

PRIMARY EFFECTS OF THE TETRACYCLINES ON
Pseudomonas aeruginosa

by

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ABSTRACT

M.Sc.

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Microbiology

PRIMARY EFFECTS OF THE TETRACYCLINES ON *Pseudomonas aeruginosa*

Pseudomonas aeruginosa grew in the presence of 10 µg/ml tetracycline (TC) or chlorotetracycline (CTC) in a minimal medium containing Mg⁺⁺. Growth is inhibited, with a six-fold increase in length of the lag phase. Cells revert to sensitivity when returned to antibiotic-free medium. Substitution of Mg⁺⁺ in the growth medium of CTC-resistant strains with Ca⁺⁺ and Sr⁺⁺ resulted in dramatic changes in growth and cell mass of cultures. Exposure of CTC-grown cells to EDTA did not result in cell lysis. SDS-PAGE of outer membrane proteins of resistant cells revealed loss of a protein band of molecular weight 73,500 D and the appearance of a 54,000 D protein band. Growth of cells resistant to CTC was hampered by subsequent exposure to penicillin G. Chelation of divalent cations from the outer membrane of sensitive cells leading to cell disruption is postulated as the primary mode of action of this antibiotic against *P. aeruginosa*.

RÉSUMÉ

M Sc.

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Microbiologie

LES EFFETS PRIMAIRES DES TETRACYCLINES SUR *Pseudomonas aeruginosa*

Dans un milieu minimal contenant de Mg^{++} , *Pseudomonas aeruginosa* s'est développée en présence de 10 $\mu g/ml$ tetracycline (TC) ou chlorotetracycline (CTC). Croissance est diminuée avec une augmentation par six fois de la période de latence. Les souches deviennent sensibles après avoir été retourné dans un milieu sans antibiotique. Le remplacement de Mg^{++} par Ca^{++} et Sr^{++} dans le milieu des souches résistantes à CTC a causé des changements dramatiques en croissance et les poids finals des souches dans ces cultures. L'addition de EDTA aux souches qui croissaient en présence de CTC n'a causé pas de dommage aux structures cellulaires. Une électrophorèse sur gels de sodium dodecyl sulfate - polyacrylamide des protéines de la membrane externe d'une souche résistante à CTC a révélé qu'une des protéines de poids moléculaire 73,500 D a été perdue, et une de poids moléculaire 54,000 D a été révélée. La croissance des souches résistantes à CTC a été diminuée après l'addition de pénicilline G. La chélation des cations bivalents de la membrane externe dans les souches sensibles causent la dislocation des souches est postulée comme la mode primaire de l'action de cette antibiotique contre *P. aeruginosa*.

DEDICATION

To my parents,
Johanne and David Sergeant,
who have shown me that it is almost possible to live on love alone
and
to Adam Steele,
with thanks for love, sustenance, and timing.

“Messieurs, c’est les microbes qui auront le dernier mot.”

Louis Pasteur

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative organism found ubiquitously in nature which can also persist as a pathogen in clinical settings, particularly in patients with cystic fibrosis or immunocompromised patients and burn victims. One of the prominent characteristics of this organism is its resistance to many different classes of antibiotics (Hancock, 1985). Elucidating the mechanisms of resistance in this organism has become one of the most active areas in modern molecular biology

Studies with different antibiotics have shown the many different mechanisms of resistance utilized by microorganisms in order to avoid cell death. It is generally believed that the area of primary resistance mechanisms in *P. aeruginosa* and other Gram-negative bacteria is the outer membrane of the cell wall (Nikaido and Vaara, 1985). This "permeability barrier" has been the object of much study, and its key components, lipopolysaccharide and proteins, have been implicated in many of the changes undergone by this cell layer in response to antibiotics (Lerve, 1974; Osborn and Wu, 1980).

The purpose of this study is to examine the effect of tetracycline on *P. aeruginosa* in order to determine a possible site of action of the antibiotic. As this field incorporates chemical, biophysical, and genetic parameters, an attempt has been made to simplify these approaches, so that resistance can be understood in terms of the simple principles of growth and survival of bacteria in a hostile environment.

LITERATURE REVIEW

ANTIMICROBIAL RESISTANCE

General Mechanisms of Resistance to Antibiotics

Bacterial resistance to antibiotics can be described in two general ways. Laboratory-derived "acquired" resistance usually involves a mechanism whereby a cellular component is altered and the antibiotic is unable to reach or react with its target site somewhere in the cell. This type of resistance is conditional on the local environmental conditions, for example, age of the bacteria, composition of the medium, and possible binding of the antibiotic to other components in the medium (Benveniste and Davies, 1973; Sabath, 1984). The other type of resistance is generally found in resistant bacteria isolated from clinical specimens. This usually involves a genetic component such as a plasmid, and results in chemical modification of the antibiotic leading to its inactivation (Benveniste and Davies, 1973). Plasmids can be transferred between bacteria through the process of transduction, via a bacterial virus (bacteriophage); by transformation, the incorporation of DNA found free in the environment, or by conjugation, the direct transfer of R ("resistance") factors between bacteria via a sex pilus. The existence of transposons, pieces of genetic material which can "jump" to different places in the bacterial genome, are important factors in the rapid and extensive transfer of resistance between bacteria by conjugation (Sande et al., 1990).

Luria and Delbruck (1943) examined the phenomenon of resistance using bacteria infected with bacteriophages. Each culture tested was

grown from a single bacterial cell, which was sensitive to virus infection. By mathematical analysis of the distribution of probabilities in subsequent generations of infected cells, they were able to show that the emergence of resistant cells in the culture was due to mutations occurring in sensitive cells, independent of their exposure to the virus. This refuted an earlier belief that resistance was due to "acquired hereditary immunity"; that is, resistance as a function of interaction with the virus, which was subsequently passed on to bacterial offspring. However, since the bacterial response to adverse conditions is often complex, mutations which are selected can be multiple and involve several genes (Demerec, 1948; Stahl, 1988).

The certainty of Luria and Delbruck's conclusions was challenged by a series of experiments considering the genetic variability of *E. coli* (Cairns et al., 1988). It is conceivable that bacteria can "direct" the expression of some, previously considered random, mutations, in response to specific growth conditions. The selection pressure favours mutants that can go on to multiply, but may also allow unmutated cells to survive and possibly respond also. This would allow cells to switch on mechanisms necessary to overcome certain environmental stresses and switch them off once the environment became more favourable. Such "inheritance of acquired characteristics" appears to be a feasible response to natural selection forces.

Laboratory-induced illustrations of the above can be taken to a higher degree, whereby organisms can be taken through a series of stronger selection pressures, and the ultimate survivors examined. An example of this is the stepwise increase in resistance as described by Demerec (1948). Strains isolated from exposure to sublethal

concentrations of penicillin were able to survive higher concentrations of the drug. This procedure could be repeated using survivors from each successive subculture, with the acquisition of resistance increasing at each step. It was also possible to collect highly resistant substrains in a single step by isolating survivors of very high concentrations of streptomycin. Examination of such substrains provides valuable insights into the nature of antibiotic resistance.

Stepwise Development of Resistance in *P. aeruginosa*

As described above, the concept that microorganisms can be "trained" to high levels of antibiotic resistance is not new (e.g. Demerec, 1948). *P. aeruginosa* has been shown to produce substrains resistant to high levels of chloramphenicol (Ingram and Hassan, 1975; Irvin and Ingram, 1980; Irvin and Ingram, 1982), polymyxin B (Brown and Watkins, 1970; Gilleland and Farley, 1982; Shand et al., 1988) several of the aminoglycosides (Galbraith et al., 1984; Daikos et al., 1990), and, more recently, some of the quinolones (Fernandes et al., 1987; Neu and Chin, 1987). While the organisms are initially inhibited by the antibiotic, an outgrowth of resistant cells appears after an extended period of incubation and these can be maintained on media containing high concentrations of the antibiotic. The fact that resistant strains of bacteria are able to develop in clinical cases upon prolonged exposure to antibiotic treatment is an important illustration of this.

When laboratory-produced resistant strains are placed in a medium devoid of the antibiotic they often revert to a level of sensitivity equal to or greater than that of the parent strain (Gilleland and Lyle, 1979; Gilleland and Farley, 1982; Shand et al., 1988). This behaviour is in direct

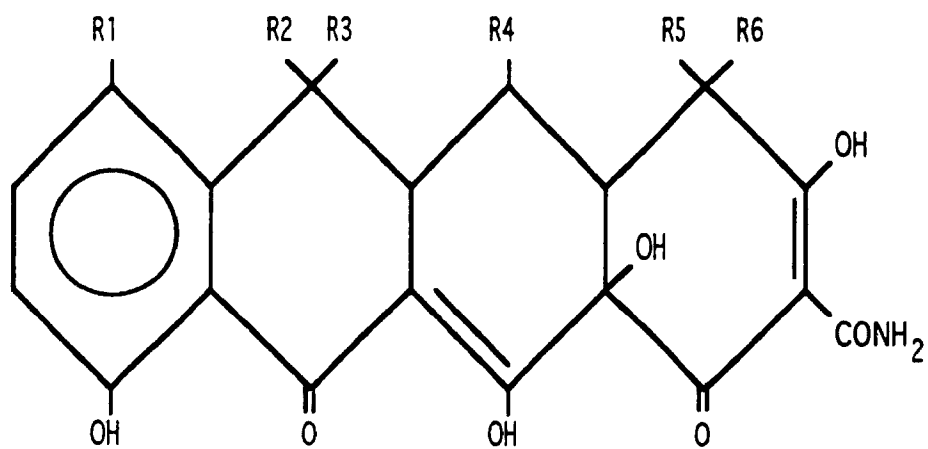
contrast to that of mutants which, by definition, are stable genetic changes which have been selected for by certain laboratory conditions. It is possible, however, to examine resistance to antibiotics in both laboratory-derived substrains and mutants isolated from clinical sources, as long as this distinction is kept in mind.

