kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>P.M. Bennet (University of Bristol, Bristol U.K.)</td>
</tr>
</tbody>
</table>
BM2 succinate agar containing 50 μg/ml of polymyxin B sulphate. Mutagenesis was carried out by suspending 0.1 ml of overnight cells in 5 ml saturated solution of ethyl sulphate in 0.06 M potassium phosphate buffer pH 6.0 for 30 min. at 25°C. Cells were then diluted 1 in 50 in PP2, allowed to grow overnight, and plated on BM2 succinate with 50 μg/ml polymyxin B. Clones were subcultured on non-selective media, then retested on polymyxin. The revertant H207 and five similar revertants of H181 and H185 were isolated by screening cultures which had been held at 4°C for several weeks for loss of polymyxin B resistance. The levels of resistance to polymyxin B on BM2 succinate agar plates were 0.8, 75, 75, and 0.8 μg/ml for H103, H181, H185 and H207, respectively.

Strain Z61, a mutant of PAO which has been shown to be highly susceptible to a wide range of antibiotics (Zimmerman, 1979), and H251, a full revertant of Z61, have been described by Angus et al. (1982).

Strain H283 is a mutant of H103 severely deficient in outer membrane protein F (porin). Isolation of this strain and three revertants of H283 with wild type levels of porin (H284, H321 and H324), is described below.

Strains AK43, AK1160 and AK1012 are LPS-altered strains obtained from A. Kropinski (Queens University, Kingston, Ontario). AK1213, a non-piliated strain, and AK1114, a non-piliated, non-flagellated strain, were also obtained from this source.
RPl was introduced into P. aeruginosa by conjugation with Escherichia coli UBl636 (RPl), kindly provided by P.M. Bennett, University of Bristol, Bristol, U.K. RPl carries resistance to ampicillin (and carbenicillin), neomycin, kanamycin, and tetracycline. Resistance to beta-lactam antibiotics is mediated by a TEM-2 type beta-lactamase (Sykes and Mathews, 1976). In the case of Z61, selection was for resistance to 100 ug/ml neomycin. For all other strains selection was on 500 ug/ml carbenicillin. Z61 (RPl) was maintained on 200 ug/ml neomycin while all other P. aeruginosa (RPl) strains were maintained on 200 ug/ml tetracycline.

A set of 17 serotype-specific strains were a kind gift from Dr. P. Liu, University of Louisville, Louisville, Kentucky. These strains were representatives of the International Antigenic Typing Scheme (IATS) (commercially marketed by Difco Ltd., Detroit, Michigan) which contains as subsets the type strains from all other commonly-used P. aeruginosa serotyping systems. They were named as follows: type 1 (ATCC 33348), type 2 (ATCC 33349), type 3 (ATCC 33350), type 4 (ATCC 33351), type 5 (ATCC 33352), type 6 (ATCC 33354), type 7 (ATCC 33353), type 8 (ATCC 33355), type 9 (ATCC 33356), type 10 (ATCC 33357), type 11 (ATCC 33358), type 12 (ATCC 33359), type 13 (ATCC 33360), type 14 (ATCC 33361), type 15 (ATCC 33362), type 16 (ATCC 33363), type 17 (ATCC 33364). Pseudomonas putida type strain (ATCC 12633) was obtained from the American Type Culture Collection (ATCC), Brockville, Maryland.
Strains H325 and H329 were independently derived mucoid derivatives of H103 selected for resistance to phage 7. These strains were not resistant to this phage and have remained mucoid after repeated subculture on PP2.

3. **Isolation of Mutants Deficient in Outer Membrane Proteins.**

Isolation of mutants was attempted using random heavy mutagenesis. This procedure was based on that of Suzuki et al. (1978), who used it with *Esherchia coli* to obtain classes of mutants for which no selection procedure is readily available.

The mutagenesis protocol used was based on that of Adelberg et al. (1965). A mid-logarithmic phase culture of H103 was collected by centrifugation, washed once in 50 mM sodium phosphate buffer (pH 6.0), and concentrated twenty-fold in the same buffer. Washed cells (0.5 ml) were added to 1 ml of 1 mg/ml N-methyl-N'-nitro-nitrosoguanidine (NTG), and held at 37°C for 30 min. Cells were then collected by centrifugation, resuspended in buffer, diluted and plated on PP2 agar. Plates were examined after 72 h growth at 30°C. Plate counts were also carried out with the untreated cell suspension. The survival rate after this mutagenesis procedure was about 0.004%.

Five hundred colonies were picked for screening. These were transferred to PP2 plates, then subcultured from single colony isolates to PP2 plates and PP2 broth. After growth at 30°C, plates were stored at 4°C and broth cultures were supplemented with dimethyl sulfoxide to 8% and stored at
-70°C. Initial screening was done using inocula from PP2 plates, and frozen stocks were used for subsequent studies.

The 500 strains were screened for outer membrane protein deficiencies using a simplified method for cell envelope preparation. Cultures were grown in 30 mL BM2 glucose (except for auxotrophs which were grown in PP2) at 30°C for 18 to 42 h, and collected by centrifugation. Cell envelopes were prepared in 30 mM Tris HCl (pH 7.4) with Mg²⁺ as described below. This method yielded envelopes which tended to be enriched in outer membrane. The envelopes were resuspended in water to approximately 10 mg protein/ml and run on sodium dodecylsulphate (SDS) polyacrylamide gels (as described below) to determine the outer membrane protein composition. Deficiencies in major proteins were easily distinguished by this procedure.

Outer membranes were prepared from strains which appeared to have deficiencies in major outer membrane protein using the one-step procedure referred to below, and these outer membranes were examined by SDS polyacrylamide gel electrophoresis to confirm the alteration.
4. **Bacteriophage and bacteriocin studies.**

(a) **Bacteriophages and partial characterization of their receptors.** All methods used in the handling of bacteriophages were described previously by Hancock and Reeves (1976). Phages were characterized using a pilus-deficient derivative of *P. aeruginosa* PA01, AK1144, and two lipopolysaccharide (LPS)-altered (rough) strains, AK43 and AK1160, obtained from A. Kropinski (Queen's University, Kingston, Ontario). Phage sources and putative receptors are summarized in Table II.

Phages were obtained from the following sources: 2, 7, 21, 44, 68, 73, 109, 352, 1214, C21, F7, F8, F10, 119X, and M6 from T.L. Pitt (Public Health Laboratory, London, U.K.); GL01, FL06, D3c+1, and D3c-1 from T. Iijima (Institute for Fermentation, Osaka, Japan); PLS27 and E79 from A. Kropinski; PB1 and B39 from D.E. Bradley (Memorial University, St. John's, Newfoundland); S1 from R. Warren (University of British Columbia); and 176p from J.D. Piguet (Institute of Hygiene, Geneva, Switzerland). These phages were purified from single plaques using H103 as a host strain except for PLS27, for which AK1160 was used as host. All other phages were isolated in the laboratory of R.E.W. Hancock, University of British Columbia, as host range mutants of phages which plated poorly on pilus- or LPS-deficient strains. The phages which were selected for ability to form plaques on AK1144 (pilus-deficient) were B6B, B6C, and B6D (independent isolates derived from 352), B9F (from M6), B5A (from 119X), C7B (from 176p), B1A (from F7), C3A (from
## TABLE II. Bacteriophage sources and receptors

<table>
<thead>
<tr>
<th>Phage</th>
<th>Lab name</th>
<th>Source</th>
<th>Putative Receptor&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>A9A</td>
<td>Pitt&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>119x</td>
<td>B5</td>
<td>Pitt</td>
<td>pilus</td>
</tr>
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<td>M6</td>
<td>B9</td>
<td>Pitt</td>
<td>pilus</td>
</tr>
<tr>
<td>B39</td>
<td>C9</td>
<td>Bradley&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>44</td>
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<td>B7</td>
<td>Pitt</td>
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<td>352</td>
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<td>D2</td>
<td>Warren&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Hancock (C27)&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>B6B</td>
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</tr>
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<tr>
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<td>B9E</td>
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</tr>
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<tr>
<td>B5A</td>
<td>B5A</td>
<td>Hancock (119x)</td>
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</tr>
<tr>
<td>C7B</td>
<td>C7B</td>
<td>Hancock (B39)</td>
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<td>A9B</td>
<td>A9B</td>
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<td>B7A</td>
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<tr>
<td>V1-V28</td>
<td>V1-V28</td>
<td>This study</td>
<td>protein</td>
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</table>

<sup>a</sup> See Nicas and Hancock, 1981.
<sup>b</sup> T.L. Pitt, Public Health Laboratory, London, U.K.
<sup>c</sup> D.E. Bradley, Memorial University, St. John's, Nfld.
<sup>d</sup> R.A.J. Warren, U. of British Columbia.
<sup>e</sup> R.E.W. Hancock, U. of British Columbia (parent strain in brackets)
<sup>f</sup> A. Kropinski, Queen's University, Kingston, Ont.
<sup>g</sup> T. Iijima, Institute for Fermentation, Osaka, Japan.
C21), and A8A (from 68). B7A was derived from 1214, selected on AK43 (an LPS-altered rough strain). A9A was a contaminant or derivative of 73 unable to form plaques on AK1144 or AK43. Phage stocks were maintained at 4°C. Stocks were checked regularly for titre and phenotype, and were repurified every 18 months.

Phages M6, B39, 73, and 119x were characterized as pilus-specific by their inability to form plaques on pilus-deficient strains. Phages 44, 109, F8, E79, 1214, PBl, S1, 352, and C3A were characterized as PAO smooth LPS-specific since they failed to form plaques on AK43 and other LPS-altered strains and could be shown to adsorb to purified LPS. Phage PLS27 has been characterized by Jarrell and Kropinski as specific for PAO rough core, and does not form plaques on smooth strains (Jarrell and Kropinski, 1981). Phages 7, 21, 68, F10, C21, F116, G101, B6B, B6C, D3c⁻¹⁺, B1A, and A8A formed plaques well on LPS altered and pilus-deficient strains and failed to adsorb to LPS and thus appear to have protein receptors. Phages 2, D3C⁺¹⁺, B5A B7A, B9E, B9F, and C7B formed plaques well on pilus-deficient and wild-type strains, but poorly on LPS-altered strains but failed to adsorb to LPS, and thus may have LPS or LPS-associated protein receptors.

(b) Aeruginocin studies. The aeruginocins used were a _P. aeruginosa_ typing set obtained from A. Kropinski, in addition to the aeruginocins from strains H41, received from J. Govan, University of Edinburgh, Scotland, and PAF41 and PAH108 from B.
The receptors of these aeruginocins are as yet uncharacterized, but they plate equally well on H103 and AK1144 although some plate only on AK43 (but not H103). Aeruginocins were prepared using the method of Kageyama (1964). Aeruginocin producing strains were grown at 30°C to an A600 of 0.6-0.8, then treated with 1 ug/ml mitomycin C (Sigma Chemical Co., MO.) to induce aeruginocin production. Culture supernatants were precipitated with 10% polyethylene glycol (PEG) then dialised to remove the PEG. Aeruginocin susceptibility was tested by spotting this preparation on bacterial lawns. Aeruginocin preparations were very unstable and tended to lose activity rapidly when stored at 4°C.

(c) **Isolation of protein-specific phages from nature.**

Isolation of phages specific for outer membrane proteins was attempted using the enrichment method of Verhoef et al. (1977). The basis of this method is the use of a strain lacking a specific receptor to adsorb out the majority of phages, leaving those phage which have the missing protein as their receptor.

The source of phages was influent sewage from the Iona Island sewage treatment plant, Vancouver, B.C. Eight collections were made, each on days following three rain-free days in the spring of 1980. Samples were centrifuged to remove debris and treated with chloroform to reduce the possibility of encountering pathogens. Undiluted chloroform-treated samples (3 ml) were mixed in equal volumes with 3.2% agar and 2% PP2
and 0.1 ml mid-logarithmic culture of H103 and spread onto PP2 plates to obtain estimates of the number of phages present. Most samples contained 10-100 plaque forming units/ml. Seven strains with apparent outer membrane protein deficiencies (isolated by heavy mutagenesis as described below) were used in these studies as the adsorbing strains.

AK1012, a LPS-deficient strain, and AK1213, a pilus-deficient strain, were used as hosts for phage propagation in order to reduce the possibility of isolating phages with LPS or pilus receptors.

For each sewage sample, 20 ml of sample was mixed with 20 ml double strength PP2 and 1 ml overnight culture of AK1012. After overnight growth, the cells were spun out and the supernatant treated with 0.5 ml chloroform. The phage titres of these preparations on H103 were $5 \times 10^3$ to $4 \times 10^5$ plaque forming units per ml. The phage preparations were then pooled, and the pooled preparations distributed in seven 8-ml aliquots. To each aliquot, cells from 15 ml of a mid-logarithmic phase culture of one strain with outer membrane protein deficiencies were added. After 10 min incubation at 37°, the cells were removed by centrifugation, and this adsorption procedure then repeated using the same strain. The supernatant was then diluted one to one in PP2, inoculated with AK1213, and grown overnight at 30° for a second cycle of phage propagation. Two further cycles of adsorption and propagation were then carried out for each of the 7 preparations. Phage preparations
were diluted to about $10^5$ to $10^6$ plaque-forming units per ml before adsorption. AK1012 was used for the third propagation and AK1213 for the fourth.

The final phage preparations were then diluted and plated with H103 on 1.6% agar overlays to obtain single plaques. Plaques were picked out of the agar using a Pasteur pipette and suspended in 1 ml PP2 broth. These phage preparations were then tested against H103, AK1012, AK1213, and the strain which had been used in the adsorption procedure. Sixty to 150 single plaque isolates were tested for each strain. Phage which plated on wild type \textit{P. aeruginosa} but not on outer membrane protein deficient strains were kept for further testing. Each of these phage was re-isolated from a single plaque. Single plaques were obtained by streaking the phage preparation onto PP2 with a loop, then slowly pouring 3 ml of PP2 with 1.6% agar onto the plate from a point near the centre of the streak. This method resulted in single plaques without carrying out dilutions of the stock. Five to 10 isolates from each phage were retested, and those with the desired host range were kept.