Bacterial Resistance to the Tetracyclines

The tetracyclines are broad-spectrum antibiotics of relatively low toxicity which have historically been put to a wide range of antibacterial uses. In clinical settings, they are often used when a complete diagnosis has not yet been made, then replaced with the drug of choice active against the organism(s) isolated. The first natural tetracycline to be put to clinical use was chlorotetracycline, in 1948, followed by oxytetracycline; tetracycline, minocycline, and several others are semisynthetic derivatives (Sande et al., 1990). The structures of tetracycline and the three analogues used in this study are given in Fig. 1.

The widespread use of tetracycline as a bactericidal agent has proven advantageous from an experimental point of view. Still, the precise mechanisms of its action are not well understood. *In vitro* investigations have concentrated on its action at the level of protein synthesis: tetracycline binds to bacterial ribosomes at a 1:1 molar ratio, namely to the 30 S subunit and prevents elongation of the nascent protein chain by disrupting codon-anticodon interaction (Chopra et al., 1981; Sande et al., 1990). Binding to the ribosome is almost completely reversible and inhibition of protein synthesis is discontinued when tetracycline is removed (Sande et al., 1990).

Figure 1: Structure of the tetracyclines.



	R1	R2	R3	R4	R5	R6
Tetracycline	H	OH	CH ₃	H	H	N(CH ₃) ₂
Oxytetracycline	H	OH	CH ₃	OH	H	N(CH ₃) ₂
Chlorotetracycline	Cl	OH	CH ₃	H	H	N(CH ₃) ₂
Minocycline	N(CH ₃) ₂	H	H	H	H	N(CH ₃) ₂

For tetracycline to exert its inhibitory effects at the ribosomal level, however, it must first gain entry to the cell. As will be discussed below, the Gram-negative cell wall possesses an outer membrane, which is an effective barrier against many toxic substances. The exact mode of passage of the tetracyclines through this membrane is still not clear. It is possible that anionic forms of the drug can bind with divalent cations such as magnesium, producing cationic chelates which then follow the Donnan equilibrium which exists across the outer membrane (Chopra and Howe, 1978). This mechanism may also play a role in the passage of tetracycline through Gram-positive cell walls (Dockter and Magnuson, 1974).

Once tetracycline reaches the cytoplasmic membrane, it is bound to the cell surface via an energy-independent step and is transported across by an active transport mechanism. This may involve a membrane-bound system, observable in laboratory-produced membrane vesicles of *E. coli* (McMurry et al., 1981); alternatively, it may be a system requiring periplasmic binding proteins (Chopra and Howe, 1978). In any event, resistance derives from a decreased uptake and accumulation of the antibiotic: this may involve decreased influx, increased efflux, or a combination of both (Chopra et al., 1981; Sande et al., 1990).

Thus, primary resistance to the action of tetracyclines in bacteria actually takes place at the cell wall level. This is borne out by the fact that while the tetracyclines are also active against host ribosomes, they are unable to enter mammalian cells to exert their action (Chopra and Howe, 1978; Sande et al., 1990). The unique characteristics of bacterial cell walls, most notably the outer membrane of Gram-negative bacteria are an important key to understanding bacterial resistance. Much recent work

has focussed on the nature of the outer membrane of *P. aeruginosa* and other Gram-negative bacteria and it is to this structure that we must now turn our attention

THE OUTER MEMBRANE OF *P. aeruginosa*

Structure of the Gram-negative Outer Membrane

Many Gram-negative bacteria are resistant to a wide range of antibiotics by which Gram-positive organisms are easily killed. This is attributable to the difference in architecture of the Gram-negative cell envelope, the outer membrane being the additional impermeability layer. It functions as the outermost boundary to the cell's external environment, existing outside both the peptidoglycan layer and the cytoplasmic membrane. The functions of the outer membrane are varied: it helps to provide mechanical stability and shock resistance for the cell; it plays a part in growth and division, as well as in defining the shape and size of the organism; is an important site for import and export of substances important to the cell, as well as for receptors and appendages; and it is largely responsible for the antigenic properties of the microorganism (Bayer, 1974). The structure of the Gram-negative outer membrane has been elucidated through a combination of electron microscopy, other high-resolution techniques and biochemical and immunological analysis. The outer membrane, like other biological membranes, is a lipid bilayer but is asymmetrically structured. Its outer leaflet consists of lipopolysaccharide (LPS), the structure of which is described lucidly by Bayer (1974) and Osborn and colleagues (1974) and which can be summarized as follows. LPS is an amphipathic molecule possessing a hydrophobic region projecting toward the internal portion of

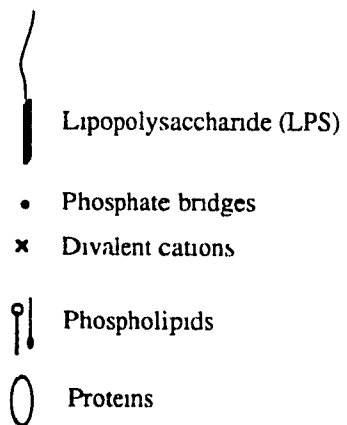
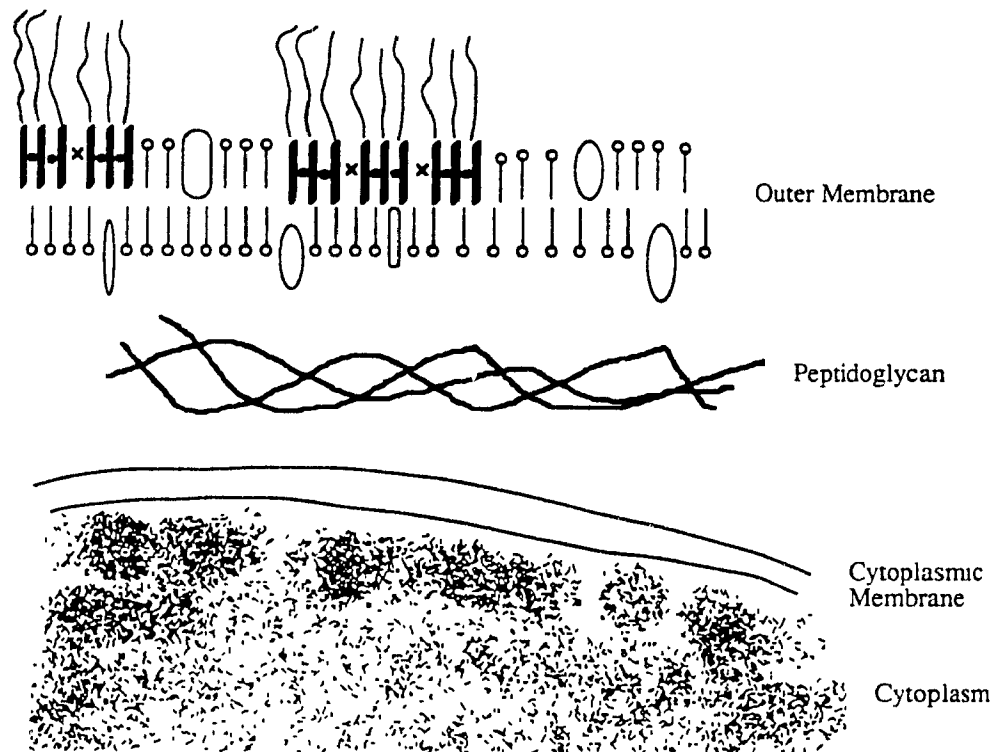
the membrane and a hydrophilic, negatively-charged region, facing the aqueous environment exterior to the cell. The hydrophobic core of the molecule is composed of Lipid A, or endotoxin, a glucosamine-containing lipid which anchors the molecule to the membrane and is covalently bound to the polysaccharide components. The next core component, the backbone, consists of two unique sugars, L-glycero-D-manno-heptose and 2-keto-3-deoxyoctonate (KDO) along with phosphate and ethanolamine. The outer core region is composed of a branched polysaccharide region containing glucose, galactose, and N-acetylglucosamine. The outermost end of the LPS molecule, the O-antigen, has a repeating oligosaccharide structure; it is these side chains which are responsible for strain-specific phenotypic characteristics in different bacteria such as serological antigenic specificity and antibiotic resistance. They are also the regions which have been shown to be nonessential to cell growth and function. Mutants deficient in these components, or rough mutants, are reduced in virulence but their growth and physiology under laboratory conditions is hardly altered. Deep rough mutant strains are blocked in synthesis of some of the backbone sugars and, while still viable, their outer membrane function is altered. These mutants have become extremely useful in that their increased sensitivity to antibiotics and other toxins can be readily studied *in vitro* (Osborn et al., 1974).

Studies by Leive (1974) on the effect of EDTA on the outer membrane of *P. aeruginosa* indicates that LPS is held in position by two mechanisms. Firstly, LPS molecules can interact strongly with each other and secondly, divalent cations play an important part in the binding of adjacent LPS molecules by ionic binding to their negatively-charged

phosphate moieties. The importance of divalent cations to the outer membrane, as well as the effects of EDTA on its integrity, will be discussed further in later sections.