(d) Bacteriophage sensitivity testing. The method of bacteriophage sensitivity testing used was based on that of Hancock and Reeves (1976). Bacterial lawns were prepared from overnight or mid-logarithmic phase cultures, diluted 1 in 10 and spread on PP2 agar, either in PP2 agar overlays (0.1 ml cells in 3 ml 1.6% agar) or by swabbing. Plates were allowed
to dry for 5 to 15 minutes at 25°C, and phage suspensions containing $10^7$-$10^{10}$ plaque forming units/ml were spotted onto the plate, either with a multiple syringe inoculator as described by Hancock and Reeves (1976), or with a micropipettor set to deliver 7.5-10 μl/spot. Plates were read after incubation for 18-24 h at 37°C or 40-48 h at 30°C.

(e) **Isolation of phage-resistant mutants.** Phage-resistant mutants were isolated by co-plating 0.1 ml mid-logarithmic phase culture with $10^7$-$10^9$ plaque forming units of phage on PP2 plates in PP2 agar overlays. After 24 h growth, colonies were picked, then taken through three series of streaking and single colony isolation. The third isolates were retested for phage sensitivity.

(f) **Phage characterization by adsorption to whole cells.** Approximately $10^6$ plaque-forming units of phage were mixed with 0.2 ml of overnight bacterial culture or PP2 and held at 37°C for 10 min. These preparations were then centrifuged to remove cells, and the supernatants were diluted and plated to determine phage titres.
5. **Antibiotic and chelator susceptibility testing.**

(a) **Antibiotics.** Gentamicin sulphate and tobramycin were gifts from Schering Co. (Pte. Claire, Quebec) and Eli Lilly Co. (Indianapolis, Indiana). Carbenicillin was purchased from Ayerst Laboratories (Montreal, Quebec). Streptomycin sulphate, neomycin sulphate, kanamycin, tetracycline hydrochloride, chloramphenicol, benzyl penicillin, rifampicin, polymyxin B sulphate (8000 U/mg) and colistin methane sulfonate (polymyxin E, 12,470 U/mg) were purchased from Sigma Chemical Co. Cefsulodin was kindly provided by Ciba Geigy A.G. (Basel, Switzerland). Nitrocefin was a generous gift from Dr. C. O'Callaghan (Glaxo Group Research Ltd., Middlesex, U.K.).

(b) **Chelator and antibiotic bacteriolysis and killing.** Testing of lysis by EDTA-Tris, ethyleneglycol-bis (2-aminoethyl ether)N,N'-tetra acetate (EGTA)-Tris and polymyxin B was carried out on cells in mid-logarithmic phase growth (absorbance at 600 nm (A600) of 0.30-0.60), which were collected centrifugation at 25°C and resuspended in 10 mM EDTA or EGTA and 10 mM Tris-HCl buffer (pH 8.5) at 25°C or 75 ug/mL of polymyxin B in 30 mM sodium phosphate buffer (pH 7.4) at 37°C. The A600 was read at timed intervals.

To test killing by EDTA-Tris, EGTA-Tris, and polymyxin, mid-log cells were centrifuged and resuspended at 100-fold
dilution in either 30 mM sodium phosphate buffer pH 7.0, with 75 ug/ml polymyxin B or either 10 mM EDTA or 10 mM EGTA in 10 mM Tris-hydrochloride, pH 8.5. After 5 min incubation at 25°C, cells were diluted and plated for viable counts on PP2 agar in PP2 agar (0.6% agar) overlays. To test killing by gentamicin, the procedure was modified slightly because gentamicin is active only on respiring cells (Hancock, 1981). Centrifuged cells prepared as above were resuspended in BM2 growth medium containing 5 ug/ml gentamicin in addition to succinate and iron at normal levels but with no other cations added, and the cells incubated at 37°C with vigorous aeration for 5 min at which time viable counts were carried out as above.

(c) **Determination of minimal inhibitory concentrations (MIC).** For determinations in defined liquid media, antibiotic resistance was measured in 1 ml volumes of BM2 succinate with the stated Mg²⁺ levels. The inoculum used was approximately 10⁵ cells of an overnight culture grown in medium identical to the test medium. The level of resistance was taken as the highest antibiotic concentration showing visible turbidity after 24 h at 37°C.

Other measurements of MIC were done on PP2 agar using the method described by Angus et al (1982). A multisyringe
Applicator was used to deliver to each plate 24 drops of approximately 2 μl which contained an estimated 1000 cells from diluted 18 h cultures. Plates were read after 18 and 48 h incubation at 37°C.

6. **Shift experiments.** Overnight cultures (1 ml) grown in BM2-succinate with 0.02 mM Mg$^{2+}$ were transferred to 200 ml of the same medium and grown to an A$_{600}$ of 0.15 to 0.20, at which point Mg$^{2+}$ was added to a final concentration of 0.5 mM. Twenty ml of culture were removed at this point, and at 15 min intervals, and these samples used to test polymyxin B and EDTA-Tris sensitivity, and for preparation of cell envelopes as described above.

7. **Membrane Isolation and Characterization of Outer Membrane, Cell Envelope, and Whole Cell Proteins.** For whole cell preparations, overnight or logarithmic phase cultures were centrifuged and the cells resuspended in 2% 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 20 min. The resulting supernatant was sonicated (1 min, setting 5, Biosonik sonicator (Bronwill Scientific, N.Y.)) to shear DNA and reduce viscosity, and the sample applied directly to the gel.
To prepare cell envelopes, cells from overnight or logarithmic phase cultures were collected by centrifugation, resuspended in 10 mM sodium phosphate buffer (pH 7.4) or 30 mM Tris-HCl, pH 7.4 containing 2 mM Mg$^{2+}$ and 10 ug/ml pancreatic deoxyribonuclease I (Sigma Chemical Co.) and broken in a French Press at 14,000 psi. Whole cells were removed by centrifugation (1000 x g, 10 min) and the resulting supernatant diluted in the same buffer and centrifuged at 160,000 x g for 2 hr. The cell envelope pellet was resuspended in deionized water.

Outer membranes were prepared using the two methods described by Hancock and Carey (1979). One, originally described by Hancock and Nikaido (1978), employs a single step and a four-step sucrose gradient and yields two outer membrane fractions with indistinguishable protein composition. The second is a more rapid method using only one sucrose gradient which yields a single outer membrane band (Hancock and Carey, 1979).

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed using the 14% acrylamide system containing 0.07 M NaCl in the running gel previously described by Hancock and Carey (1979). Only in the presence of 0.07 M NaCl or with high acrylamide monomer concentrations are outer membrane proteins H1 and H2 separated (Hancock and Carey, 1979). Solubilization conditions were 88°C for 10 min in reduction mix containing 2-mercaptoethanol.

Ratios of protein H1 to H2 were calculated from dens-
itometer tracings made on a Helena Quick Scan Jr. Densitometer (Helena Laboratories, Beaumont, Texas) of Coomasie brilliant blue R50-stained gels. Outer membrane protein H2 was used as a reference since it was one of the major proteins of the cell and its level varied with little growth conditions as judged by SDS gel electrophoresis of whole cell proteins.

8. **Determination of cell envelope cation levels.** Cell envelopes prepared as described above were extracted by the method of Kenward *et al.* (1978) and cation levels determined by atomic absorption spectroscopy carried out by Susan Jaspar and Susan Liptak (Department of Civil Engineering, University of British Columbia), Canadian Microanalytical Corp., Vancouver, B.C., or Dr. S. Ma and Dr. D.H. Copp (Department of Physiology, University of British Columbia).

9. **Displacement of Mg^{2+} from the cell envelope by polymyxin B and aminoglycosides.** Cells grown to an A_{600} of 0.60 were incubated with 1 mM KCN (to prevent aminoglycoside uptake) for 15 min at 37° with aeration. Streptomycin, gentamicin or polymyxin was then added and the cells incubated a further 10 min. Cell envelopes were then prepared as described above, and analysis of divalent cations was carried out, as described above, on lyophilized envelopes.
10. **Streptomycin uptake assays.** [H]-dihydrostreptomycin (1.2 Ci/mmol) (Amersham Searle, Oakville, Ontario) was diluted by the addition of nonradioactive streptomycin to a specific activity of 50 uCi/mg streptomycin. Cells were grown by shaking at 37°C to an $A_{600}$ of 0.5-0.6 in unmodified nutrient broth, and the uptake assay started by the addition, to the growing cells, of streptomycin to the desired final concentrations. At defined times, 1.0 ml samples were removed and the cells collected by filtration onto nitrocellulose filters (0.45 um, Millipore, Bedford, Mass.) which had been presoaked in 0.1 M LiCl prior to use. The filtered cells were then washed twice with 3 ml of 0.1 M LiCl, dried at 60°C for one hour, then assayed for radioactivity in a toluene-based scintillant. This method was based on those of Holtje (1978) and Bryan and colleagues (1976) for streptomycin uptake assays. Trial experiments demonstrated that the filter preparation and washing techniques were critical, as previously suggested (Holtje, 1978; Bryan, 1976) but that the methods of either of the above authors were satisfactory.

Nutrient broth was chosen as the medium for uptake assays because it has relatively low Mg$^{2+}$ levels (Nicas and Bryan, 1978), and has previously been shown suitable (Bryan et al., 1976). BM2, the phosphate buffered minimal medium used elsewhere in this study was judged unsuitable as high phosphate was found to inhibit aminoglycoside uptake, necessitating the use of large amounts of label.
11. Enhancement of nitrocefin permeability by aminoglycosides and chelators. The assay used was modified from that of O'Callaghan et al. (1972). H103 growing in the presence of 0.2 mg/ml benzyl penicillin or H103 (RPl) growing in the presence of 20 ug/ml tetracycline was grown to an A600 of 0.50 to 0.60, harvested by centrifugation at 25°C and resuspended in 20 mM sodium phosphate buffer pH 7.0 at an A600 of 1.50 to 2.0.

For studies with chelators, the cells were then diluted 1 in 10 in EDTA-Tris or EGTA-Tris at a final concentration of 10 mM EDTA or EGTA and 10 mM Tris-HCl pH 8.5. After 2 min at 25°C, 0.1 ml of cell suspension was quickly mixed with 0.65 ml of nitrocefin (12.5 ug/ml, in phosphate buffer) and the hydrolysis of nitrocefin monitored spectrophotometrically by measurement of the increase in absorbance at 540 nm. Rates of hydrolysis for untreated cells were also measured, and hydrolysis rates were expressed as the ratio of hydrolysis rates in treated cells to rates of untreated cells. In studies with gentamicin, 1 ml of gentamicin was added to 0.1 ml of cell suspension to give a final concentration of 10-100 ug/ml. After 2 min at 25°C, 0.6 ml of nitrocefin (250 ug/ml) was added and hydrolysis of the nitrocefin monitored as above.

12. Measurement of outer membrane permeability by nitrocefin hydrolysis. A method based on the technique of Zimmermann and Rosselet (1980) as modified by Angus et al. (1982) was developed, since neither the original technique nor the modification
enabled a measurement of outer membrane permeability for strain H283. In particular, a relatively substantial release of periplasmic beta-lactamase was found during the resuspension of cells after centrifugation. Therefore, the technique was further modified as follows: *P. aeruginosa* or *E. coli* strains containing the RPl plasmid were grown overnight in PP2 broth at 37°C in the presence of 200 ug/ml tetracycline or in the case of Z61 (RPl) with 20 ug/ml tetracycline to ensure retention of the plasmid. (Retention of the plasmid under these conditions was confirmed by comparing plate counts on PP2 and PP2 with 200 ug/ml carbenicillin or 200 ug/ml neomycin.) The overnight cultures were diluted 1 in 20 into fresh PP2 broth and grown to an A<sub>600</sub> of 0.6 to 0.8. A 0.1 ml sample of cells was placed in the sample cuvette of a Perkin-Elmer (Oak Brook, Ill.) Lambda 3 dual beam spectrophotometer. Another 1.5 ml sample was taken at the same time and centrifuged for 1 min at 9000 x g in an Eppendorf microcentrifuge model 5412 (Brinkman Instruments, Westbury, N.Y.). The cell-free supernatant was decanted and 0.1 ml added to the reference cuvette of a Perkin-Elmer Lambda 3 spectrophotometer. To both reference and sample cuvettes, 0.8 ml of a 0.1 mg/ml solution of the chromogenic beta-lactam nitrocefin (O'Callaghan et al., 1972) was added and the differential rate of conversion of nitrocefin to nitrocefoic acid followed over time at an absorbance of 540 nm using a coupled Perkin-Elmer model 581 strip chart recorder. Since both sample and reference cuvettes contained supernatants, the differential
rate of hydrolysis was a measure of whole cell hydrolysis of nitrocefin. Control experiments showed that the rate of hydrolysis did not increase over time, indicating that cell breakage was not occurring.

In all experiments with the outer membrane protein mutants H283 (RPl) and H181 (RPl), the culture used was checked for protein F deficiency or Hl overproduction by examining whole cell protein profiles on SDS polyacrylamide gels after each experiment.

13. Other assays. The protein assay used was that of Schacterle and Pollack (1973). Levels of 2-keto-3-deoxyoctonate were estimated by the method of Osborn et al. (1963).
CHAPTER ONE

SUSCEPTIBILITY TO EDTA-TRIS, POLYMYXINS AND AMINOGLYCOSIDES

1. Susceptibility to polymyxin B and EDTA-Tris in Mg$^{2+}$ sufficient and deficient media. Wild type P. aeruginosa PA01 strain H103, grown on Mg$^{2+}$-sufficient medium (0.5 mM Mg$^{2+}$) was sensitive to polymyxin B and EDTA killing (Table III) and lysis (Fig. 1). In contrast, H103 grown under Mg$^{2+}$-deficient (0.02 mM Mg$^{2+}$) conditions was 70 to 700 fold more resistant to these agents (Fig. 1; Tables III and V), in agreement with previously published results (Brown and Melling, 1969; Gilleland et al., 1974). This resistance could be reversed by culturing H103 on Mg$^{2+}$-sufficient medium for a few generations.

All lysis and killing experiments were done on mid-logarithmic phase cells after control experiments showed that susceptibility to lysis by EDTA-Tris and by polymyxin B varied with the growth phase of the culture. When 18 h (late stationary phase) cultures grown in Mg$^{2+}$ sufficient medium were transferred to fresh medium, susceptibility to lysis increased throughout early logarithmic phase, and reached a maximum in middle and late logarithmic phase. As cells entered stationary phase, susceptibility decreased, so that 18 h cultures were highly resistant to lysis, exhibiting only 5 to 20% of the level of lysis seen in mid-log cells. Cells grown in Mg$^{2+}$ deficient medium followed a similar pattern, except that de-
Figure 1. Effect of adaptation on Mg\(^{2+}\)-deficient medium, and of mutation to polymyxin B resistance, on susceptibility of cells to lysis by polymyxin B and EDTA-Tris. A, lysis by EDTA-Tris; B, lysis by polymyxin B.

Symbols: Wild type strain H103 grown in Mg\(^{2+}\)-deficient (0.02) mM medium (▲); strain H103, (▲), polymyxin B resistant mutants H181, (●) and H185, (▲) and revertant H207, (○) grown in Mg\(^{2+}\)-sufficient (0.5 mM) medium.
### TABLE III. Resistance to killing by EDTA-Tris and polymyxin B of H103 and its polymyxin B resistant mutant H181. Effect of Mg$^{2+}$ concentration in the growth medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mg$^{2+}$ concentration during growth (mM)</th>
<th>Survivors (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EDTA-Tris</th>
<th>Polymyxin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103</td>
<td>0.02</td>
<td>65</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.9</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>H181</td>
<td>0.02</td>
<td>77</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>76</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were treated for 5 minutes in 10mM EDTA in 10mM-Tris-HCl (pH 8.5) or 75 ug/ml polymyxin B in phosphate buffer (pH 7.4).
crease in susceptibility began somewhat earlier, in late logarithmic phase growth, and these cells were more resistant than cells in Mg\textsuperscript{2+} sufficient medium during all phases of growth. The growth rates of cells growing in Mg\textsuperscript{2+}-sufficient and Mg\textsuperscript{2+}-deficient medium were identical in log phase, although lower levels of Mg\textsuperscript{2+} (below 0.01 mM) resulted in decreased growth rate. For cultures grown in 0.015-0.05 mM mg\textsuperscript{2+}, the growth yield was proportional to the amount of Mg\textsuperscript{2+} added.

2. **Susceptibility of polymyxin-resistant mutants.** Two polymyxin B resistant mutants of H103, strains H181 and H185, were resistant to EDTA-Tris and polymyxin B killing (Table III) and lysis (Fig. 1), irrespective of the medium Mg\textsuperscript{2+} concentration. The mutant phenotype was stable for up to 12 consecutive single colony isolations on Mg\textsuperscript{2+}-sufficient medium. Six spontaneous revertants of H181 and H185 (e.g., strain H207) had regained all of the wild type properties of strain H103 (see Fig. 1 and below). These data suggest that H181 and H185 each have a single mutation resulting in phenotypic alterations mimicking those of the Mg\textsuperscript{2+}-limited, adaptively resistant strain H103.

3. **Aminoglycoside resistance.** Since Mg\textsuperscript{2+} has been previously shown to antagonize the action of aminoglycosides, as well as polymyxins (Zimelis and Jackson, 1973), the mutants were tested for enhanced resistance to three representative aminoglycoside antibiotics, gentamicin, streptomycin and tobramycin. Strains
H181 and H185 were consistently 4-fold more resistant to gentamicin and streptomycin and 2-fold more resistant to tobramycin (Table IV). In contrast they were equally susceptible to carbenicillin and tetracycline when compared to strain H103.

Strain H103 grown in Mg\(^{2+}\)-deficient medium was also shown to be much more resistant to gentamicin than H103 grown in Mg\(^{2+}\)-sufficient medium (Table V) when resistance was measured in a common assay medium. Studies reported elsewhere (Hancock, Raffle and Nicas, 1981) have shown that H103 grown in Mg\(^{2+}\)-deficient medium shows a marked decrease in sensitivity to killing by gentamicin over a broad range of antibiotic concentrations. The level of resistance achieved was shown to be similar to that of the polymyxin resistant mutants H181 and H185.

4. **Substitution of other cations for Mg\(^{2+}\)**. Results of supplementation of Mg\(^{2+}\) deficient medium with other cations are shown in Table V. These results confirmed and extended the findings of Boggis *et al.* (1979) with respect to the effects of different metal cations on susceptibility to polymyxin and EDTA-Tris. Cells grown with Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\) or Sr\(^{2+}\) were at least 1000 times more sensitive to polymyxin B, 100 times more sensitive to EDTA-Tris and 10 times more sensitive to gentamicin than were cells grown in low Mg\(^{2+}\); susceptibility to lysis in EDTA-Tris was also much enhanced in such cells. In contrast, cells grown with the several other cations (Ba\(^{2+}\), Zn\(^{2+}\), Sn\(^{2+}\),
TABLE IV. Resistance of H103, its polymyxin B resistant derivatives H181 and H185 and a revertant H207 to various antibiotics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance (ug/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gentamicin</td>
<td>Streptomycin</td>
<td>Tobramycin</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>H103</td>
<td>1</td>
<td>8</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>H181</td>
<td>4</td>
<td>32</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>H185</td>
<td>4</td>
<td>32</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>H207</td>
<td>1</td>
<td>8</td>
<td>0.5</td>
<td>16</td>
</tr>
</tbody>
</table>

a Resistance levels were determined as described in Methods, using liquid BM2 medium with 0.5 mM Mg$^{2+}$.

b The differences observed, while not large, were reproducibly obtained in 6-10 individual experiments.
Al³⁺, Na⁺), were indistinguishable from cells grown in low Mg²⁺ in their relatively low susceptibility to polymyxin B, EDTA-Tris and gentamicin, providing that a common assay medium was used.

5. **Minimal inhibitory concentrations of polymyxin at various Mg²⁺ levels.** Although growth of _P. aeruginosa_ in media with low Mg²⁺ increases resistance to polymyxin when susceptibility is tested in a common assay medium, the presence of added external Mg²⁺ can protect both resistant and susceptible cells from the polymyxin action (Newton, 1964; Klemperer _et al._, 1979). The MIC to polymyxin B and colistin (polymyxin E) shown in Table VI reflect these Mg²⁺ effects. Thus, although cells grown in 5 mM Mg²⁺ are clearly highly susceptible to killing by polymyxin B in the absence of Mg²⁺ (Table III), they appear to be relatively resistant in MIC measurements done in the presence of Mg²⁺ (Table VI). Since at this Mg²⁺ concentration there was a large molar excess of Mg²⁺ over polymyxin B (e.g., 60-fold at 100 ug/ml polymyxin B, 1000-fold at 4 ug/ml polymyxin B), the results may be explained by competition for a site on the LPS normally occupied by Mg²⁺ (see Fig. 10 and Discussion). Competition between Mg²⁺ and the polymyxins could also explain the 2.5-fold difference in the resistances of H181 and H185 grown and tested in 0.5 mM Mg²⁺ relative to H103 grown in 0.02 mM Mg²⁺, (Table VI) whereas the resistances of the strains grown at these Mg²⁺ concentrations were similar when tested in
**TABLE V.** Effect of growth in various divalent cations on induction of outer membrane protein H1, lysis and killing by EDTA-Tris, and killing by polymyxin B and gentamicin.

<table>
<thead>
<tr>
<th>Mg(^{2+}) during growth (mM)</th>
<th>Other cations during growth (mM)</th>
<th>Induction of protein H1(^a)</th>
<th>% Lysis by EDTA-Tris(^b)</th>
<th>% Survivors(^c) Polymyxin B</th>
<th>EDTA-Tris</th>
<th>Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>-</td>
<td>+</td>
<td>11.5</td>
<td>15.0</td>
<td>65.0</td>
<td>11.0</td>
</tr>
<tr>
<td>0.02</td>
<td>Ba(^{2+}) (0.5)</td>
<td>+</td>
<td>6.0</td>
<td>20.3</td>
<td>14.6</td>
<td>21.5</td>
</tr>
<tr>
<td>0.02</td>
<td>Sn(^{2+}) (0.5)</td>
<td>+</td>
<td>16.0</td>
<td>20.5</td>
<td>11.7</td>
<td>10.5</td>
</tr>
<tr>
<td>0.02</td>
<td>Zn(^{2+}) (0.5)</td>
<td>+</td>
<td>14.0</td>
<td>20.2</td>
<td>13.0</td>
<td>19.5</td>
</tr>
<tr>
<td>0.02</td>
<td>Ca(^{2+}) (0.5)</td>
<td>-</td>
<td>51.5</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
<td>1.56</td>
</tr>
<tr>
<td>0.02</td>
<td>Mn(^{2+}) (0.5)</td>
<td>-</td>
<td>53.5</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
<td>1.65</td>
</tr>
<tr>
<td>0.02</td>
<td>Sr(^{2+}) (0.5)</td>
<td>-</td>
<td>51.7</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
<td>0.68</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>52.3</td>
<td>≤0.01</td>
<td>&lt;0.1</td>
<td>2.01</td>
</tr>
</tbody>
</table>

\(a\) Induction of H1 as judged from sodium dodecyl sulphate polyacrylamide gels of cell envelope proteins, see Fig. 3.

\(b\) Lysis was measured as decrease in \(A_{600}\) after 15 min in 10 mM EDTA, 10 mM Tris-HCl pH 8.5.

\(c\) Cells were treated for 5 min with 75 ug/ml polymyxin B in phosphate buffer, 10 mM EDTA in 10 mM Tris-hydrochloride, or 5 ug/ml gentamicin in growth medium without added divalent cations.
**TABLE VI.** Levels of outer membrane protein H1, cell envelope Mg\(^{2+}\) concentration and resistance to polymyxins of H103 and its polymyxin B resistant derivatives H181 and H185 and a revertant H207: effect of varying Mg\(^{2+}\) concentrations in the growth medium.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mg(^{2+}) concentration during growth (mM)</th>
<th>Outer Membrane Proteins: Ratio H1:H2(^a)</th>
<th>Cations in cell envelope (ug/mg protein)(^b)</th>
<th>Resistance (ug/ml)(^d) Polymyxin B</th>
<th>Resistance (ug/ml)(^d) Colistin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103</td>
<td>0.02</td>
<td>4.7</td>
<td>4.8</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.7</td>
<td>17.1</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.2</td>
<td>20.5</td>
<td>0.8</td>
<td>4</td>
</tr>
<tr>
<td>H181</td>
<td>0.02</td>
<td>6.3</td>
<td>3.8</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4.7</td>
<td>8.9</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.4</td>
<td>11.6</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>H185</td>
<td>0.02</td>
<td>6.4</td>
<td>2.9</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>-c</td>
<td>8.8</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>2.9</td>
<td>12.6</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>H207</td>
<td>0.5</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Cell envelopes were isolated and subjected to SDS polyacrylamide gel electrophoresis. The ratios of proteins H1 to H2 were calculated from densitometer tracing of stained gels loaded with a standard amount of protein. Outer membrane protein H2 was used as a reference since it was one of the major proteins of the cell and its levels varied very little with growth conditions.

\(^b\) Levels obtained by atomic adsorption spectroscopy.

\(^c\) "-" means not done.

\(^d\) Resistance was determined in liquid BM2 succinate using the given Mg\(^{2+}\) concentrations.
the absence of Mg$^{2+}$ (Table III). The relationship of the
resistance shown in Table VI and properties of the outer
membrane is described below (Chapter Two).

6. **Streptomycin uptake and binding in susceptible and
resistant strains.** The pattern of streptomycin uptake in both
strains H103 and H181 (illustrated by a typical experiment Fig.
2) followed three phase kinetics as described by Bryan and
colleagues for other strains (Bryan and Van Elzen, 1976): an
instantaneous binding phase, an early slow uptake phase (EDP-I)
and a later rapid uptake phase (EDP-II). An extensive series
of experiments was performed in an attempt to demonstrate
differences in the apparent amount of streptomycin binding to
cyanide-treated [i.e., non-streptomycin transporting (Bryan and
Van Den Elzen, 1977)] or -untreated wild type strain H103 or
mutant H181 cells. For five separate experiments done at eight
different concentrations of streptomycin (data not shown),
statistical analysis of the data suggested that there was no
significant different ($P > 0.5$) in aminoglycoside binding to the
two strains. Thus, any apparent differences in streptomycin
binding to the two strains (e.g., as seen at 10 µg/ml in Fig.
2) were shown by more careful analysis to be not significant.
Scatchard analysis of the data from one experiment suggested in
the order of $2 - 5 \times 10^7$ potential binding sites for strepto-
mycin per cell. The large number of non-specific binding sites
on the cell (since there are only about $2 - 4 \times 10^6$ molecules
Figure 2. Uptake of $[^3H]$ streptomycin at two concentrations by the wild type strain H103 and the outer embrane protein H1 overproducing strain H181. Symbols: $\circ$ H103, 2 ug/ml streptomycin; $\bullet$ H103, 10 ug/ml streptomycin; $\triangle$ H181, 2 ug/ml streptomycin; $\blacktriangle$ H181, 10 ug/ml streptomycin.
Figure 3. Time required for initiation of rapid uptake of streptomycin (EDP-II) in the wild type strains H103 (○) and the outer membrane protein H1 overproducing strain H181 (△). The points represent the means of three experiments; the given lines were drawn by linear regression analysis of the points with correlation coefficient \( r^2 \) of 0.98 for H103 and 0.97 for H181.
of LPS per *P. aeruginosa* cell), and the high background filter adsorption of streptomycin even under the stringent washing procedures used may well have acted together to obscure expected differences in aminoglycoside binding.

The major alteration in the kinetics of streptomycin uptake seen in the resistant strain, H181, was that at all concentrations of antibiotic used, transition from the early slow phase of uptake EDP-I to the later rapid phase EDP-II was delayed in the resistant strain (Fig. 3). This difference was consistently observed in seven separate experiments, each using several levels of antibiotic.