The inner leaflet of the outer membrane is composed predominantly of glycerophospholipids, their acyl chains extending towards the interior of the membrane. Interspersed throughout the outer membrane are lipoproteins and other structural or enzymic glycoproteins; some of the lipoproteins are responsible for the attachment of the membrane to the cell's underlying peptidoglycan layer. LPS is also bound through hydrophobic and ionic interactions with these proteins (Osborn et al., 1974). Many different binding states between these molecules probably exist in an equilibrium as this, like other membranes, is a fluid medium through which molecules can move fairly easily (Singer and Nicholson, 1972). This may be important in the passage of low-molecular weight solutes or antibiotics, but more experiments are necessary to clarify the nature of the permeability of the outer membrane (Osborn et al., 1974). Assembly of the outer membrane originates with components synthesized within the cell which are secondarily translocated to the outer membrane and integrated into the membrane structure. This process allows for sites of interaction between the cytoplasmic membrane and the outer membrane, or zones of adhesion further illustrating that, for an understanding of many cellular processes, the entire cell envelope must also be considered. The above information has led to the postulation of a structure for the cell envelope of *P. aeruginosa* and other Gram-negative bacteria (Fig. 2). It must be kept in mind that, as new discoveries are made, this model is likely to change.

Figure 2: Postulated structure of the *P. aeruginosa* cell envelope.



Outer Membrane Proteins of *P. aeruginosa*

The proteins of the Gram-negative outer membrane have been extensively studied in *Escherichia coli* and *Salmonella typhimurium* (Osborn and Wu, 1980; Lugtenberg and Van Alphen, 1983, Nikaido and Vaara, 1985). Resolution of the outer membrane proteins of *P. aeruginosa* has not been reviewed in such detail and only a few different laboratories have concentrated their efforts on isolating individual protein components. Visualization of proteins and polypeptides in cellular extracts has been greatly facilitated by the technique of sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) by which these components are treated with a detergent (SDS) to give them a net negative charge, subsequently run in an electric field and the migration pattern characterized (Laemmli, 1970). The actual molecular weights of the proteins identified in the literature vary with the methods used to isolate them and with the concentrations of SDS and polyacrylamide used in the gel preparations (Stinnett and Eagon, 1973, Hancock and Nikaido, 1978).

Hancock and Nikaido (1978) perfected a method for the separation of the inner and outer membranes of *P. aeruginosa* strain PAO1 which did not require the use of ethylenediamine-tetraacetic acid (EDTA), which was used in earlier techniques and to which the organism is extremely sensitive. After sucrose density gradient centrifugation four separate membrane fractions were obtained, two of which were found to be virtually identical outer membrane fractions. Analysis of the OM fraction by SDS-PAGE showed four major protein bands with molecular weights of approximately 37,000 D, 35,000 D, 21,000 D, and 17,000 D (Hancock and Nikaido, 1978). Subsequent work with higher

concentrations of acrylamide in the gel systems provided better resolution of protein bands such that induction of different proteins under varying chemical conditions could be observed (Hancock and Carey, 1979). Proteins D1 and D2, with apparent molecular weights of 46,000 D and 45,500 D, respectively, were found to be glucose-induced and heat-modifiable. Protein E, observed at approximately 44,000 D, was partially induced in glucose-containing medium but was not heat-modifiable. Protein F, previously described as the major porin protein in *P. aeruginosa*, responsible for the passage of hydrophilic molecules such as sucrose through the outer membrane (Hancock et al., 1979), had an apparent molecular weight of 39,000 D, and was also heat-modifiable. Proteins G (25,000 D) and H2 (20,500 D) were also found to be heat-modifiable; whereas protein H1, at approximately 21,000 D, was not. Protein I, known to be Braun's lipoprotein, appeared at 9,000 to 12,000 D on these gels (Hancock and Carey, 1979). Several of these proteins were later determined to be noncovalently linked with the underlying peptidoglycan of the bacterium, namely proteins F, H2, and I (Hancock et al., 1981a).

Bacterial Resistance and the Outer Membrane

As stated earlier, the response of a microorganism to the presence of an antibiotic in the growth medium can be complex and take place at many different levels of cell function. While there is relatively little literature on the action of the tetracycline antibiotics on *P. aeruginosa*, many groups have examined the effects of other classes of antibiotics on this organism. Since the mechanisms of action of the antibiotics used

differ a great deal, results obtained with each class will be considered separately below.

Polymyxin B exerts its effect on *P. aeruginosa* by interacting with and disrupting the phospholipids of its membranes, causing leakage of cell contents, which leads to cell death (Sabath, 1984). The cell wall of a strain of *P. aeruginosa* trained to be resistant to 6000 units/ml polymyxin showed a 70 percent increase in readily-extractable lipid, a 75 percent decrease in phospholipid, and a 90 percent decrease in Mg^{++} from that of sensitive bacteria (Brown and Watkins, 1970). Chemical analysis of strains of *P. aeruginosa* resistant to different levels of polymyxin B revealed a decrease in the amount of carbohydrate and KDO in the LPS when compared with sensitive cells. An increase in cell wall total protein was observed but SDS-PAGE revealed a loss in concentrations of three specific proteins, with molecular weights of 47,000 D, 36,500 D, and 24,000 D which appeared to be responsible for polymyxin resistance (Gilleland and Lyle, 1979). Electron microscopy of the outer membrane of a *P. aeruginosa* strain adapted to growth on a high level of polymyxin B illustrated no significant changes upon further exposure to the drug (Gilleland and Farley, 1982). The isolated cytoplasmic membrane remained sensitive to the antibiotic, as did the outer membranes of both the wild-type parent strain and strains reverting to sensitivity.

Nicas and Hancock (1980) found that wild-type *P. aeruginosa* grown under conditions of Mg^{++} limitation overproduced protein H1. Two mutants resistant to polymyxin B also possessed high levels of this protein, which varied little in media of different Mg^{++} concentrations. The opposite was found in PAO1 adapted stepwise to resistance to 6000 units/ml of polymyxin B (Shand et al, 1988). Protein H1 was lost at an

early level of polymyxin treatment and proteins D, E, F, and G were reduced in quantity at subsequent levels. These characteristics were retained even when the medium was depleted of Mg^{++} and the resistant variants were grown in lower concentrations of the antibiotic.

The aminoglycoside antibiotics are large polycationic molecules which bind to negatively-charged portions of the core LPS in the outer membrane under certain conditions (Day, 1980). Exposure to the aminoglycoside gentamicin has a disruptive effect on the *P. aeruginosa* outer membrane. Examination of cells trained to a high level of gentamicin resistance indicated a distorted, undulating surface and loss of some sugars of the O-specific saccharide region of the LPS, giving it the appearance of a leaky rough mutant (Galbraith et al., 1984). Another study employing electron microscopy showed blebbing and wrinkling of the outer membrane after brief incubation with the antibiotic, followed by disruption of the peptidoglycan layer and cytoplasmic layer leading to cell lysis. Total protein and KDO were also lost from the outer membrane and a decrease in protein F was observed. In addition, a new protein band appeared just below the 20,100 D marker. Some Mg^{++} and Ca^{++} was also lost from the gentamicin-treated cells (Martin & Beveridge, 1986). Similar results were obtained after amikacin treatment of sensitive cells but the loss of protein was found to be uniform among all protein species. LPS was also removed and its electrophoretic profile revealed no preferential loss of any particular LPS species (Walker and Beveridge, 1987). Another aminoglycoside, tobramycin, also caused immediate damage to the *P. aeruginosa* cell wall, although this could be prevented by the presence of Mg^{++} in the medium. Material released from tobramycin-treated cells contained an increased amount of protein when

compared with unexposed cells. Specifically, a 29,000 D protein seen in SDS-PAGE profiles of this supernatant was identified as the enzyme β -lactamase, released from the periplasmic space (Raulston and Montie, 1989).

Work with resistance to the β -lactam antibiotics in *P. aeruginosa* has focussed mainly on the production of the enzyme β -lactamase or alterations in penicillin-binding proteins; these will not be covered here. Work with other outer membrane proteins resulting from β -lactam resistance is less prevalent. One group of researchers found additional outer membrane proteins in a carbenicillin-resistant clinical isolate when grown in both the presence and absence of 600 $\mu\text{g}/\text{ml}$ of the drug. These had apparent molecular weights of 53,000 D and 49,000 D; two bands with molecular weights between 10,000 D and 29,000 D observed in sensitive strains appeared in increased amounts in the resistant strain. Revertants regained the banding pattern of the sensitive strain (Pataryas et al., 1982). In a study of *P. cepacia*, a reduced amount of a 36,000 D protein and a loss of a 27,000 D protein in the outer membrane was noted in both a mutant strain and two clinical isolates (Aronoff, 1988).

A new category of β -lactams, the carbapenems, came into wide use during the last decade. One of these, imipenem, is a very broad-spectrum antibiotic initially described as being extremely effective against *P. aeruginosa* (Braveny, 1984). However, descriptions of strains resistant to imipenem quickly became prominent. While no induction of β -lactamase was observed, resistant strains showed a decrease in an outer membrane protein species with a molecular weight between 45,000 D and 49,000 D (Lynch et al., 1987; Buscher et al., 1987). This was also found in clinical isolates resistant to treatment with meropenem, another

carbapenem found to be effective on strains of *P. aeruginosa* resistant to imipenem (Margaret et al., 1989). This protein was later identified as the outer membrane protein D2 (Trias and Nikaido, 1990). Alterations in outer membranes were also observed in strains of *P. aeruginosa* resistant to two other classes of fairly new antibiotics: the quinolones, in which several protein bands were lost (Daikos et al., 1988; Chamberland et al., 1989; Yamano et al., 1990) and norfloxacin, in which a new protein band with a molecular weight between 50,000 D and 54,000 D was observed (Hirai et al., 1987; Fukuda et al., 1990).