7. **Permeabilization of the outer membrane by aminoglycosides.**

The ability of EDTA and polymyxin to interact with and disrupt the outer membrane of gram-negative bacteria is well known (Leive, 1965; Cooperstock, 1974; Gilleland and Murray, 1976; Michael and Eagon, 1966; Rosenthal and Strom, 1977). The ability of aminoglycosides to interact with the outer membrane was investigated by examining their ability to enhance outer membrane permeability to other agents. It has been reported elsewhere (Hancock, Raffle and Nicas, 1981) that treatment with gentamicin made *P. aeruginosa* susceptible to lysis by lysozyme, an enzyme normally unable to penetrate the outer membrane to reach its site of activity, peptidoglycan. Conditions known to block aminoglycoside transport and killing did not affect gentamicin-lysozyme lysis. These conditions
included treatment with KCN, the uncouplers dinitrophenol and sodium azide, inhibitors of protein synthesis (chloramphenicol and tetracycline) and the \textit{str A} mutation. Gentamicin-promoted lysozyme lysis was however completely inhibited by 1 mM Mg$^{2+}$ (Hancock, Nicas and Raffle, 1981).

Permeabilization of the outer membrane by gentamicin in strain H103 was also examined by measuring the hydrolysis of a chromogenic beta-lactam, nitrocefin. An increased rate of hydrolysis in intact non-growing cells would indicate increased permeation of the beta-lactam through the outer membrane to the periplasmic beta-lactamase. The rate of hydrolysis could be increased 3.5 fold over that of untreated strain H103 cells by pretreatment with 100 ug/ml gentamicin and 1.7-fold with 10 ug/ml gentamicin [the actual concentrations of gentamicin present during the assay were in fact 15 ug/ml and 1.5 ug/ml, respectively]. Treatment with 50 mM EDTA increased the rate of hydrolysis 10-fold. As in the case of gentamicin-mediated lysozyme activity, permeabilization to nitrocefin could be totally inhibited by 1 mM Mg$^{2+}$.

8. \textbf{Other properties of polymyxin resistant strains}

(a) \textbf{Resistance to chloramphenicol and other antibiotics.}

The reference strain used, \textit{P. aeruginosa} PA01 strain H103, was previously mutated to high chloramphenicol resistance (MIC of 200-400 ug/ml compared to 10-20 for most isolates; B. Holloway, personal communication). The polymyxin resistant
mutants H181 and H185 were found to have lost this high level of chloramphenicol resistance, and had MIC of 20 ug/ml. The revertants of H181 and H185 regained the same high levels of chloramphenicol resistance as the parent. Strains H181 and H185 did not differ from H103 with regard to their susceptibility to rifampicin, cefsulodin, ticarcillin, Cu\(^{2+}\) and Ag\(^{2+}\), as well as carbenicillin and tetracycline as shown in Table IV.

(b) **Ability to accept RPl.** The plasmid RPl could be conjugally transferred easily into H103 from either *E. coli* or other *P. aeruginosa* strains with a transfer frequency of 1.0-2.4 x 10\(^{-4}\) transconjugants per recipient. In contrast, the polymyxin resistant mutant H181 accepted the plasmid at 10\(^{3}\) to 10\(^{4}\)-fold lower frequency, 2.0 x 10\(^{-8}\) to 6.7 x 10\(^{-7}\). Frequencies of conjugation did not differ according to the antibiotic used for selection.

(c) **Loss of viability in cold storage.** It was found that cultures of H181 and H185 which had been stored at 4°C for several weeks gave rise to a high proportion of revertant clones when subcultured and tested for polymyxin resistance. In an attempt to explain this phenomenon, the loss of viability of wild type and polymyxin resistant mutants after storage was compared. It was found that after one week in liquid growth medium at 4°C, viable counts of H103 showed 15-45% survival, in contrast to 1.2-4.6% survival of H181. Of the surviving H181
clones, only 0.3-1% remained polymyxin resistant. Cultures stored for 1-2 weeks at -70°C in 10% dimethyl sulfoxide showed a similar difference in viability between H103 and H181.

9. Summary. Cells grown in Mg$^{2+}$-deficient medium (0.02 mM Mg$^{2+}$) were more resistant to the action of EDTA, polymyxin B, and aminoglycosides than were cells grown in Mg$^{2+}$-sufficient medium. Ca$^{2+}$, Sr$^{2+}$, or Mn$^{2+}$ could substitute for Mg$^{2+}$ in reversing resistance while several other cations could not. Mutants selected for polymyxin resistance resembled cells grown in low Mg$^{2+}$ in their resistance to cationic antibiotics and EDTA. These mutants also showed altered uptake of aminoglycosides. It was shown that aminoglycosides could interact with the outer membrane in wild type cells so as to make it more permeable to other substances. Aminoglycoside-mediated permeabilization could be inhibited with Mg$^{2+}$. 
CHAPTER TWO

OUTER MEMBRANE CHARACTERIZATION

1. Outer membrane protein patterns. Outer membrane protein H1 was shown to be increased up to 24-fold in Mg\(^{2+}\)-limited H103 cells (Fig. 4, compare gel E with F; Table VI), while the protein G level was depressed 3-fold. Under these conditions, H1 was by far the major cellular protein as judged by SDS poly-acrylamide gel electrophoresis of whole cell proteins. The polymyxin B resistant mutants H181 and H185 had constitutively high levels of protein H1 (Fig. 4, gels B and C) which varied only 2-fold with changing Mg\(^{2+}\) concentrations in the medium (Table VI). The level of protein G was also depressed in both H181 and H185. Since other strains with greatly depressed levels of protein G (e.g., Hancock and Carey, 1979) had no alteration in polymyxin B or EDTA resistance, or in levels of protein H1, it was concluded that the decrease in this protein is unrelated to the resistance observed in the adapted or mutant strains. Similarly, a variety of growth conditions (e.g., pyruvate as a carbon source, limiting NH\(_4^+\)) could depress levels of protein G without effect on protein H1 levels. Levels of protein D2 also appeared to be slightly lower. The level of porin (protein F) appeared to be somewhat reduced in membranes with higher levels of protein H1. However, porin function appeared to be unaltered in these strains (see Chapter Four).
Figure 4. Effect of adaptation on Mg\(^{2+}\)-deficient medium and mutation to polymyxin B resistance on levels of protein H1. Gels A and B - whole cell preparations of strains H103 and H185, respectively, grown with 5 mM Mg\(^{2+}\); Gels C and D - outer membrane preparations of strains H181 and H103, respectively, grown with 0.5 mM Mg\(^{2+}\); Gels E and F - cell envelopes of H103 grown with 0.02 mM Mg\(^{2+}\) and 5 mM Mg\(^{2+}\), respectively. In the protein patterns of whole cells, cell envelopes or outer membranes of H103 grown on low (0.02 mM) Mg\(^{2+}\) or the polymyxin B resistant mutants H181 and H185 grown on high (5 mM) Mg\(^{2+}\), a large increase in outer membrane protein H1 was observed, while protein G was somewhat decreased compared to H103 grown on high Mg\(^{2+}\). The high molecular weight protein seen in gel E but not in gel F cell envelopes is an inner membrane protein of unknown function induced in either H103 or H181 grown in low Mg\(^{2+}\).
An inner membrane protein of 75,000 daltons was also observed in all strains (e.g., Fig. 4, Gel E) grown on Mg\(^{2+}\)-deficient medium but never in strains grown on Mg\(^{2+}\)-sufficient medium. Thus, it was concluded that this protein also is unrelated to the phenomena reported here. All six revertants had regained wild type membrane protein patterns at all Mg\(^{2+}\) concentrations (e.g., H207, Table VI). Although pilin has a similar molecular weight to protein H1 (Parynychych, 1979), they are distinct proteins as determined using a pilin sample kindly provided by W. Parynychych (University of Alberta, Edmonton, Alberta).

It was determined that the amount of protein per mg (dry weight) of cell envelope (54 ± 16%) and the amount of 2-keto-3-deoxyoctonate per mg of protein (87 ± 7 ug/mg) did not vary significantly between wild type cells grown in Mg\(^{2+}\)-sufficient medium and either polymyxin resistant mutants or cells grown in Mg\(^{2+}\) deficient medium (P >0.5 by Student's t test).

The change in the levels of protein H1 in the outer membrane were reflected in part by the degree of resistance to two polymyxin antibiotics and by the Mg\(^{2+}\) concentration of the cell envelopes of strains H103, H181, H185 and H207 grown at various medium Mg\(^{2+}\) concentrations (Table VI). Thus, a 7-fold increase in H1 levels between strains H103 and H181, grown in BM2 minimal succinate medium containing 0.5 mM Mg\(^{2+}\) was associated with a 25-fold increase in polymyxin B and colistin resistance. Similarly, strain H103 grown on Mg\(^{2+}\)-deficient medium had 7
fold higher levels of protein H1 than the same strain grown on Mg$^{2+}$-sufficient medium, and was 10-fold more resistant to polymyxins. It is well established that high Mg$^{2+}$ concentrations in the medium inhibit the action of polymyxins (Newton, 1954). In the experiments described in Table VI, an increase in polymyxin resistance was observed in the presence of 5 mM Mg$^{2+}$ despite levels of protein H1 similar to that of cells grown in 0.5 mM Mg$^{2+}$. As described above (Chapter One, Section 5), this effect could be accounted for by competition between Mg$^{2+}$ and polymyxin B for binding sites. The large molar excess of Mg$^{2+}$ over polymyxin at 5 mM polymyxin could account for the higher MIC in high Mg$^{2+}$. This could also explain why the MIC of H181 and H185 in 0.5 mM Mg$^{2+}$ indicate higher resistance than H103 grown in 0.02 mM Mg$^{2+}$ despite the high degree of similarity between these cells in their level of H1 (Table VI) and resistance to polymyxin killing in the absence of Mg$^{2+}$ (Table III).

Results of supplementation of Mg$^{2+}$-deficient medium with other divalent cations are shown in Fig. 5. Induction of protein H1 was prevented in Mg$^{2+}$-deficient media supplemented with 0.5 mM Ca$^{2+}$, Mn$^{2+}$, or Sr$^{2+}$, as well as 0.5 mM Mg$^{2+}$, but not with 0.5 mM Zn$^{2+}$, Sn$^{2+}$, Ba$^{2+}$ (Fig. 5), Al$^{3+}$, or 1.0 mM Na$^{+}$ (data not shown). As shown in Table V, cells grow in cations which prevent H1 induction are highly susceptible to killing by polymyxin, EDTA-Tris and gentamicin, whereas cells grown in cations which failed to prevent induction of H1 were indistinguishable from cells grown in low Mg$^{2+}$ in their relatively low susceptibility to these agents.
Figure 5. Effect of growth in different divalent cations on induction of protein H1. Sodium dodecyl sulphate polyacrylamide gel of cell envelopes from cells grown in the presence of different divalent cations. Lane A, 0.02 mM Mg\(^{2+}\); Lane B, 0.5 mM Mg\(^{2+}\); Lanes C-H, 0.02 mM Mg\(^{2+}\) plus 0.5 mM Ca\(^{2+}\) (C), 0.5 mM Mn\(^{2+}\) (D), 0.5 mM Sr\(^{2+}\) (E), 0.5 mM Ba\(^{2+}\) (F), 0.5 mM Sn\(^{2+}\) (G) or 0.5 mM Zn\(^{2+}\) (H). In order to ensure that all the protein H1 ran at the head modified position, solubilization was carried out at 100°C for 10 min (as described by Hancock and Carey, 1979). This results in partial heat modification of protein F to run with a lower relative mobility at the position F* (Hancock & Carey, 1979).
2. Effect of shift from low to high \(\text{Mg}^{2+}\). To further confirm the relationship between the presence of outer membrane protein H1 and resistance, shift experiments were carried out. Cells growing in 0.02 mM \(\text{Mg}^{2+}\) in early logarithmic phase growth (\(A_{600}\) of 0.15-0.2) were supplemented with \(\text{Mg}^{2+}\) to 0.5 mM, and levels of H1 in the cell envelope and susceptibility to polymyxin B and EDTA-Tris were followed over time. As described above, 0.02 mM \(\text{Mg}^{2+}\) allows the same growth rate as 0.5 mM up to an \(A_{600}\) of 0.6, so these cells could not be considered \(\text{Mg}^{2+}\) limited for growth. Control experiments demonstrated that cells grown in 0.02 mM \(\text{Mg}^{2+}\) showed increasing levels of protein H1 as the medium became depleted in \(\text{Mg}^{2+}\). However, the shift to high \(\text{Mg}^{2+}\) was performed on early logarithmic phase cells, which showed only moderately higher levels of protein H1 (2- to 3-fold) in order to avoid any non-specific effects of \(\text{Mg}^{2+}\) starvation [e.g., stringent response (St. John and Goldberg, 1980), ribosome effects (e.g., Gestland, 1966; Schlessinger et al., 1967)]. Addition of \(\text{Mg}^{2+}\) did not alter the growth rate (about 42 min generation time), and the cells remained in logarithmic growth phase throughout the sampling period. The ratio of outer membrane protein H1 to protein H2, calculated from densitometer tracings of Coomassie blue stained gels was used to estimate relative levels of protein H1 in cell envelopes. The time required for the relative level of protein H1
Figure 6. Effect of shift from low to high Mg\(^{2+}\) on levels of protein H1 and susceptibility to EDTA-Tris and polymyxin B. Cells growing in 0.02 mM Mg\(^{2+}\) received Mg\(^{2+}\) to a final concentration of 0.5 mM at time 0. Samples of cultures were subsequently removed at intervals and assayed for protein H1 levels and sensitivity. A. Decrease in ratio of protein H1:H2 measured from stained gels of cell envelopes (levels of protein H2 are constant under the conditions used). B. Increase in sensitivity to bactericidal action of EDTA-Tris (O) or polymyxin B (●). Cells sampled at the given times were treated for 5 min with either 10 mM EDTA in 10 mM Tris-HCl pH 8.5 or 75 µg/ml polymyxin B in phosphate buffer, then plated for viable counts. C. Increase in sensitivity to bacteriolytic action of EDTA-Tris. Lysis was measured as the decrease in A\(_{600}\) after 15 min treatment with 10 mM EDTA-Tris in 10 mM Tris-HCl pH 8.5
to decrease by one-half was estimated as about 38 min, close to
the time for one cell division. After 45 to 60 min, the pro-
tein H1 level stabilized at the levels previously seen in cells
grown in Mg\(^{2+}\) sufficient medium. Increase in susceptibility to
killing by polymyxin B and EDTA-Tris and lysis by EDTA-Tris
followed a very similar time course (Fig. 6). There was a
short lag before the cells increased in sensitivity to the
bactericidal action of EDTA. However, sensitivity to lysis by
EDTA more closely paralleled decrease in protein H1.

3. Divalent cation concentration of cell envelopes and dis-
placement of cations by aminoglycosides and polymyxin B.

As indicated in Table VI, decrease in levels of pro-
tein H1 was accompanied by an increase in cell envelope Mg\(^{2+}\)
content. In experiments with wild type cells and three levels
of Mg\(^{2+}\), there was a reciprocal relationship between Mg\(^{2+}\)
levels in the cell envelope and protein H1 levels (correlation
coefficient of 0.99 by linear regression). For the protein H1
overproducing mutants grown in Mg\(^{2+}\)-sufficient medium, protein
H1 levels were about 7-fold greater than wild type levels,
while envelope Mg\(^{2+}\) was reduced about 2-fold.

The relationship between decrease in protein H1 lev-
els and increase in cell envelope Mg\(^{2+}\) suggested that H1 may
act by replacing Mg\(^{2+}\) at a site susceptible to chelator and
antibiotics. This was tested by examining whether polymyxin or
aminoglycoside could act to displace Mg\(^{2+}\) in whole cells. Cells were pre-treated with KCN to prevent inner membrane uptake of aminoglycosides. When cells grown in 0.5 mM Mg\(^{2+}\) were treated with 50 ug/ml of polymyxin B, cell envelope Mg\(^{2+}\) content was reduced by about 10%. Treatment with 25 ug/ml gentamicin or 50 ug/ml streptomycin reduced Mg\(^{2+}\) levels by about 3.5 - 5%. This suggests that these agents can act to displace Mg\(^{2+}\), but that a relatively small number of sites are involved.

Table VII shows that when cells were grown with Ca\(^{2+}\), Mn\(^{2+}\), or Zn\(^{2+}\) as the major divalent cation, high levels of the major divalent cation were incorporated into the cell envelope. The levels of Zn\(^{2+}\) were significantly lower than the levels of Ca\(^{2+}\) or Mg\(^{2+}\) (P <0.05 by Student's unpaired t-test) in cells grown with 0.5 mM Ca\(^{2+}\) and Mg\(^{2+}\), respectively.

4. Comparison of EGTA and EDTA susceptibility of Ca\(^{2+}\) and Mg\(^{2+}\) grown cells. Since cells were always provided with some Mg\(^{2+}\) as a growth factor, and cell envelopes all contained significant levels of Mg\(^{2+}\) (Table V), it was attempted to determine whether EDTA was exerting its effect on Ca\(^{2+}\) grown cells by removal of the small amounts of Mg\(^{2+}\) or by removal of the major cation Ca\(^{2+}\) (Table III). Cells grown in Ca\(^{2+}\) were equally susceptible to lysis by EGTA (which can be regarded as a Ca\(^{2+}\)-specific chelator) and EDTA (which chelates both Ca\(^{2+}\)
**TABLE VII.** Divalent cations of cell envelopes after growth in the presence of different cations.

<table>
<thead>
<tr>
<th>Mg²⁺ present during growth (mM)</th>
<th>Other cations present during growth (mM)</th>
<th>Divalent cations in cell envelope (nmol/mg dry wt)</th>
<th>Total b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td></td>
<td>Mg²⁺ 54.7</td>
<td>54.7</td>
</tr>
<tr>
<td>0.02</td>
<td>Ba²⁺ (0.5)</td>
<td>Ca²⁺ &lt;18</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Sn²⁺ (0.5)</td>
<td>Mn²⁺ &lt;0.4</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Zn²⁺ (0.5)</td>
<td>Zn²⁺ &lt;1</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>Ca²⁺ (0.5)</td>
<td>Ca²⁺ 26.3</td>
<td>173.3</td>
</tr>
<tr>
<td>0.02</td>
<td>Mn²⁺ (0.5)</td>
<td>Mn²⁺ 25.0</td>
<td>136</td>
</tr>
<tr>
<td>0.02</td>
<td>Sr²⁺ (0.5)</td>
<td>Sr²⁺ 26.0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>Ca²⁺ 76.5</td>
<td>192</td>
</tr>
<tr>
<td>0.5</td>
<td>Ca²⁺ (0.5)</td>
<td>Ca²⁺ 76.5</td>
<td></td>
</tr>
</tbody>
</table>

a Determined by atomic absorption spectroscopy; means of up to 5 separate determinations on 2 to 3 separate samples.

b Mg²⁺ plus other cation present during growth.
and Mg$^{2+}$ at high efficiency). EGTA had no measurable effect on cells grown on 0.5 mM Mg$^{2+}$ as the sole divalent cation. EGTA-Tris had some bactericidal activity on Ca$^{2+}$ grown cells, but none on Mg$^{2+}$ grown cells. However, the bactericidal activity of EGTA was several orders of magnitude lower than that of EDTA. Permeabilization of the outer membrane as a result of chelator treatment was also measured, by examining hydrolysis of a chromogenic beta-lactam nitrocefin. An increase in the rate of hydrolysis indicates increased permeation of the beta-lactam through the outer membrane to the periplasmic beta-lactamase (Angus et al., 1982), and thus provides a sensitive and specific technique for demonstrating disruption of the outer membrane permeability barrier. EGTA- and EDTA-Tris treatment were of similar efficiency in permeabilizing Ca$^{2+}$ grown cells to nitrocefin, producing hydrolysis rates about 30 times higher than those seen in unbroken cells. Mg$^{2+}$ grown cells were similarly affected by EDTA, but were not affected by EGTA. Furthermore, if Ca$^{2+}$ grown cells which had been treated with EGTA were subsequently treated with EDTA, only a small increase (5-10%) in the rate of hydrolysis was observed. A similar increase was seen in EDTA treated Ca$^{2+}$ grown cells subsequently treated with EGTA. All of the above experiments were repeated using an RPl plasmid-encoded beta-lactamase, with essentially identical results.
TABLE VIII. Effects of EGTA-Tris and EDTA-Tris on cells grown in Mg$^{2+}$ and Ca$^{2+}$.

<table>
<thead>
<tr>
<th>Cations during growth (mM)</th>
<th>% Lysis$^a$</th>
<th>% killing$^a$</th>
<th>Increase in nitrocefin hydrolysis$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGTA</td>
<td>EDTA</td>
<td>EGTA</td>
</tr>
<tr>
<td>Mg$^{2+}$ 0.5</td>
<td>0</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>Mg$^{2+}$ 0.02; Ca$^{2+}$ 0.5</td>
<td>40</td>
<td>36</td>
<td>37</td>
</tr>
</tbody>
</table>

$^a$ See Table V.

$^b$ Ratio of rate of nitrocefin hydrolysis in cells treated for 2 min with 10 mM EGTA or EDTA in 10 mM Tris-HCl pH 8.5 to rate of hydrolysis in untreated cells.
5. **Protein Hl induction in other Pseudomonas strains.** The 17 strains in a collection representing the 17 *P. aeruginosa* serotypes of the International Antigenic Typing Scheme (IATS) have been shown to have outer membrane protein patterns extremely similar to that of *P. aeruginosa* PAO H103 (Mutharia, Nicas and Hancock, 1982). All these strains have a protein equivalent to protein Hl with respect to heat modifiability and molecular weight. When these strains and a type strain of *P. putida* were grown in Mg$^{2+}$ deficient BM2 succinate all but one, the IATS 6 strain, showed large increases in levels of protein Hl similar to that seen in H103 (Fig. 7).

6. **Summary.** Outer membrane protein Hl was present as the major cellular protein both in cells which acquired resistance to polymyxin B, aminoglycosides and EDTA by growth in Mg$^{2+}$-deficient medium and in polymyxin-resistant mutants. Those cations which could substitute for Mg$^{2+}$ in reversing resistance to these agents also prevented induction of protein Hl. In shift experiments the time course of loss of protein Hl correlated with that of increase in susceptibility to cationic antibiotics and chelators. Increase in protein Hl was associated with a decrease in cell envelope Mg$^{2+}$. Cells grown in Ca$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$ had levels of those cations in their cell envelopes similar to the levels of Mg$^{2+}$ seen in cells grown in Mg$^{2+}$-sufficient medium. In cells grown in Ca$^{2+}$, but not cells
Figure 7. Induction of protein H1 in Pseudomonas strains grown in low Mg\(^2+\). SDS polyacrylamide gels of cell envelopes of cells grown in BM2 succinate with 0.02 mM Mg\(^2+\). A. H181; B. Pseudomonas putida type strain; 1-16 IATS serotype strains of P. aeruginosa types 1-16. All strains were inducible for protein H1 except for the type 6 strain. The type 17 strain is not shown.
grown in Mg$^{2+}$, the calcium specific chelator EGTA had effects similar to those of EDTA. Both aminoglycosides and polymyxin B could be shown to displace Mg$^{2+}$ from the cell envelope.
1. Isolation of outer membrane protein deficient strains by random heavy mutagenesis and isolation of phage specific for protein receptors. Isolation of mutants was carried out by random heavy mutagenesis followed by screening of cell envelopes for protein alterations. This method has been used in E. coli to obtain classes of mutants for which no selection is readily available such as mutants in penicillin binding proteins, lipoprotein and cardiolipin synthetase (Suzuki et al., 1978). This approach was adopted after preliminary studies showed that selection procedures for outer membranes protein mutants in other organisms were often inapplicable to P. aeruginosa and were generally slow, laborious and inefficient. The mutagenesis procedure was designed to generate multiple mutations in the survivors.

It was anticipated that any outer membrane protein deficient mutants isolated by this method could then be used to identify potentially useful selective agents such as bacteriophage and bacteriocins (see below). NTG was chosen as the mutagen after preliminary experiments showed that mutagenesis with diethyl sulphate to similar survival levels yielded fewer auxotrophic mutants. In order to avoid selecting mainly strains resistant to the mutagen, the treatment time chosen was
within the range where treatment time was still linearly related to survival. Preliminary experiments showed a linear relationship for up to 1 h treatment. Under the conditions chosen (30 min treatment with 1 mg/ml NTG) the survival was 0.004%, suggesting a high mutation frequency.

High frequency of mutation among the 500 survivors was also indicated by preliminary screening which showed 19% unable to grow on minimal medium, 15% unable to grow at 42°C, 3% unable to serve as host for pilus specific phages (M6 and B39), 23% unable to serve as host for LPS specific phage (Phage 44), 6% carbenicillin resistant, and 0.4% with the relatively rare mutation of resistance to high levels (0.1 mg/ml) of streptomycin.

Screening of the 500 strains for membrane protein alterations yielded mutants severely deficient in 4 of the 7 major outer membrane proteins, F, G, H1 and H2 (Fig. 8). Two were found which appeared to have protein F with a slightly altered molecular weight present in somewhat reduced amounts. The outer membrane protein alterations were confirmed on SDS polyacrylamide gels of purified outer membranes. No strains deficient in proteins I, D1, or D2 were found.

A number of strains appeared to have moderately high molecular weight (50,000-80,000) proteins present in large amounts (e.g., T133 Fig. 8, Gel D). It was not established whether these were inner or outer membrane proteins.
Figure 8. Membranes of heavily mutagenized strains with apparent protein alterations. Arrows indicate alterations. Gels A, H, M - H103 (wild type) outer membrane (OM); Gel B - T316 cell envelope (CE) (H1 deficient); Gel C - T509 CE (G deficient); Gel D - T133 CE (extra protein); Gel E - T513 CE (H2 deficient); Gel F - H283 CE (F deficient); Gel G - T129 (H1 deficient); Gels I and K - T817 OM (F altered); Gels J and L T941 OM (F altered); Gels K, L and M were solubilized without 2-mercaptoethanol so that F runs in the F* position.
Bacteriocins and bacteriophages have outer membrane receptors. Strains resistant to these agents are usually deficient in those receptors, and bacteriophage and bacteriocins which use specific outer membrane components can often be used as a means of selecting outer membrane protein deficient mutants (for examples see reviews by Osborne and Wu, 1980; and DiRienzo et al., 1978). An attempt was made to use 7 strains with altered outer membrane proteins isolated by random mutagenesis to identify agents which could then be used to select for specific outer membrane protein mutants. The 7 strains were tested for sensitivity to a set of 22 aeruginicins and 32 phages. Partial characterization of the receptors of these phages had shown that 19 of them probably had outer membrane protein receptors (Table II; Nicas and Hancock, 1980). However, no bacteriophage with obvious potential utility was found in the collection, and none of protein deficient mutants was resistant to any aeruginocin.

A second, more direct approach attempted was the isolation of phage of the desired specificity from nature using the enrichment technique of Verhoef et al. (1977). This method is based on the use of bacteria lacking a specific receptor to adsorb out the majority of phages. Adsorption is followed by propagation, and the process repeated for several cycles.

Each of the 7 outer membrane protein-altered strains isolated was used as the adsorbing strain in separate experiments.
Results of these enrichments are summarized in Table IX. The procedure was successful in isolating phage unable to plate on 3 of the 7 protein-deficient or protein-altered strains used. No attempt was made to establish whether the 3 groups of phages unable to form plaques on specific outer membrane-altered mutants each represented a set of multiple isolates of the same phage or several different phages with the same host range.

Four phage isolates were found which did not form plaques on the G deficient strain T509. These isolates did form plaques on the second G deficient strain, T817. Nine mutants resistant to this group of phages were isolated. None had any membrane protein alterations apparent on SDS polyacrylamide gels. Nine phage isolates (V20–V28) were found which did not form plaques on T316, a strain deficient in both protein H1 and LPS. These phages were able to form plaques on other LPS-altered strains. However, 19 of 20 mutants resistant to these phages had altered LPS as judged by inability to form plaques on LPS receptor-specific phages. Of the 14 resistant strains examined on SDS polyacrylamide gels, 3 had apparently reduced levels of H1 (data not shown). These were named H317, H318 (isolated against phage V28) and H319 (isolated against V27). A spontaneous revertant of H318, named H318b, was also found.