The Importance of Divalent Cations to the Outer Membrane

The necessity of magnesium as a component of growth media for bacteria has been known for some time, the Mg^{++} -requirement for Gram-negative bacteria being approximately one-tenth that of Gram-positive bacteria (Webb, 1949). Lysis of *P. aeruginosa* by EDTA due to its chelation of divalent cations led to an understanding that cross-linking of certain components of the cell wall by divalent cations is essential in maintaining the integrity of the cell (Eagon and Carson, 1965). Chemical assays identified these cations as, in decreasing quantitative order, Mg^{++} , Ca^{++} , and Zn^{++} (Eagon et al., 1965) and these were implicated in the reaggregation of LPS components liberated upon EDTA treatment of *P. aeruginosa* (Asbell and Eagon, 1966a) and the restoration to osmotic stability of EDTA-induced osmoplasts (Asbell and Eagon, 1966b). It was later shown that *P. aeruginosa* grown in low concentrations of Mg^{++} became resistant to the action of EDTA (Brown and Melling, 1969a). The importance of Mg^{++} in the outer membranes was further illustrated by its necessity in the preparation of spheroplasts (osmotically-stable,

peptidoglycan-free cell preparations) of *P. aeruginosa*; previous use of EDTA in this procedure had been unsuccessful, resulting in cell lysis (Cheng et al., 1970a; Cheng et al., 1970b). Further, the LPS of *P. aeruginosa* has been found to change in composition in response to changes in the concentration of Mg^{++} in the growth medium (Day and Marceau-Day, 1982).

The role of divalent cations in the development of resistance in *P. aeruginosa* has also been examined. It appears that Mg^{++} can protect *P. aeruginosa* from the lytic effects of polymyxin, most likely by competing for anionic sites on the cells (Newton, 1953). Low concentrations of Mg^{++} in the growth medium prevented uptake of polymyxin thus increasing cellular resistance to the drug (Brown and Melling, 1969b). This was also shown in cultures of *P. cepacia*, another nosocomial pathogen, in its response to several antibacterial agents (Cozens and Brown, 1983). Mg^{++} -limitation was also effective in altering the cellular composition of *P. aeruginosa* and in decreasing its sensitivity to two chlorinated phenols (Gilbert and Brown, 1978). Chemostat-grown cells of *P. aeruginosa* limited in both glucose and Mg^{++} developed resistance to polymyxin B and EDTA (Finch and Brown, 1975). Kenward et al. (1979) also found that resistance to polymyxin B and EDTA was increased in *P. aeruginosa* grown in Mg^{++} -depleted medium and several components of the cell wall were altered.

Manipulation of the cationic content of growth media can also affect expression of outer membrane proteins in resistant cells. Overproduction of protein H1 corresponding with resistance to polymyxin B, EDTA and gentamicin occurred in *P. aeruginosa* grown in low concentrations of Mg^{++} and was prevented by supplementation with

higher concentrations of Mg^{++} and other divalent cations. These results illustrate that divalent cations occupy important sites in the outer membrane and that chelation by EDTA or competition for these sites by polymyxin and the aminoglycosides are important (Nicas and Hancock, 1980; Nicas and Hancock, 1983). However, other researchers found that Mg^{++} -limitation was not so critical in the expression of this protein (Shand et al., 1988).

The influence of magnesium on *P. aeruginosa* was, however, more pronounced when dealing with the aminoglycoside antibiotics. Gentamicin bound to the core region of LPS of a wild-type strain of *P. aeruginosa* in a partially ionic manner. Mg^{++} and Ca^{++} were able to interfere with this reaction, whereas monovalent cations did not (Day, 1980). Loss of Mg^{++} and Ca^{++} , as determined by atomic absorption spectroscopy, was observed in gentamicin-treated *P. aeruginosa* (Martin and Beveridge, 1986). The displacement of these ions from the outer membrane by gentamicin and amikacin appears to destabilize the cells, accounting for some of the ultrastructural changes observed (Martin and Beveridge, 1986; Walker and Beveridge, 1987).

Work with chloramphenicol resistance in *P. aeruginosa* under different growth conditions indicated that certain growth substrates acted as chelators of divalent cations and thus increased the sensitivity of cells to the drug (Irvin and Ingram, 1982). Manipulation of the Mg^{++} concentration in citrate medium indicated that cells resistant to a higher concentration of chloramphenicol had a higher requirement for Mg^{++} . Growth of wild-type cells was affected very little by variations in Mg^{++} concentration of the medium. Experiments with glucose-grown cells gave similar results. In addition, attempts were made to evaluate

whether other divalent cations could substitute for a percentage of the Mg^{++} requirement. Partial supplementation with Sr^{++} and Mn^{++} equalled the resistance levels of Mg^{++} alone, whereas Ca^{++} supplementation surpassed that of Mg^{++} . However, none of these cations can replace Mg^{++} as an essential growth factor in *P. aeruginosa*. Other cations, such as Zn^{++} , Be^{++} , and Ba^{++} , as well as the monovalent ions Na^+ and K^+ , were not able to substitute for Mg^{++} to any degree. Thus, divalent cations which are important in the structure of the *P. aeruginosa* cell wall appear to play a part in its ability to resist the deleterious effects of antibiotic action.

The Effect of EDTA on the Outer Membrane

EDTA has a bactericidal effect on *P. aeruginosa*. This characteristic was, at one time, suggested as a taxonomic tool for helping to identify organisms of this species (Wilkinson, 1966). Rapid lysis, as measured by a decrease in optical density at 660 nm, occurs in the organism when treated with EDTA alone, as opposed to the combination of EDTA, lysozyme and Tris responsible for lysis of other Gram-negative organisms. The use of EDTA was shown to enhance the action of lysozyme against Gram-negative organisms (Repaske, 1958). The presence of EDTA in culture media was also shown to increase the activity of polymyxin B and several quaternary ammonium detergents on *P. aeruginosa* (Brown and Richards, 1965, Voss, 1967). Previous growth of the cultures in medium containing either Mg^{++} or Ca^{++} corrected for this effect. Since these cations were found to interfere with the action of the above antibacterials on the cell wall, chelation of the cations with EDTA provided an effective means to enhance antibiotic

activity (Brown and Richards, 1965). Loss of viability due to leakage of cell contents caused by EDTA was thought to be due to the removal or displacement of metal cations in the cell wall of *P. aeruginosa* (Gray and Wilkinson, 1965a). This led to chemical analysis of the cell walls of EDTA-sensitive *P. aeruginosa* which suggested that LPS was released from the cells upon treatment with EDTA (Gray and Wilkinson, 1965b) possibly in the form of a complex with other cell wall components (Cox and Eagon, 1968). Fragments of released material were observed in electron micrographs of cells lysed with EDTA (Eagon and Carson, 1965).

As discussed in the previous section, the concentration of divalent cations in the growth medium can affect the activity of EDTA on *P. aeruginosa*. Resistance to EDTA was found to increase as the amount of Mg^{++} decreased (Brown and Melling, 1969a). The presence of Mg^{++} in the *P. aeruginosa* cell wall is an important consideration, since EDTA is a potent chelator of this cation. However, substitution of other divalent cations for Mg^{++} by the same researchers showed that Ca^{++} had the greatest effect in restoration of EDTA sensitivity with Zn^{++} having a lesser effect, and Be^{++} , Sr^{++} , and Ba^{++} no effect at all (Brown and Melling, 1969b). In another study, Ca^{++} was also shown to replace Mg^{++} and increase its sensitivity to EDTA, while Ba^{++} did not (Finch and Brown, 1975). Later work showed that Mn^{++} was able to perform this function to some degree as well (Kenward et al., 1979). Results with Ca^{++} , Mn^{++} , and Sr^{++} were corroborated by Nicas and Hancock (1983). Their use of EGTA, a chelator specific for Ca^{++} has been useful in examining the role of Ca^{++} in these systems, although EGTA does not have a bactericidal effect on *P. aeruginosa*.

Examination by electron microscopy of freeze-etched *P. aeruginosa* cells treated with EDTA-Tris in Mg^{++} -deficient and Mg^{++} -sufficient media revealed several ultrastructural changes which were compared with observed chemical and physical changes. EDTA-resistant cells showed a loss of phosphorus combined with an increase in total carbohydrate and KDO when compared with sensitive cells. In addition, a change in the conformation of cell wall proteins and their distribution between the inner and outer membranes occurred in resistant cells. SDS-PAGE of cell envelope preparations revealed the loss of a 100,000 D protein in these cells, as well as the appearance of a 50,000 D protein not present in cells grown under Mg^{++} -sufficient conditions (Gilleland et al., 1974).

A model system exists for the study of the effects of EDTA on *P. aeruginosa*, as well as certain synthesis processes. Addition of EDTA to cells in the presence of hypertonic sucrose results in the formation of osmotically stable rods (osmoplasts). These retain some of the properties of the original cells and can self-repair the outer membrane damage when incubated in sucrose and Mg^{++} as well as the protein-LPS complex released during EDTA treatment. These restored cells are no longer able to grow, confirming the toxic effects of EDTA on *P. aeruginosa* (Stinnett and Eagon, 1975). Work with EDTA and cell permeability has been well-reviewed by Leive (1974) but most of this was carried out using *E. coli*. As we have seen, the effect on *P. aeruginosa* is somewhat more pronounced. However, reports of the effect of EDTA on other Gram-negative bacteria have been extremely useful in defining the outer membrane permeability barrier implicated in the resistance of an organism such as *P. aeruginosa* to so many antibacterial agents.

Interaction Between the Tetracyclines and Divalent Cations

Evidence for the chelation of cations by the tetracyclines has been observed over many years in clinical settings: their absorption is impaired if ingested with dairy products, aluminum hydroxide, or salts of calcium, magnesium, iron, and bismuth, and administration of the antibiotic in children results in the formation of tetracycline-orthophosphate complexes in growing teeth and bones (Sande et al., 1990). *In vitro*, chlorotetracycline (CTC) has been shown to bind Mg^{++} , Ca^{++} , Sr^{++} , and Zn^{++} , resulting in enhanced fluorescence when located in biological tissues (Caswell and Hutchison, 1971a).