A number of phage isolates which did not form plaques on the porin deficient strain H283 were also obtained (phages
TABLE IX. Results of enrichment procedure for isolation of outer membrane protein receptor-specific phages.

<table>
<thead>
<tr>
<th>Adsorbing Strain</th>
<th>Outer Membrane Alteration&lt;sup&gt;a&lt;/sup&gt;</th>
<th># Isolates Tested After Enrichment</th>
<th># Isolates Not Plating on Deficient Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>H283</td>
<td>F-deficient</td>
<td>161</td>
<td>15 (V1-V15)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T817</td>
<td>F-altered; G-deficient</td>
<td>60</td>
<td>9 (V16-V19)</td>
</tr>
<tr>
<td>T941</td>
<td>F-altered</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>T509</td>
<td>G-deficient</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>T129</td>
<td>Hl-deficient</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>T316</td>
<td>Hl-deficient; LPS altered</td>
<td>120</td>
<td>9 (V20-V28)</td>
</tr>
<tr>
<td>T513</td>
<td>H2-deficient</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Fig. 8.

<sup>b</sup> Isolate designations.
V1-V15). However, revertants of H283 which appear to have normal levels of porin were also resistant to these phages, strongly indicating that their receptor is something other than porin (see below).

2. **Characterization of a porin deficient isolate.** As the protein F deficient strain H283 was isolated after heavy mutagenesis, more than one mutation might be expected in this strain. To circumvent this problem, 3 independent spontaneous revertants were isolated. Two of these, H284 and H324 were isolated fortuitously after growth on BM2 glucose. The third, H321, was selected for by enriching for strains with higher growth rates by repeated subculture of cells growing in liquid medium. The isogenicity of the revertants and H283 was confined by their phage sensitivity patterns (see below). Transfer of the specific mutant gene responsible for porin deficiency was not attempted since the only available method of screening was SDS polyacrylamide gel electrophoresis of cell envelopes, and frequencies of gene transfer in *P. aeruginosa* tend to be quite low.

Protein F has been shown to be present in about 1 - 3 $\times 10^5$ copies/cell of H103. The revertants H284 (Fig. 9, Gel C), H321, and H324 (data not shown) contained apparently normal levels of protein F. In contrast, the outer membranes of the mutant strain H283 did not contain observable levels of protein F (Fig. 9, Gels B and E). The pattern of other outer membrane proteins was the same in the mutant H283 and its revertants.
Figure 9. Cell envelopes of wild type strain H103, its porin deficient mutant H283, and a revertant, H284. Gel A, D. - H103; Gel B, E. - H283; Gel C. - H284. Cells envelopes for gels A, B, and C were solubilized in normal reduction mix before application to the gel. For gels E and D, 2-mercaptoethanol was omitted from the solubilization mix, so that protein F runs in the $F^*$ position. The amount of protein F detectable in H283 is less than 1% that of H103 or H284.
When the electrophoretic mobility of protein F from H103 and the revertants was increased by omission of 2-mercaptoethanol from the solubilization buffer (Hancock and Carey, 1979), no outer membrane polypeptide from strain H283 was similarly 2-mercaptoethanol modified (c.f. Fig. 9, Gels D and E).

All other major outer membrane proteins appeared to be present in H283 in quantities comparable to those of the revertant, although the absence of the large protein F band on SDS polyacrylamide gels resulted in apparent enhancement of minor bands. Strains H283 and its revertants were similar to the parent strain H103 in their ability to produce two inducible outer membrane proteins: D1, a glucose-inducible porin (Hancock and Carey, 1980), and protein H1.

3. Bacteriophage sensitivity of outer membrane protein mutants, mucoid isolates, and serotype strains. As bacteriophages and bacteriocins have outer membrane receptors, testing the susceptibility of strains to these agents is a useful method of revealing outer membrane alterations. No differences were found in the susceptibilities of the polymyxin B-resistant mutants H181 and H185 to 24 phages and 22 aeruginocins when compared to the wild type strain, H103 (Table X). The porin deficient strain H283 also showed no differences in sensitivity when tested against the aeruginocins and 33 phages. Since some of the phages were smooth LPS or pilus receptor specific, it can be concluded that H181, H185 and H283 had smooth LPS and were piliated. The 3 revertants of H283 were also sensitive to
all these phages, except for H284 which was resistant to phages with pilus receptors. This strain was also non-motile, suggesting it was defective in flagellar function, as well as pili. A set of 15 phages (V1-V15) able to plate on the wild-type H103, a pilus-deficient strain, AK1213, and an LPS-deficient strain, AK1012, but unable to form plaques on H283 was isolated from nature (see above). Revertants of H283 were also resistant to these phages. Two of these phages, V4 and V7, could be shown to adsorb efficiently to whole cells of the wild type H103 (95-100% reduction in phage titre), but relatively poorly to the porin deficient mutant H283 or its revertant H284 (2-10% reduction in phage titre). This suggests that H283 and its derivatives lack a membrane receptor for these phages which is present in wild-type cells.

The species P. aeruginosa can be divided into 17 serotypes based on antigenic differences in their LPS (Brokopp and Farmer, 1979; Lanyi et al., 1979). These differences reflect chemical variations in the LPS 0 side-chain (Koval and Meadow, 1975). Pili also vary from strain to strain (Brinton, 1981). As bacteriophages are thought to interact with chemically unique sites on specific cell surface components (e.g., Braun and Keiger-Bauer, 1975), the ability of a given phage to form plaques on several strains indicates a receptor common to these strains. It should however be noted that lack of sensitivity to a bacteriophage does not necessarily imply absence of the receptor protein since single amino acid substitutions in
such proteins has been shown to result in resistance to phages which normally use this protein as a receptor (e.g., E. coli CR63; Braun and Keiger-Bauer, 1977). Receptors of the phages used had been partially characterized allowing them to be separated into five groups (see Table II, Methods). Results of sensitivity testing are listed in Table X and summarized in Table XI. The results with the 13 phages that had uncharacterized protein receptors indicated that these receptor sites were quite well conserved. For example, phage 7 formed plaques on all 17 P. aeruginosa serotyping strains tested, while phages B9F and F116 plated on 16 and 11 strains, respectively.

None of the bacteria studied appeared rough as tested either with a bacteriophage which used rough but not smooth LPS as its receptor, or by examination of gross colony morphology. With the exception of IATS type 11 bacteria, all typing strains plated one or more smooth LPS specific phages (Tables X and XI). Indeed, 9 of the 17 serotypes plated 7 or more of the 9 smooth LPS-specific phages. This indicated that there is some homology (i.e., the receptor site) in the 0 antigens of different serotyping strains despite the prior observation of significant chemical variations. It was further observed that only 3 of the 17 strains plated phages specific for PA01 pili in agreement with the finding that pili differ from strain to strain as demonstrated for P. aeruginosa and other bacteria.

Taken overall, the results suggested that the surfaces of P. aeruginosa cells were moderately well conserved since
| Phage | A | B | C | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | D | E | F | G | H | I | J | K |
| 119x  | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| M6    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B39   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 44    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| E79   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| F8    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 109   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 1214  | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| PB1   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| C3A   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| S1    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 352   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| PLS27 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 2     | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| D3c+1 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B5A   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B7A   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| C7B   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B9E   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B9F   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 7     | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 21    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 68    | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| C21   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| F116  | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| G101  | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B1A   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| A8A   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B6B   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| D3c-1 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| B6C   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| F10   | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| V1-V15| S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

a A: H103 (wild type); B: AKL213 (pilus-deficient); C: AKL012 (LPS-deficient); 1-17: IATS serotype strains 1-17; D,E: protein H1 overproducers, H181 and H185; F: H283 (porin-deficient); G, H, I: revertants of H283 (H284, H321, H324); J, K: mucoid strains, H329 and H325.

"S" means sensitive; "-" means not tested; no entry means resistant.
TABLE XI.  Bacteriophage susceptibility of serotype strains of *P. aeruginosa* performed using phages propagated and characterized on *P. aeruginosa* PA01 strains.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Smooth LPS specific phages (9)</th>
<th>Rough LPS specific phage (1)</th>
<th>Pilus specific phages (2)</th>
<th>Possible LPS receptor phages (6)</th>
<th>Possible protein receptor phages (13)</th>
<th>Cumulative susceptibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>13</td>
<td>100%</td>
</tr>
<tr>
<td>Pilus deficient</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>13</td>
<td>90%</td>
</tr>
<tr>
<td>LPS altered</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>13</td>
<td>50%</td>
</tr>
<tr>
<td>O Serotype 1</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>83%</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>12</td>
<td>83%</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>11</td>
<td>83%</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>12</td>
<td>83%</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>13</td>
<td>83%</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>60%</td>
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<tr>
<td>7</td>
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<td>0</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>60%</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7%</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>27%</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>7%</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>20%</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>37%</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>53%</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>30%</td>
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<tr>
<td>16</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>60%</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>23%</td>
</tr>
</tbody>
</table>

Nos. of phages reacting with 45% of serotype strains: 9/9 0/1 0/2 2/6 11/13 73%
9 of the 17 strains plated 53% or more of the phages screened. The antigenic and physical similarity of outer membrane proteins of *P. aeruginosa* has been further studied by Mutharia et al. (1982).

Two mucoid derivatives of H103 originally isolated by selection for resistance to phage 7 were also tested. One of these was identical to the parent in its phage sensitivity, while the other was sensitive to over 60% of the phages, indicating that mucoidy does not necessarily impede phage sensitivity, in agreement with previous results (Martin, 1973).

4. **Summary.** Random heavy mutagenesis with nitrosoguanidine was used to isolate mutants deficient in outer membrane proteins F, G, H1, and H2. Three revertants of the protein F-deficient strain were also isolated. Attempts to isolate phage with outer membrane protein specific receptors yielded one group of phages which could be used to select for mutants deficient in protein H1 and a group of phages unable to form plaques on the protein F-deficient strain or its revertants. Revertants of the protein F-deficient strain had a protein band on SDS polyacrylamide gels which was indistinguishable from protein F in its electrophoretic mobility both in the presence and absence of 2 mercaptoethanol, while no such protein was detectable on gels of the protein F-deficient strain. Phage sensitivity of wild type, protein F-deficient and H1 overproducing mutants were essentially the same, indicating that the
mutants were piliated, had smooth LPS, and lacked any gross surface alterations. Phage studies with a set of strains representing the 17 serotypes of P. aeruginosa indicated considerable conservation of phage receptors.
MEASUREMENT OF OUTER MEMBRANE PERMEABILITY

Outer membrane protein F has been characterized as a porin on the basis of in vitro studies (Hancock et al., 1979; Benz and Hancock, 1981). Such studies have indicated that although protein F forms substantially larger channels than the porins of enteric bacteria (Hancock et al., 1979; Nikaido, 1980), the in vitro activity of protein F is relatively low in that only a small proportion of the porin proteins form functional channels (Angus et al., 1982; Benz and Hancock, 1981). Low outer membrane permeability of wild-type P. aeruginosa cells has also been shown in vivo (Angus et al., 1982). The low activity of protein F in vitro and in vivo raises the possibility that the effects observed could be due to a minor protein co-purifying with protein F rather than protein F itself. This hypothesis was tested by examining the permeability of a strain severely deficient in protein F, H283. Isolation of this strain and its revertants (which have normal levels of protein F) is described above.

The technique employed was based on that of Zimmerman and Rosselet (1977). This technique is based on the concept that, providing enough beta-lactamase is present in the periplasm, then the beta-lactamase activity of intact cells will be limited by the rate of diffusion of beta-lactam across the
outer membrane to the periplasmic beta-lactamase. Thus, the equilibrium rate of hydrolysis of beta-lactam by intact cells ($V_{\text{intact}}$) is equal to the rate of diffusion ($V_{\text{Diff}}$). This allows calculation of an outer membrane permeability coefficient $C$ using the following equation of Zimmermann and Rosselet:

$$V_{\text{int}} = V_{\text{Diff}} = C(S_{\text{out}} - S_{\text{in}})$$

where $S_{\text{out}}$ is the beta-lactam concentration added and $S_{\text{in}}$ is the periplasmic concentration of beta-lactam [which can be calculated from the Michaelis-Menten equation]. The beta-lactam used in these measurements was the chromogenic cephalosporin nitrocefin (O'Callaghan, 1972). The beta-lactamase used was the periplasmic TEM-2 enzyme encoded by the plasmid RPl, which was introduced into each of the strains used by conjugation. Measurement of total beta-lactamase activity in cells broken by passage through a French press was carried out to confirm that periplasmic activity was in excess. Beta-lactamase activity of broken cells varied little from strain to strain and was 7 to 500-fold higher than the activity of whole cells. Other studies (Angus et al., 1982) have demonstrated that the temperature coefficient of nitrocefin hydrolysis by intact cells ($V_{\text{Diff}}$) is consistent with nitrocefin entering via a hydrophilic pathway. In addition, it was established here that nitrocefin hydrolysis in intact cells was directly proportional to the concentration of nitrocefin added ($S_{\text{out}}$) for strains H103 (RPl) and E. coli
UB1636 (RPI) over an 8-fold range of substrate concentrations (0.025-0.02 mg/ml), as predicted by the above diffusion equation.

The results shown in Table XII revealed that strain H283 was significantly less permeable than its parent strain H103 or the revertant H284. While the standard deviations of these results were high, the range of rates of hydrolysis of nitrocefin in intact cells of H283 and H103 or H284 did not overlap and the means were clearly different as judged by the Student t-test. As an additional control, previous results demonstrating that the antibiotic super-susceptible mutant Z61 is significantly more permeable than H103 or its full revertant H251 (Angus et al., 1982) were confirmed using this modified assay. In contrast, there was no significant alteration in permeability of the aminoglycoside and polymyxin resistant mutant H181. An E. coli K-12 strain UB1636 had much greater permeability than any of the P. aeruginosa strains studied here.

Summary. Outer membrane permeability was measured using the rates of hydrolysis, in intact cells, of a chromogenic beta-lactam, nitrocefin, by periplasmic beta-lactamase. It was shown that the protein F-deficient mutant H283 had reduced outer membrane permeability relative to its parent or revertant, indicating that this protein is the major outer membrane porin. Protein H1 overproducing strains were not altered in outer
TABLE XII. Rate of nitrocefin hydrolysis by intact cells, and outer membrane permeability coefficients C, of *P. aeruginosa* strain H103 (RPl), its RPl plasmid-containing derivatives, Z61 (RPl) and *E. coli* strain C127 (RPl).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Nos. of Determinations</th>
<th>Rate of nitrocefin hydrolysis in intact cells (pmol nitrocefin min⁻¹ mg cell dry wt⁻¹)ᵃ</th>
<th>Outer Membrane permeability coefficient C (sec⁻¹ mg cell dry wt⁻¹ x 10⁴)</th>
<th>Significance, by Student t-test, of difference from H103 (RPl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H103 (RPl)</td>
<td>Parent</td>
<td>12</td>
<td>60 ± 17</td>
<td>4.1</td>
<td>p&gt;0.5</td>
</tr>
<tr>
<td>H181 (RPl)</td>
<td>GM, PX, EDTA resistant, protein H1 overproducing</td>
<td>9</td>
<td>62 ± 36</td>
<td>4.2</td>
<td>p&gt;0.5</td>
</tr>
<tr>
<td>H251 (RPl)</td>
<td>Revertant of Z61</td>
<td>7</td>
<td>58 ± 13</td>
<td>3.9</td>
<td>p&gt;0.5</td>
</tr>
<tr>
<td>H284 (RPl)</td>
<td>revertant of H283</td>
<td>7</td>
<td>59 ± 30</td>
<td>4.0</td>
<td>p&gt;0.5</td>
</tr>
<tr>
<td>H283 (RPl)</td>
<td>protein F-deficient</td>
<td>5</td>
<td>9.8 ± 7.6</td>
<td>0.7</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Z61 (RPl)</td>
<td>antibiotic super-susceptible</td>
<td>12</td>
<td>360 ± 170</td>
<td>24.9</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>UB1636 (RPl)</td>
<td><em>E. coli</em></td>
<td>13</td>
<td>740 ± 390</td>
<td>50.6</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

ᵃ mean ± standard deviation
membrane permeability. An antibiotic supersusceptible strain of *P. aeruginosa* was found to be significantly more permeable than its revertant or the wild type. An *E. coli* strain had much greater permeability than any of the *P. aeruginosa* strains used here.
DISCUSSION

This study demonstrates that the outer membrane of Pseudomonas aeruginosa plays a major role in the antibiotic resistance of this organism. The results of the permeability studies (Table XII) were consistent with the hypothesis that antibiotic resistance can be explained on the basis of low permeability of the P. aeruginosa outer membrane due to the properties of protein F. Although in vitro experiments have indicated that the area of individual protein F channels is up to 3-fold larger than the area of E. coli porin channels (Benz and Hancock, 1981), and that the number of porin molecules per cell is about the same in E. coli and P. aeruginosa (Benz and Hancock, 1982; Rosenbusch, 1974), P. aeruginosa was shown to have an outer membrane permeability coefficient (C) significantly lower than that of E. coli. This was especially striking when it is considered that the size of nitrocefin (520 daltons) approaches the exclusion limit of E. coli porins. It would thus be expected that diffusion of nitrocefin would be slowed by frictional and steric interactions with the walls of the channel as discussed by Nikaido and Rosenberg (1981), so that the diffusion constant C would be decreased when compared to a larger channel such as protein F (Hancock and Nikaido, 1978). In order to correct for the apparent reduction in C due to smaller channels, the permeability to nitrocefin of single pores of P. aeruginosa porin compared to those of E. coli
porins may be corrected theoretically (Nicas and Hancock, 1982) using Fick's law and the Renkin equations (Renkin, 1954; Lakshminarayananiah, 1969, p.325). This calculation can be made assuming a hydrated radius of 0.44-0.53 nm for nitrocefin (similar to that of a disaccharide) and using previous estimates for the radii of *E. coli* porin Ib and *P. aeruginosa* protein F (0.65 nm and 1.1 nm, respectively; Benz and Hancock, 1981; Nikaido and Rosenberg, 1981). Using such values one arrives at the conclusion that the steric effect of interaction of nitrocefin with *E. coli* porin channel walls will result in an apparent 11-fold decrease in the C value of *E. coli* pores relative to the C value for single pores of *P. aeruginosa*. Taken together with the 12-fold difference in C value shown in this study, the calculated total difference in outer membrane pore area available for the diffusion of nitrocefin in *P. aeruginosa* was 132-fold lower. Since the actual area of a *P. aeruginosa* channel is three times that of *E. coli*, the total number of active and functional porin channels (per cell dry weight) would be less than 1/400 of that of *E. coli* or about 100 per cell assuming 4 x 10⁴ porin trimers per cell (Rosenbusch, 1974). This difference in permeability correlates well with the low porin activity of protein F which has been observed both *in vitro* and *in vivo* (Angus et al., 1982; Benz and Hancock, 1981) and also correlates with the known high intrinsic resistance of *P. aeruginosa* to hydrophilic antibiotics.
It has previously been demonstrated (Angus et al., 1982) that the apparent number of functional channels can be increased by LPS mutation in strain Z61, but no explanation has emerged as to why more than 99% of protein F molecules do not form functional pores in wild type cells. The evidence reported here from studies with protein F-deficient cells argues in favour of the possibility that the actual porin is protein F rather than a minor contaminating protein. The protein F-deficient mutant had a significantly lower outer membrane permeable coefficient C compared to its parent or to revertant strains. Since strain H283 does have a measurable C value, it may well not be totally porin-deficient, but rather porin protein F-deficient, in that other porin proteins may be present at lower levels in the outer membrane. Black lipid bilayer studies of fractions from porin deficient mutants of E. coli (Benz et al., 1978) have provided evidence that a more cation-selective channel is responsible for residual porin activity in these strains. In the case of P. aeruginosa, two other inducible porin proteins, P and D1 (Hancock and Carey, 1980; Hancock et al., 1982), are possible candidates for providing the residual porin activity of the protein F-deficient strain H283.

The low level of active porin in wild-type cells suggests that the hydrophilic pathway is a relatively inefficient means of traversing the outer membrane of P. aeruginosa. It would thus be expected that hydrophilic antibiotics, including aminoglycosides, would be relatively ineffective against
P. aeruginosa. However, this study offers evidence that highly cationic antibiotics effective against P. aeruginosa, i.e., aminoglycosides and polymyxins, may cross the membrane by means of an alternate "self-promoted" pathway.

This study demonstrates that the outer membrane is a major determinant of the activity of polymyxins, aminoglycosides and EDTA. A close correlation between levels of outer membrane protein H1 and susceptibility to chelators, polymyxin B, and gentamicin was shown under a variety of different growth conditions. In shift experiments, the decrease in protein H1 brought about by increasing Mg$^{2+}$ in the growth medium closely paralleled increase in susceptibility to EDTA-Tris and polymyxin B. When other divalent metal cations were substituted for Mg$^{2+}$, only cells grown in those cations which prevented induction of protein H1 were susceptible to EDTA, polymyxin B and gentamicin.

It has been suggested that polymyxins, chelators (Brown, 1975; Nicas and Hancock, 1980), and aminoglycosides (Hancock, 1981; Hancock et al., 1981) act at a common site on the outer membrane. A model for the action of these agents on the outer membrane is illustrated in Fig. 10. It is proposed that these agents act at a site on the outer membrane, possibly a polyphosphate site on the lipopolysaccharide and that divalent cations bound at this site would be required for the stability of the outer membrane. Thus EDTA would act to remove divalent cations by chelation, while the cationic antibiotics, polymyx-
Figure 10. Model illustrating the proposed mechanism of resistance to aminoglycosides, polymyxin B, and EDTA-Tris in P. aeruginosa with high levels of protein H1. O.M. outer membrane; R core—the heptose, KDO, rough core region of the LPS; O Ag—the somatic antigen of P. aeruginosa LPS; (P)$_n$—the polyphosphate portion of the P. aeruginosa LPS; H1—major outer membrane protein H1, which appears in large amounts in specific mutants and in cells grown in Mg$^{2+}$ deficient medium. It is proposed that the crosslinking of the negatively charged polyphosphate regions of the LPS by Mg$^{2+}$ is important for outer membrane stability in sensitive cells. EDTA by removing Mg$^{2+}$, and the highly cationic antibiotics aminoglycosides and polymyxin B by displacing Mg$^{2+}$, lead to disruption of the outer membrane permeability barrier. It is proposed that in cells with high levels of protein H1, this protein replaces Mg$^{2+}$ at specific sites in the outer membrane. Protein H1 thus protects the polyphosphate site on the LPS from attach by aminoglycosides, polymyxin B and EDTA and makes the cell relatively resistant to these agents.
ins and aminoglycosides, would act by competing for the lipopolysaccharide binding site. Protein H1 is proposed to act by replacing divalent cations at this site on the lipopolysaccharide, thus protecting it from attack by these agents.

Both polymyxin and EDTA are known to interact with *P. aeruginosa* LPS (Cooperstock, 1974; Michaels and Eagon, 1966). An early consequence of polymyxin B and EDTA action is permeabilization of the outer membrane (Brown and Melling, 1969; Iida and Koike, 1974; Laporte *et al.*, 1977; Rosenthal and Strom, 1977) and polymyxin B has been shown to cause blebbing of the outer monolayer of the outer membrane, which is the sole location of cellular LPS (Gilleland and Murray, 1976; Schindler and Teuber, 1975). Schindler and Osborn (1979) have demonstrated that the 2-keto-3-deoxyoctonate-lipid A region of *Salmonella typhimurium* LPS has high affinity binding sites for polymyxin B (Kd = 0.3 to 0.5 μM - approximately 10-fold higher than for polymyxin-phospholipid interactions) and for Mg²⁺ and Ca²⁺. Earlier studies by Newton (1954) demonstrated that Mg²⁺ and polymyxin B competed for a *P. aeruginosa* cellular site, which he postulated to be polyphosphate in nature. *P. aeruginosa* LPS has been demonstrated to have an especially high phosphate concentration (Dewry *et al.*, 1971), and may have up to 8 moles of phosphate per mole of LPS in the heptose-KDO region (A. Kropinski, personal communication). There is evidence that some of this phosphate is present as triphosphate (Wilkinson, 1981). Since phosphoryl and phosphodiester groups
are negatively charged at neutral pH, this could contribute to the relatively high Mg\(^{2+}\) content of \textit{P. aeruginosa} cell envelopes (Brown and Woods, 1972). Aminoglycosides are also highly cationic, and have been shown to bind to \textit{P. aeruginosa} LPS (Day et al., 1978). The ability of aminoglycosides to interact with the outer membrane and promote a significant alteration in its permeability was shown by both cell lysis in the presence of lysozyme and aminoglycosides (Hancock, Raffle and Nicas, 1981), and increased hydrolysis of nitrocefin in the presence of gentamicin. Lysozyme is normally inactive on gram-negative bacteria as it is unable to penetrate the outer membrane to reach its site of activity, the peptidoglycan. It was shown that gentamicin and streptomycin acted to overcome this penetration barrier, allowing lysozyme to attack the peptidoglycan and lyse the cells. Lysozyme itself is known to bind to outer membranes (Day et al., 1978), and this ability may have contributed to the efficiency of its permeation in aminoglycoside-treated cells. This is further suggested by the relatively inefficient gentamicin-mediated permeabilization of outer membranes to nitrocefin, a chromogenic beta-lactam. In fact, this implies that aminoglycoside-mediated permeabilization has some specificity for cationic substrates such as lysozyme and aminoglycosides. The permeabilization observed cannot be attributed to aminoglycoside killing as it was shown that it occurs under conditions in which aminoglycosides are known not to be transported or lethal (Bryan and Van Den Elzen,
Aminoglycosides are clearly able to promote the passage of other molecules through the outer membrane, and it seems likely that they are also capable of promoting their own transport. It is proposed that aminoglycoside uptake and killing in *P. aeruginosa* requires interaction with a divalent cation binding site at the outer membrane. This interaction promotes uptake of the antibiotic into the periplasm, permitting further transport at the level of the cytoplasmic membrane. It was observed that Mg\(^{2+}\) was able to inhibit aminoglycoside mediated permeability to both nitrocefin and to lysozyme. This inhibition, coupled with the results of killing experiments with protein H1-overproducing strains, strongly suggests that the site of aminoglycoside activity at the outer membrane is a Mg\(^{2+}\) binding site. Competition at the level of the outer membrane may well explain the unusually high antagonism of aminoglycosides by divalent cations (Madeiros et al., 1971; Zimelis and Jackson, 1973) in *P. aeruginosa*, although it is probable that other sites of competition also exist. It has been shown that those aminoglycosides which are highly active against *P. aeruginosa*, such as gentamicin, are much more efficient at inducing permeabilization to lysozyme than are less active aminoglycosides such as streptomycin (Hancock, Nicas and Raffle, 1981). These results also suggest that the ability of aminoglycosides to promote permeabilization of the
outer membrane may in fact be a major determinant in their activity. In this light, it is interesting that some of the new anti-Pseudomonal aminoglycosides differ from their parent compounds largely in their efficiency of transport (Lee et al., 1978) rather than their inhibition of ribosomal function. Improved ability to interact with the outer membrane could, in part, account for their increased transport.