The uptake of the tetracyclines by bacterial cells probably involves two phases: adsorption to the surface followed by transport across membranes (Sande et al., 1990). Taking advantage of the chelating properties of CTC, a fluorescence technique was developed to follow the initial uptake stage (Dockter and Magnuson, 1974). These experiments were carried out using *Staphylococcus aureus*, but results were also found to hold for *E. coli*. The fluorescence of CTC was enhanced when the drug chelated membrane-bound cations such as Ca^{++} and Mg^{++} , and increased markedly when observed within an apolar environment such as within the membrane (Caswell and Hutchison, 1971b; Dockter and Magnuson, 1974). That CTC was able to bind with divalent cations of the outer membrane was confirmed by the ability of EDTA to quench the fluorescence at the initial stage of binding, since CTC is first bound to cations at the external surface of the cell. Further, exogenously added magnesium ions were found to compete with initial fluorescence

enhancement. The ability of the CTC-Mg⁺⁺ chelate to pass through the membrane was temperature-dependent: below a certain temperature, the membranes were found to become rigid and semi-crystalline, while above these temperatures, fluidity was restored, leading to increased lipid permeability (Dockter and Magnuson, 1974).

THE ROLE OF THE OUTER MEMBRANE AS A PERMEABILITY BARRIER

We have seen how experimental evidence has illustrated the importance of the outer membrane in Gram-negative bacteria. It is clear that this cell wall layer provides the first level of protection against deleterious substances in the extracellular environment. For example, the outer membrane can effectively slow down the diffusion of an antibiotic into the bacterial cell in order to give the cell a chance to inactivate the small amount entering at any given time. In this way, the organism will not be as easily overwhelmed by the large amount of a toxic substance present in the medium (Nikaido and Vaara, 1985). Thus, the identification and description of the outer membrane as a "permeability barrier" has become increasingly important in the literature.

As we have seen, the LPS molecule plays a very important role in the barrier function of the outer membrane. However, much of the literature has concentrated on describing the proteins found in this layer as key components in permeability. Those which do not appear to contribute to the shape-determining function of the outer membrane have been designated as "porins". These proteins exist in dimeric and trimeric forms in the outer membrane, and have been described as water-filled channels through which hydrophilic solutes may pass in order to

enter the internal cellular environment. Some porins appear to be specific for certain substances, since their sizes will allow only the passage of solutes of particular molecular weights, electrical charges, and hydrophobicities (Nikaido and Vaara, 1985).

It is felt that *P. aeruginosa* displays intrinsic resistance to many different antibiotics due to poor permeability of the outer membrane. Protein F has been described as the major outer membrane porin in this organism. While it is present in many copies in the cell, it has been postulated that only a very small percentage of these available porin channels are open at any given time (Hancock, 1984). The intrinsic resistance to any given solute can be measured as its rate of penetration, or the permeability coefficient, which allows for comparison of penetration of different substances. Other modes of penetration into cells are designated "non-porin" pathways (Nikaido, 1989).

Bacteria resistant to certain antibiotics often show changed permeability characteristics to other antibiotics (Gilleland and Farley, 1982; Shand et al., 1988). Thus, it has become important to define the nature of permeability of different types of antibiotics through the outer membrane of *P. aeruginosa*, in order to come up with a model that could explain the penetration of many different classes of molecules and shed more light on the structure of this cellular component.

SYNERGISTIC EFFECTS OF COMBINATIONS OF ANTIBIOTICS

Given that *P. aeruginosa* has been shown to develop resistance to many antibacterial agents, some at high levels and at a rapid rate, the possibility of combining antimicrobials must be considered. This has become an accepted practice *in vivo*, as clinical situations often warrant

the use of more than one antibiotic in the treatment of persistent infections or those for which more than one bacterium is responsible (Sande et al., 1990). However, the use of antibiotics in combination with other antibacterial agents *in vitro* in order to further understand their mechanisms of action on *P. aeruginosa* has remained rather limited to date.

Since the mechanisms of action of different classes of antibiotics vary, combinations of two of them may have an additive or an antagonistic effect. Depending on the specific mode of action of each drug, bactericidal and bacteriostatic drugs may or may not be used in combination. In terms of antibiotics which act on the bacterial cell wall, the action of one may facilitate the uptake of the second (Sande et al., 1990). With respect to development of resistance, a combination of the possible frequencies of mutations conferring resistance to each drug could give an indication of the decreased likelihood that a microorganism would exhibit resistance to both (Demerec, 1948).

Most strains of *P. aeruginosa* are susceptible *in vitro* to the synergistic combination of a broad-spectrum antipseudomonal penicillin plus an aminoglycoside; alternative drug choices include aztreonam or cephalosporins (Sande et al., 1990). The mechanism of action of combinations of carbenicillin-gentamicin and moxalactam-tobramycin were examined by Scudamore and Goldner (1982). Synergism was observed at all concentration ratios tested, but the addition of EDTA did not increase the synergistic effect, except at a 16:1 ratio of carbenicillin to gentamicin. This indicates that the synergistic effect of these two antibiotics is not due to one promoting the entry of the other through the outer membrane.

As microorganisms such as *P. aeruginosa* become resistant to more of the standard antibiotics in use, many new agents (especially the β -lactams and cephalosporins) or combinations of agents are being investigated. Examples of these include Timentin, a combination of ticarcillin, a β -lactam antibiotic, and clavulanic acid, which was found to be more effective than piperacillin alone in treating urinary tract infections and osteomyelitis caused by several different Gram-negative bacteria (File et al, 1985; Gentry et al., 1985). Certain peptides, such as polymyxin B nonapeptide (PMBN), have been examined with regard to their role in the disruption of the Gram-negative outer membrane, either alone (Vaara and Viljanen, 1985; Viljanen et al., 1988) or in combination with other antibiotics (Lam et al., 1986). Another group of polycationic peptides, called defensins, has been isolated from leukocytes; these also act in concert with some antibacterials against *P. aeruginosa*, but to a lesser degree than PMBN (Viljanen et al., 1988). The activity of some antipseudomonal agents was also increased using sodium hexametaphosphate (Vaara and Jaakkola, 1989), as well as Lys-5, a polymer of the amino acid lysine (Vaara, 1990). Many other chemical agents have been assessed for their ability to assist standard antibiotics in their antibacterial action against *P. aeruginosa*. Since many of these appear to enhance the permeability of the outer membrane, they have been named "permeabilizers" (Hancock and Wong, 1984). Given the extent to which these substances have been used medically and the wide range of chemical activities among them, the search for other antimicrobial combinations for use in the future appears almost limitless.

MATERIALS AND METHODS

BACTERIAL STRAINS

Stock cultures of *P. aeruginosa* strains PAO1 (Holloway, 1969) and ATCC 9027 were maintained on Pseudomonas Agar P (Difco) slants at room temperature and transferred every 2 to 4 weeks. Stock cultures were periodically checked for purity and colony morphology on plates of Pseudomonas Agar P.

STEPWISE DEVELOPMENT OF RESISTANCE TO TETRACYCLINE (TC)

A chemically defined medium (Eagon and Phibbs, 1971) was used in order to provide a uniform growth medium in which the organisms could be subjected to increasing concentrations of tetracycline (Sigma Chemicals, St. Louis, Missouri). *P. aeruginosa* PAO1 and 9027 were each transferred from stock slants to Eagon-Phibbs slants devoid of TC and incubated at 37 C for 24 h. Subsequent transfers to TC-containing slants were incubated overnight at 37 C followed by an additional 24 h at room temperature. For efficient stepwise transfer, intermediate concentrations of TC were necessary: the method of transfer being from 0 µg/ml TC to 5 µg/ml, the passing through 10, 20, 30, 50, 100, up to 150 µg/ml. Test strains (10, 50, 100 and 150 µg/ml) were maintained by transfer every 2 weeks onto an Eagon-Phibbs slant containing the corresponding concentration of TC.

LIQUID MEDIA

A chemically defined medium (Eagon and Phibbs, 1971) was used in order to examine changes in growth characteristics with varying cation

and antibiotic concentrations. Glucose was used as the carbon source, at a concentration of 5 g/L, and was added after autoclaving the basal salts. Stock solutions of divalent cations were autoclaved separately and added to the flasks prior to inoculation. Stock solutions of the tetracyclines were prepared (10 or 20 mg/mL), filter-sterilized, and added to the flasks after the addition of divalent cations, immediately before inoculation. Oxytetracycline and minocycline were from Sigma Chemicals, St. Louis, Missouri, and chlorotetracycline was from ICN Biochemicals, Cleveland, Ohio.

All media were prepared using glass-distilled water.

GROWTH CONDITIONS

The organisms were grown at 37°C with constant agitation (250 rpm) in a Psycrotherm Incubator Shaker (New Brunswick Scientific Instruments, New Brunswick, New Jersey). Growth was measured as an increase in optical density at 660 nm, measured in cuvettes of 1 cm light path, on a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

"Starter" cultures were prepared in order to standardize inoculum sizes for growth determinations. Starters consisted of a generous inoculum from a stock slant added to 50 mL medium in a 250 mL Erlenmeyer flask. Aliquots were taken from cultures having reached an optical density at 660 nm of 0.10 to 0.30 (mid-exponential growth) and transferred to 100 mL of medium in a 500 mL Erlenmeyer flask to equal 0.5% inoculum. The ratio of liquid to total flask volume was kept constant at 1:5.

CHLOROTETRACYCLINE INACTIVATION

Two sets of test flasks were prepared: one set (A) by adding glucose, MgSO_4 (400 μM) and 10 $\mu\text{g}/\text{mL}$ chlorotetracycline (CTC) to basal salts medium, and the other set (B) by adding glucose and 10 $\mu\text{g}/\text{mL}$ CTC but withholding the MgSO_4 . Both sets were divided into two further groups: those incubated at 4 C and those at 37 C. Within each of these groups, half of the flasks were left exposed to light and the other half kept in darkness. All flasks were left uninoculated for 24 h, at which point MgSO_4 was added to set B and all flasks inoculated with a mid-exponential culture of cells grown in the presence of 10 $\mu\text{g}/\text{mL}$ CTC. Growth was monitored as described in the previous section.

EFFECT OF EDTA ON PAO1

Starter cultures containing either no CTC or 10 $\mu\text{g}/\text{ml}$ CTC were prepared as described above and inoculated with PAO1 from a stock slant. Once starters had reached mid-exponential growth, a 0.25-ml aliquot was transferred to 50 ml Eagon-Phibbs medium devoid of CTC. Cultures were once again allowed to reach mid-log phase, at which point they were harvested by centrifugation at $10,000 \times g$ for 10 min. The pellets were resuspended into 50 ml of Eagon-Phibbs medium without MgSO_4 to which either EDTA at a concentration of 10 μM or 50 μM or 10 $\mu\text{g}/\text{ml}$ CTC had been added. Growth measurements were taken over 24 h as described previously.

OUTER MEMBRANE PROTEINS

Separation of the inner and outer membranes of *P. aeruginosa* strain PAO1 was carried out using the method of Hancock and Nikaido

(1978). Both wild type and CTC-treated cells were treated identically. Three litres of cells were harvested at late exponential phase by centrifugation at $12,000 \times g$ for 10 minutes. Cell preparations were kept at 4 C for this and all subsequent stages of the procedure. The pellet was washed once using one tenth of the original culture volume of 30 mM tris (hydroxymethyl)aminomethane-hydrochloride (Tris buffer) at pH 8.0. The washed pellet was resuspended in 15 mL of 20% (w/v) sucrose in Tris buffer, which also contained 1 mg each of deoxyribonuclease and ribonuclease. This suspension was homogenized in a glass homogenizer and passed three times through a chilled French pressure cell held at a steady pressure of 15,000 psi. The lysate was incubated for 10 min in the presence of 0.5 mL lysozyme (1 mg/mL) and then centrifuged at $1000 \times g$ for 10 minutes to remove cell debris and unlysed cells. Tris buffer was added to dilute the supernatant to 12 mL.

Six mL were layered onto a sucrose step gradient consisting of 1 mL of 70% (w/v) sucrose and 5 mL of 15% (w/v) sucrose in Tris buffer. The tubes were centrifuged for 1 h at 39,000 rpm in a Beckman SW40 rotor (Beckman Instruments, Palo Alto, California). The bottom 2 mL was removed from each gradient and further applied to a density gradient consisting of 1 mL 70% (w/v), 3 mL 64% (w/v), 3 mL 58% (w/v), and 3 mL 52% (w/v) sucrose in Tris buffer. These tubes were centrifuged at 39,000 rpm in the SW40 rotor for 14 hours. The uppermost layer was removed using a Pasteur pipette and discarded. The bottom layer (outer membrane fraction) was diluted in distilled water and washed twice at 45,000 rpm in a Beckman T_i 55.2 rotor for 30 minutes. The pellets were resuspended in a small amount of distilled water and stored at -20 C.

12% SLAB SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis of outer membrane proteins was carried out on a two-level slab gel system (Protean II, Bio-Rad Inc., Richmond, California). The upper, stacking, gel was made up of 4% w/v acrylamide /N'N'-Bis-methylene-acrylamide (Bis), 2.5 mL 0.5 M Tris-HCl at pH 6.8, and 100 μ L 10% (w/v) SDS. Polymerization was initiated by adding 50 μ L 10% ammonium persulfate and 10 mL N,N,N,N'-tetramethylethylenediamine (TEMED). The lower, running, gel consisted of 12% (w/v) acrylamide/Bis, 12.5 mL 1.5 M Tris-HCl at pH 8.8, and 0.5 mL 10% SDS. To polymerize the gel, 250 μ L 10% ammonium persulfate and 25 μ L TEMED were added.

Electrode buffer

Once polymerized, the gels were immersed in electrode buffer consisting of 3 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS.

Digestion buffer

Outer membrane proteins were digested in a buffer composed of 0.5 M Tris HCl at pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 5% 2- β -mercaptoethanol, and 0.05% (w/v) bromophenol blue. A ratio of 1:1 of sample protein and digestion buffer was used and the mixture was heated at 95 C for 5 min in a Temp-block module heater (CANLAB), then cooled to 4 C. Once digested, the protein samples were loaded onto the stacking gel immersed in electrode buffer. A 50 μ L Hamilton syringe was used to deliver 20 μ L of sample mixture to each well, which corresponded to a concentration of 50 μ g of protein.

Protein standards

A mixture of protein standards (Bio-Rad) was treated identically to the outer membrane fractions and electrophoresed concurrently. This consisted of myosin (200,000 D), *E. coli* β -galactosidase (116,250 D), rabbit muscle phosphorylase b (97,400 D), bovine serum albumin (66,200 D), hen egg white ovalbumin (45,000 D), bovine carbonic anhydrase (31,000 D), soybean trypsin inhibitor (21,500 D), and hen egg white lysozyme (14,400 D). These were used to determine apparent molecular weights of the outer membrane proteins.

Electrophoresis conditions

Electrophoresis was carried out at a constant voltage of 400 V (25 ma) for 12 to 15 h, or until the tracking dye had run off the bottom of the gel. The temperature of the electrode buffer and gel was maintained at 15 C.

Fixing and staining of electrophoresed proteins

Once electrophoresis was completed, gels were separated from the gel apparatus and placed in a mixture of 10% acetic acid and 30% methanol at 65 C for 1 h. This acts as a protein fixative while also removing SDS, which would interfere with staining. Gel proteins were stained in 0.1% Coomassie blue in 10% acetic acid/30% methanol for 1 h at 65 C. Destaining was carried out in 10% acetic acid at 65 C for 2 h, using a synthetic sponge to absorb excess dye.

GROWTH OF PAO1 IN THE PRESENCE OF CHLOROTETRACYCLINE
AND PENICILLIN G

A starter culture of PAO1 was prepared and mid-exponential aliquots taken to inoculate 4 flasks each containing 100 mL Eagon-Phibbs medium, in the same manner as described previously. When growth in each flask reached mid-exponential phase, cultures were centrifuged at $10,000 \times g$ for 10 minutes and resuspended in 100 mL of the same medium to which $10 \mu\text{g/mL}$ CTC had been added. After 2 h (approximately one doubling), penicillin G was added at a concentration of $100 \mu\text{g/mL}$ to one-half of the flasks. Optical density readings of all flasks were taken at 660 nm, over a period of 24 h.

RESULTS

GROWTH OF *P. aeruginosa* ON SOLID MEDIA

Cultures of *P. aeruginosa* ATCC 9027 on Pseudomonas agar P slants achieved maximum growth after 48 h at room temperature. A greenish-blue pigment (pyocyanin) was evident, permeating the bottom half of the slant, and cultures had a distinct musty odour. Strain PAO1 also grew to confluence after 48 h under the same conditions. A pale green pigment was produced, and a slightly sweet odour was characteristic of these cultures.

STEPWISE ACQUISITION OF RESISTANCE IN *P. aeruginosa*

Cultures of *P. aeruginosa* ATCC 9027 and PAO1 were "trained" to grow on concentrations of up to 150 µg/ml TC in minimal media slants. The chemically-defined medium of Eagon and Phibbs (1971) was chosen in order to fulfill minimum requirements for growth of *P. aeruginosa*. Glucose was added as the sole carbon source and magnesium, for which the organism has an absolute dependence, was provided.

The organisms grew best at each step after initial incubation overnight at 37 C followed by an additional 24 h at room temperature. Storage at room temperature had no further effect on culture characteristics. Both strains lost pigment production upon growth in TC-containing media and characteristic odours were diminished. TC-resistant substrains were characteristically mucoid, adhering to the transfer loop and making manipulation of cultures more difficult.

EFFECT OF THE TETRACYCLINES ON *P. aeruginosa*

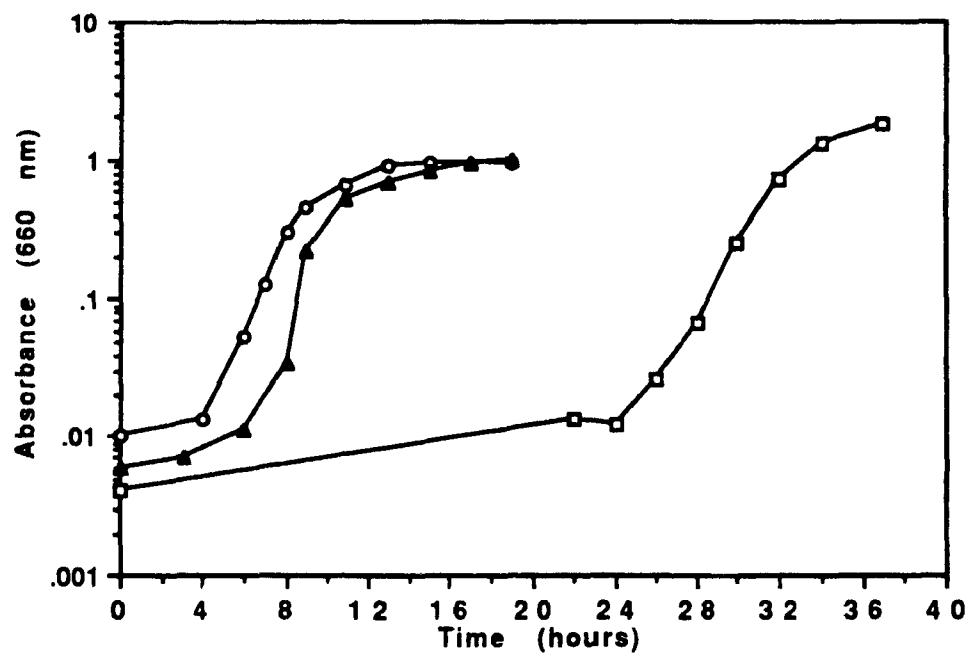
In view of the fact that stepwise acquisition of resistance yields substrains able to grow normally on subsequent exposure to the same concentrations of tetracycline, characterization of the growth of each individual substrain was attempted. Initial experiments were carried out using TC; results are shown in Appendix I. The protocol for growing these cultures varied somewhat from that given for CTC in Materials and Methods. Here, each test flask was inoculated with a "trained" substrain from the Eagon-Phibbs slant containing the corresponding concentration of TC. Starter cultures were grown to stationary phase, so cells used for inoculating media for growth determinations were older. Total volume of culture was 150 ml in a 500-ml Erlenmyer flask, giving a liquid to flask volume ratio of slightly more than 1:3. Thus, these results cannot legitimately be compared with those shown below.