Studies of aminoglycoside transport in H1 overproducing strains also provide evidence that the outer membrane plays a major role in aminoglycoside activity. Aminoglycoside uptake in both *E. coli* and *P. aeruginosa* has been shown to occur in three consecutive phases (Bryan and Van Den Elzen, 1975 and 1976): an initial rapid electrostatic binding, followed by an early slow uptake phase (EDP-I), and a later rapid uptake phase (EDP-II). The binding is energy independent, while the two latter phases are energy requiring and occur only in presence of an energized cytoplasmic membrane and electron transport (Bryan and Van Den Elzen, 1975 and 1976). EDP-II may coincide with or follow the onset of loss of viability since both EDP-II and lethality can be inhibited by chloramphenicol (Bryan and Van Den Elzen, 1975; Hurwitz and Rosano, 1961), and do not occur ribosomally resistant (*strA*) strains (Bryan and Van Den Elzen, 1975). Alteration of ribosomal affinity in other mutants also affects uptake (Ahmad et al., 1980). Comparison of streptomycin uptake in wild type strains with uptake in resistant mutants which overproduce protein H1, showed that in
the resistant strains the late rapid phase EDP-II was always delayed (Fig. 2, 3). It would thus appear that the outer membrane has a critical influence on the events required to initiate EDP-II. Somewhat similar delays in the onset of EDP-II have been observed for certain *E. coli* mutants with altered ribosomal affinity to aminoglycosides (Ahmad *et al.*, 1980). However, since the mutant was selected for resistance to polymyxin and EDTA, and since ribosomal effects are usually specific to given aminoglycosides (Hancock, 1981), it is unlikely that our mutants also have ribosomal alterations. Furthermore, it was demonstrated that single step revertants of strains H181 and H185 to polymyxin sensitivity had low levels of protein H1 on Mg²⁺-sufficient medium as well as wild type aminoglycoside and EDTA susceptibilities, suggesting that the different phenotypic alterations in the mutants had a common basis.

The increase in resistance to aminoglycosides conferred by protein H1 is smaller than that seen for polymyxin. This could be due to alternative binding or uptake sites for aminoglycosides, a requirement for fewer binding sites for aminoglycoside activity, or to a lower affinity of aminoglycosides for the proposed LPS binding site. Schindler and Osborne (1979) have demonstrated that polymyxin has a higher affinity for *Salmonella typhimurium* LPS than Mg²⁺, while others have demonstrated that moderately high levels of Mg²⁺ are necessary to inhibit polymyxin activity (Davis, 1974). In the case of aminoglycosides, antagonism by Mg²⁺ occurs at quite low
Mg\(^{2+}\) levels, and this competition tends to mask the protective effects of protein Hl when the protein is induced by growth in low Mg\(^{2+}\). Thus, in MIC measurements of aminoglycoside susceptibility, \textit{P. aeruginosa} cells in low Mg\(^{2+}\) were apparently more susceptible than cells in high Mg\(^{2+}\). When the differential competitive effects of Mg\(^{2+}\) were eliminated by comparison of loss of viability in a common assay medium (Tables III and V; Hancock, Raffle and Nicas, 1981), cells grown in Mg\(^{2+}\)-deficient medium were actually more resistant to aminoglycoside killing than cells grown in Mg\(^{2+}\)-sufficient medium.

The increase in resistance seen in outer membrane protein Hl overproducing strains cannot be attributed to a general decrease in outer membrane permeability. The major pore-forming protein (protein F) was only slightly reduced in amount in protein Hl overproducing strains. Furthermore, sensitivity to both carbenicillin and tetracycline [which use the so-called hydrophilic (porin-mediated) pathway in \textit{E. coli} (Nikaido and Nakae, 1974)] was not altered in these strains (Table IV) and their growth rate on Mg\(^{2+}\)-deficient media was unaffected suggesting that the porin is functionally normal. The finding that the Hl overproducing mutants were unchanged in their sensitivity to phages, including LPS receptor-specific phages, also suggested that there are no major surface alterations. Normal porin function was confirmed by the lack of significant difference in permeation of nitrocefin
between wild type and protein H1 overproducing strains (Table XII). This suggests that the hydrophilic pathway (Nikaido and Nakae, 1974) of passive permeation through the hydrophilic pores formed by porin (Hancock and Nikaido, 1978; Nikaido and Nakae, 1979) may not be the major route taken by aminoglycoside antibiotics in \textit{P. aeruginosa}. Although the large pore size of \textit{P. aeruginosa} (Hancock and Nikaido, 1978) would not be expected to offer any barrier to permeation of such antibiotics, there is evidence that most of the pores at any given time are not in an active, open state (Benz and Hancock, 1981; and above). However, it is possible that in other organisms where a greater proportion of porins are in the active state (Benz et al., 1980) and there are less Mg$^{2+}$ binding sites on the cell surface (Brown and Melling, 1969), the hydrophilic pathway of antibiotic uptake may offer an alternative, efficient means of streptomycin permeation. Evidence for this is provided by the finding of Foulds and Chai (1978), who demonstrated that a porin Ia deficient mutant of \textit{E. coli} defective in the hydrophilic uptake pathway was somewhat more resistant to kanamycin and gentamicin, and studies with \textit{E. coli} \text{omp B} mutants (which are deficient in both major porins) have shown a 4-fold reduction in aminoglycoside resistance (V. Raffle, E. Buenaventura, and R.E.W. Hancock, unpublished results). Furthermore, streptomycin action in other organisms such as \textit{E. coli} should be, and is, less affected by Mg$^{2+}$ antagonism (Madeiros et al., 1971) as would be predicted.
An alternative explanation for some of the results presented here might be that protein H1 is a magnesium binding outer membrane protein which also binds gentamicin and streptomycin and thus specifically limits access of aminoglycosides to porin. However, significant differences in the binding of streptomycin to mutant strain H181 with high protein H1 levels, when compared to our wild type strain H103, could not be demonstrated. In addition, at a streptomycin concentration where we could demonstrate a large difference in killing of strains H181 and H103 (Hancock, Raffle and Nicas, 1981) less than 0.5% of the added streptomycin become bound to cyanide-treated cells, suggesting that binding did not significantly alter the effective concentration of streptomycin in the medium. Also, mutant strains had less Mg$^{2+}$ in their outer membranes, suggesting that protein H1 is unlikely to be a specific Mg$^{2+}$ binding protein. Finally, the above alternative does not explain aminoglycoside-mediated permeabilization of outer membranes or the unusually high Mg$^{2+}$ antagonism of aminoglycosides in wild type *P. aeruginosa* (Madeiros et al., 1971). Thus, the above alternative model seems unlikely, although we cannot rigorously exclude that binding of aminoglycosides to protein H1 contributes to the phenotype of the mutants.

Resistance to polymyxin acquired during growth under conditions other than low divalent cations has been reported by other workers. Gilleland and colleagues (Gilleland and Murray, 1976; Gilleland and Lyle, 1979) and Brown and Watkins (1970)
studied \textit{P. aeruginosa} trained to grow on very high levels (750 ug/ml) of polymyxin. Such strains exhibit a large number of alterations in phospholipid content, readily extractable lipid, wall phosphorous, LPS and envelope protein (Brown and Watkins, 1970; Gilleland and Lyle, 1979). In addition, ultra structural alterations in these strains differ from those of cells grown in Mg\textsuperscript{2+}-deficient medium (Gilleland and Murray, 1976).

Thus, this type of resistance cannot be compared to the resistance reported here. \textit{P. aeruginosa} may also acquire resistance to polymyxin B by growth on branched-chain amino acids and branched-chain acyl derivatives as sole carbon source (Conrad et al., 1979). This resistance appears to be related to changes in fatty acid composition of readily extractable lipids (Conrad et al., 1981), and differs from the resistance reported here in that it does not affect aminoglycoside susceptibility.

Alterations in Mg\textsuperscript{2+} content, readily extractable lipid and reduction in phospholipid have also been observed in polymyxin resistant clinical isolates of \textit{P. aeruginosa} and other gram-negative bacteria (Brown and Wood, 1972). Yet another mechanism of acquired polymyxin B resistance has been shown in \textit{P. fluorescens}, where growth under phosphate-limiting conditions reduces the amount of membrane phospholipids and brings about the synthesis of a novel cationic lipid, ornithine amide lipid. It is thus clear that there are a variety of mechanisms for acquisition of polymyxin resistance, as would be expected for an antibiotic which interacts with both outer and inner
membrane components to exert its lethal effects (Storm et al., 1977). The common factor in most of these forms of resistance may be the reduction of the amount or availability of negatively charged membrane components, either on phospholipid or on LPS, with which the antibiotic may interact. The model presented here for protein H1 mediated resistance shares this property. In agreement with this, Vaara and co-workers (Vaara et al., 1979; Vaara, 1981) have reported a class of LPS mutants in Salmonella typhimurium (pmrA) which are polymyxin resistant and have LPS with reduced binding affinity for polymyxin. No information is available on the aminoglycoside resistance of these strains. Membrane changes similar to those reported in this study may also be responsible for the gentamicin resistance of clinical isolates of P. aeruginosa with reduced aminoglycoside transport reported by Bryan et al. (1976) and the adaptive resistance to gentamicin and EDTA reported by Pechey and James (1974).

Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Sr\textsuperscript{2+} were able to substitute for Mg\textsuperscript{2+} both in preventing H1 induction and in allowing susceptibility to these agents. These four cations showed very similar effects, implying that they are equivalent in their ability to regulate protein H1. Regulation of protein H1 production by cations could be mediated by a common receptor for these cations. Similar receptor specificity has been seen for the chemotaxis receptor for Mg\textsuperscript{2+} in S. typhimurium and E. coli (Koshland, 1979). Alternatively, the expression of H1 may be
sensitive to the total amounts of divalent cation in the outer membrane itself, or to divalent cations bound at specific sites in the outer membrane. Alteration of outer membrane protein composition in response to changes in other outer membrane components (Van Alphen et al., 1976; DiRienzo and Inouye, 1979) is known to occur in *E. coli*. The ability to respond to conditions at the outer membrane by alteration of outer membrane protein is also seen in the modulation of levels of major outer proteins of *E. coli* in response to an osmotic pressure gradient across the outer membrane (Kawaji et al., 1979).

As cell envelopes isolated from cells grown with divalent cations other than Mg$^{2+}$ contained some Mg$^{2+}$, we considered the possibility that the site of activity of aminoglycosides, polymyxins and EDTA could involve this small amount of Mg$^{2+}$ rather than a site occupied by other cations. The effect of EGTA on Ca$^{2+}$ grown cells, however, strongly suggested that Ca$^{2+}$ is replacing Mg$^{2+}$ at the target site. EGTA chelates Ca$^{2+}$ efficiently but Mg$^{2+}$ poorly ($K_{eff}$ 10.4 vs. 4.7, Roberts et al., 1970). Ca$^{2+}$ grown cells appeared as sensitive to the outer membrane effects of EGTA as they are to those of EDTA, as shown both by the direct measurement of lysis and by increase in the rate of hydrolysis of nitrocefin. EGTA, however, did not have the high bactericidal activity of EDTA, suggesting that additional sites are involved in the bactericidal action of EDTA. Other workers (Boggis et al., 1979) have noted that sensitivity to lysis by
EGTA is dependent on the amount of Ca\(^{2+}\) present in the growth medium, but that sensitivity to EGTA is decreased if Mg\(^{2+}\) as well as Ca\(^{2+}\) is present, suggesting that either cation may occupy the site attacked by the two chelators.

The number of cation binding sites involved in the action of EDTA, polymyxin and gentamicin on the outer membrane may well represent a small proportion of the total number of sites. Schindler and Osborn (1979) have demonstrated at least two classes of Ca\(^{2+}\) and Mg\(^{2+}\) binding sites on the lipopolysaccharide of *Salmonella typhimurium* with widely differing affinities, while only high affinity binding sites for polymyxin B were found. The involvement of a small number of sites is suggested by the displacement of only 3.5 to 10% of the cell envelope Mg\(^{2+}\) from cells treated with aminoglycosides or polymyxin B. A relatively small proportion of critical target sites for polymyxins, EDTA-Tris, and gentamicin, could also explain why cells grown in Zn\(^{2+}\) are not sensitive despite the high levels of Zn\(^{2+}\) in cell envelopes and the very high efficiency of Zn\(^{2+}\) chelation by EDTA. It was observed that Zn\(^{2+}\) grown cells had high levels of protein H1, and that their level of divalent cations was 15-35% lower than the level found in cells grown in Ca\(^{2+}\) or Mn\(^{2+}\) and 8% lower than cells grown in 0.5 mM Mg\(^{2+}\). It may be that only those divalent cation binding sites which are sensitive to attack by cationic antibiotics are protected by protein H1, and the remaining sites which can be occupied by Zn\(^{2+}\) (or indeed
any of the divalent cations studied) are not required for membrane stability. This would suggest that these critical divalent cation binding sites can only be occupied by specific divalent cations, i.e., Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, or Mn$^{2+}$. Alternatively, protein H1 could only displace divalent cations from these sites when induced by limitation of these divalent cations. Although there appears to be a linear reciprocal relationship between the decrease in Mg$^{2+}$ levels and increase in protein H1 in the cell envelope of wild type cells, it would appear that resistance to chelators and cationic antibiotics correlates more closely with the presence and amounts of protein H1 than with absolute levels of divalent cations in the cell envelope. This is supported by studies of the mutant strains resistant to these agents, which overproduce protein H1 to about 7-fold wild type levels in 0.5 mM Mg$^{2+}$ but show a reduction of less than 2-fold in their envelope Mg$^{2+}$ levels. Protection of a relatively small proportion of divalent cation binding sites would thus appear to be sufficient to confer resistance.

In summary, it is concluded that the outer membrane of *P. aeruginosa* is a major determinant of the antibiotic resistance of this bacterium. Two separate lines of evidence support this conclusion: 1) Alteration of the outer membrane, for example, by growth in low Mg$^{2+}$ or by mutation to H1 overproduction, alters resistance to cationic antibiotics and chelators. The data presented here suggest that these agents
are effective against *P. aeruginosa* because they are able to actively disrupt the outer membrane; and 2) The outer membrane of *P. aeruginosa* has been shown to be relatively impermeable despite the greater channel size of its porins, possibly due to there being a relatively small number of active, functional, channels. Previous studies with other bacteria have suggested two major pathways for antibiotic uptake across the outer membrane. These are the hydrophilic or porin-mediated pathway and the hydrophobic pathway which is apparently restricted to deep rough mutants of *E. coli* and *Salmonella*. The work described here strongly suggests the existence of a third major pathway in *P. aeruginosa*, self-promoted uptake in which polycationic antibiotics interact with and disrupt the outer membrane promoting uptake of further molecules of antibiotic.
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