Figure 3 shows the growth of PAO1 in Eagon-Phibbs medium supplemented with glucose and 400 μM Mg^{++} . Wild-type cells exhibit a lag phase of approximately 4 h before undergoing exponential growth. The stationary phase is reached after approximately 12 h. Cells transferred from a starter culture containing 10 $\mu\text{g}/\text{ml}$ of CTC into growth-determination medium of the same composition (designated RT) show approximately a six-fold increase in the length of the lag phase. The exponential phase of growth is also extended, with a more gradual transition into stationary growth. At this point, growth continues, although no longer at an exponential rate, suggesting that the stationary phase *per se* is not achieved by 36 h. The difference in absorbance at the end of the exponential phase in resistant versus wild-type cells indicates

Figure 3

Fig. 3. Growth of PAO1 in minimal medium.

Symbols: (●) Wild type (WT)
 (◻) Resistant to 10 μ g/ml CTC (RT)
 (▲) Reverted to sensitivity in absence of CTC (RS)



an increase in cell mass for cells resistant to CTC.

In order to determine whether the effect of CTC on PAO1 is permanent, cells exposed to one growth cycle in the presence of the drug and transferred to growth-determination medium without CTC were examined, and the results are also shown in Fig. 3. While reversion to sensitivity appears to occur, it is not complete. When compared with wild-type, untreated cells, the lag phase of the revertants (RS) is slightly extended and the transition to stationary phase is more gradual. It is possible that these differences are due to residual CTC remaining bound to the cells. Regardless of the quantity of residual CTC, it should be acknowledged that the drug affects the organism by delaying, but not entirely hampering, its growth.

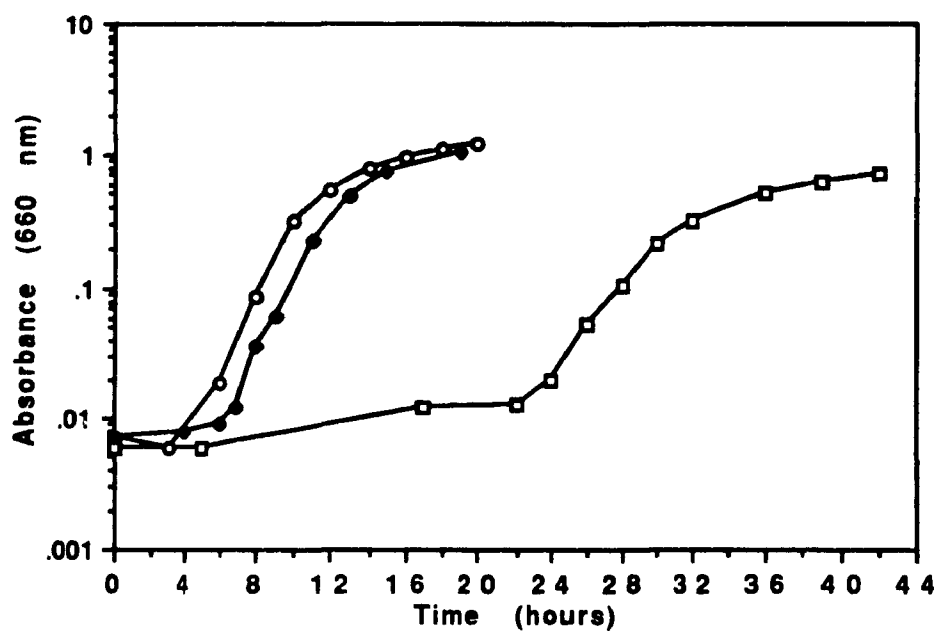
Growth of *P. aeruginosa* 9027 is shown in Fig. 4. While sensitive and revertant cells show an almost identical pattern to that of strain PAO1, the effect of CTC on 9027 is more pronounced. While the length of the lag phase is extended to the same degree as for PAO1, in 9027 logarithmic growth is not achieved. Therefore, CTC more effectively inhibits the growth of 9027.

An attempt was made to use tetracyclines of various hydrophobicities in order to examine possible alternate mechanisms of resistance in *P. aeruginosa* (Leive et al., 1984; Sompolinsky and Krausz, 1973). Preliminary experiments undertaken here showed that the growth of both 9027 and PAO1 exposed to oxytetracycline in starter cultures paralleled that seen in experiments using TC. Both of these forms of tetracycline are light-and heat-sensitive, making their use in experiments

Figure 4

Fig. 4. Growth of ATCC 9027 in minimal medium.

Symbols: (o) Wild type (WT)
 (□) Resistant to 10 μ g/ml CTC (RT)
 (◇) Reverted to sensitivity in absence of CTC (RS)



requiring much manipulation more difficult. Minocycline proved to be insoluble in water, requiring preparation using methanol; since the experiments described herein utilize aqueous media and both strains of *P. aeruginosa* grew extremely slowly in the presence of minocycline, work with this analog was discontinued. Growth characteristics of both 9027 and PAO1 were more reliably reproduced when CTC was used; in view of its stability and chelating properties (to be discussed later), it was used in all subsequent experiments.

Due to the fact that *P. aeruginosa* strain 9027 did not exhibit characteristic logarithmic growth upon exposure to CTC, combined with the fact that PAO1 displayed much more versatility in its response to different growth conditions, strain PAO1 was used exclusively throughout the rest of this study.

CHLOROTETRACYCLINE INACTIVATION

To determine whether differences in growth rates of CTC-treated cultures were due to inactivation of the drug by exposure to heat or light, or by chelation of CTC upon exposure to Mg^{++} , an experiment was designed to test each of these growth parameters as a variable. From Table 1 it appears that uninoculated media kept at 4 C do not lose any CTC activity since exponential growth of PAO1 begins following a 24-h lag period after inoculation. This is analagous to the growth observed in cultures inoculated immediately after the media are prepared. However, preincubation of uninoculated cultures at 37 C results in growth of CTC-treated cells after a lag lasting between 6 and 12 h, indicating a loss of

Table 1. Effect of incubation conditions on CTC.

37 C					4 C			
Light			Dark		Light		Dark	
Normal		Mg ⁺⁺ late	Normal	Mg ⁺⁺ late	Normal	Mg ⁺⁺ late	Normal	Mg ⁺⁺ late
Growth								
6 h	-	-	-	-	-	-	-	-
12 h	+	+	+	+	-	-	-	-
20 h	+	+	+	+	-	-	-	-
24 h	+	+	+	+	+	+	+	+

Cultures were prepared as described in Materials and Methods. CTC was added with MgSO₄ (normal) and without MgSO₄ (Mg⁺⁺ late) and stored uninoculated for 24 h. Inoculation took place at 0 h.

+ = growth (turbidity) observed in flask

- = no growth observed in flask

activity of the drug upon exposure to incubation temperature. In addition, CTC does not appear to be light-sensitive.

SUBSTITUTION OF MAGNESIUM WITH OTHER DIVALENT CATIONS

Irvin (Ph.D. thesis, 1984) observed that strains of *P. aeruginosa* resistant to chloramphenicol have a greatly increased requirement for Mg^{++} in order to grow optimally. It is possible to manipulate the concentration of Mg^{++} and other divalent cations in minimal medium in order to emphasize the relative responses of the organism to changes in availability of these ions. Cells of Pu 21 resistant to chloramphenicol were found to be completely inhibited in minimal medium containing glucose and 10 μM Mg^{++} . However, the addition of other cations, such as Ca^{++} , Sr^{++} , and Mn^{++} , at a concentration of 90 μM were shown to spare the organism's requirement for Mg^{++} and were thus compared to its growth in 100 μM Mg^{++} .

In wild-type PAO1, growth in Eagon-Phibbs minimal medium is significantly decreased in the presence of 10 μM Mg^{++} compared with that in 100 μM Mg^{++} (Fig. 5). Supplementation of medium containing 10 μM Mg^{++} with 90 μM Ca^{++} appears to "correct" for the deficiency in Mg^{++} , and growth under these conditions slightly surpasses that in 100 μM Mg^{++} . While the Ca^{++} -supplemented culture reaches stationary phase sooner, final cell densities in the presence of Ca^{++} are not as great.

Substitution of Ca^{++} with Sr^{++} , however, results in a decrease in the growth rate of the culture, with growth characteristics similar to those of cultures grown in 10 μM Mg^{++} alone. Thus, it appears that Ca^{++} is able to effectively substitute for Mg^{++} in the cell wall of the organism.

Figure 5

Fig. 5. Divalent cation substitution in wild type PAO1.

Symbols: (□) 100 μM Mg^{++}
(●) 90 μM Ca^{++} plus 10 μM Mg^{++}
(◇) 90 μM Sr^{++} plus 10 μM Mg^{++}
(▣) 10 μM Mg^{++}

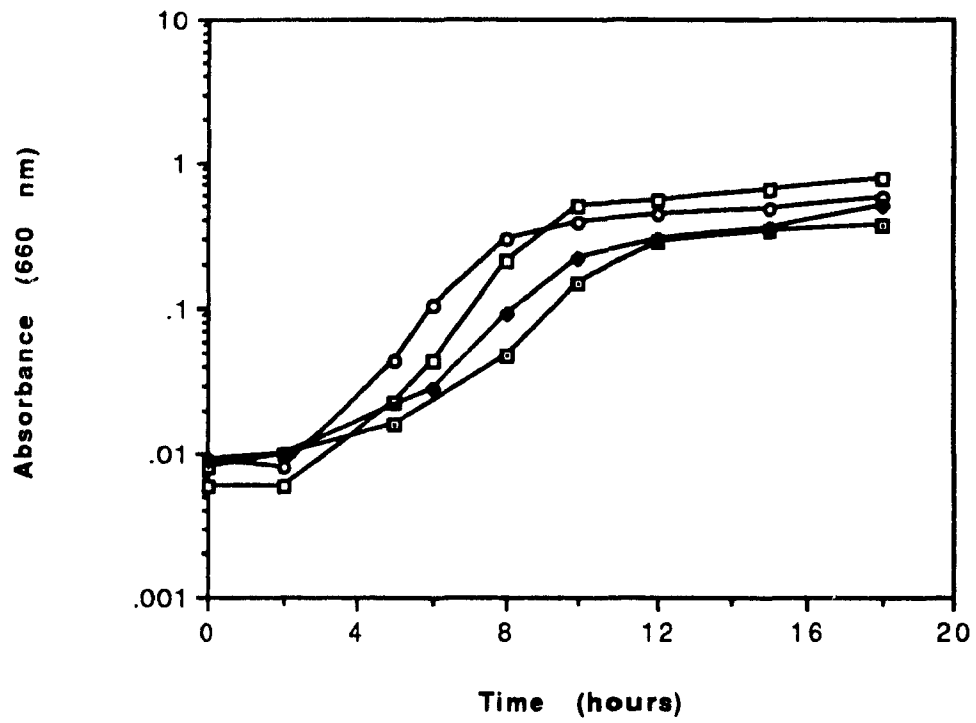


Figure 6

Fig 6. Elimination of Mg^{++} from medium for wild type PAO1.

Symbols: (■) no Mg^{++}
(●) 100 μM Ca^{++}
(◆) 100 μM Sr^{++}

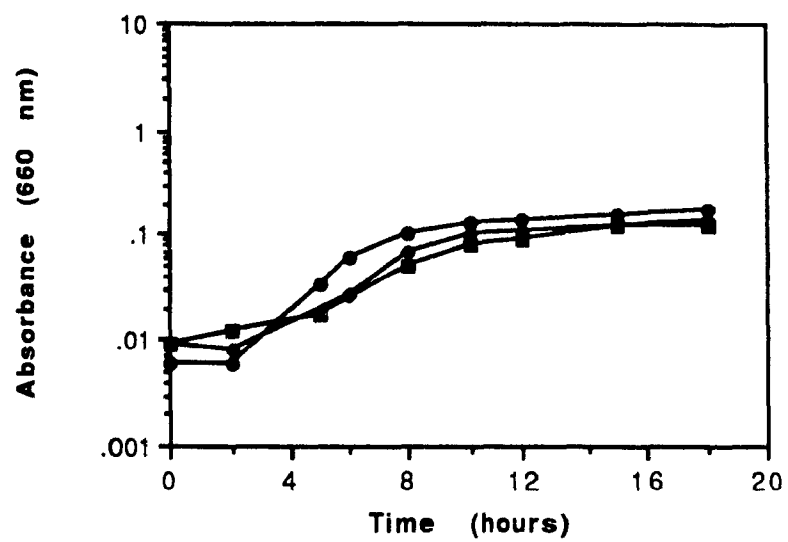
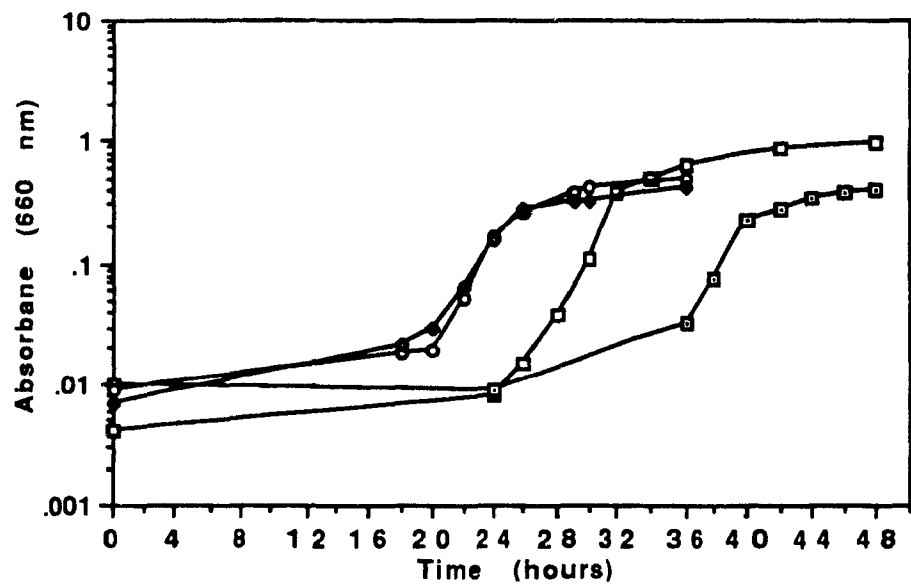


Figure 7

Fig. 7. Divalent cation substitution in CTC-resistant cells (RT)

Symbols: (▣) 100 μM Mg^{++}
 (●) 90 μM Ca^{++} plus 10 μM Mg^{++}
 (◇) 90 μM Sr^{++} plus 10 μM Mg^{++}
 (⊖) 10 μM Mg^{++}



In addition, growth of PAO1 in the absence of any Mg^{++} was examined in order to verify the organism's absolute requirement for this ion (Fig. 6). Growth proceeds at a much slower rate than in a medium containing $100\ \mu M\ Mg^{++}$ whereas the addition of Ca^{++} and Sr^{++} at a concentration of $100\ \mu M$ improved the growth rate

In contrast, the effect of divalent cation manipulation in CTC-treated cells is more dramatic (Fig. 7). Reduction of Mg^{++} to a concentration of $10\ \mu M$ increases the length of the lag phase beyond the 24-h lag observed in cultures exposed to $10\ \mu g/ml$ CTC in the presence of $100\ \mu M\ Mg^{++}$. Final cell densities in $10\ \mu M\ Mg^{++}$ are reduced by more than half. Addition of either Ca^{++} or Sr^{++} at a concentration of $90\ \mu M$ reduces the length of the lag phase to approximately 20 h but final culture densities at 36 h are considerably lower than those of cells grown in $100\ \mu M\ Mg^{++}$ at 48 h (a similar point in the growth pattern, given this "shift to the left"). This effect is also marked when comparing CTC-treated PAO1 grown in the absence of Mg^{++} with cultures supplemented with $100\ \mu M$ of either Ca^{++} or Sr^{++} (Fig. 8). Final cell densities are similar but Ca^{++} and Sr^{++} are again able to reduce the length of the lag phase appreciably (here, from 36 to approximately 20 hours).

Revertant cultures show changes in growth patterns closer to those of wild-type cells than CTC-treated cells (Figs. 9 and 10). Here again, Ca^{++} is the divalent cation to which the organism responds most effectively. As before, cells are able to grow in the absence of Mg^{++} with $100\ \mu M\ Ca^{++}$ improving the growth rate more than that observed with Sr^{++} substitution or the absence of Mg^{++} altogether.

Figure 8

Fig 8. Elimination of Mg^{++} from medium for CTC-resistant cells (RT)

Symbols: (■) no Mg^{++}
(●) 100 μM Ca^{++}
(◆) 100 μM Sr^{++}

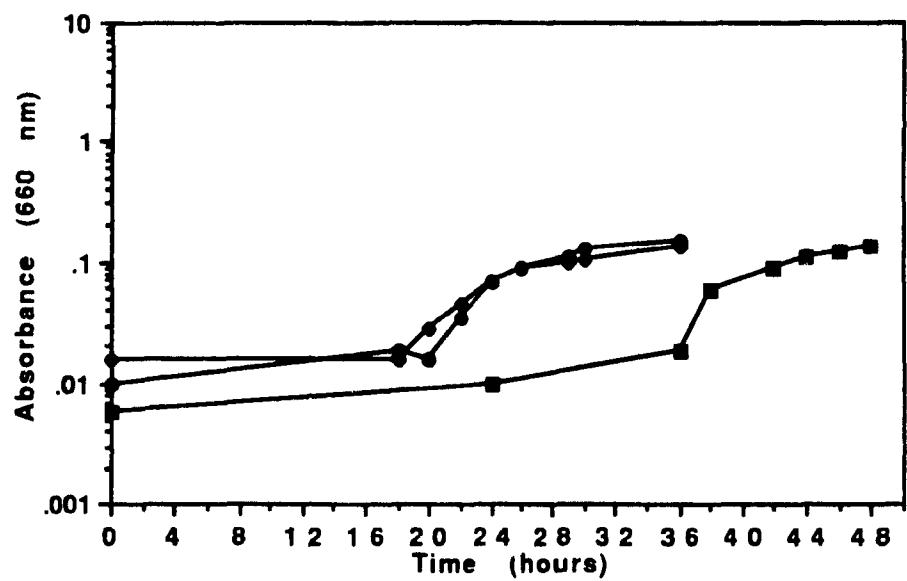


Figure 9

Fig. 9. Divalent cation substitution for revertant cells (RS).

Symbols: (◻) 100 μM Mg^{++}
 (○) 90 μM Ca^{++} plus 10 μM Mg^{++}
 (◇) 90 μM Sr^{++} plus 10 μM Mg^{++}
 (◼) 10 μM Mg^{++}

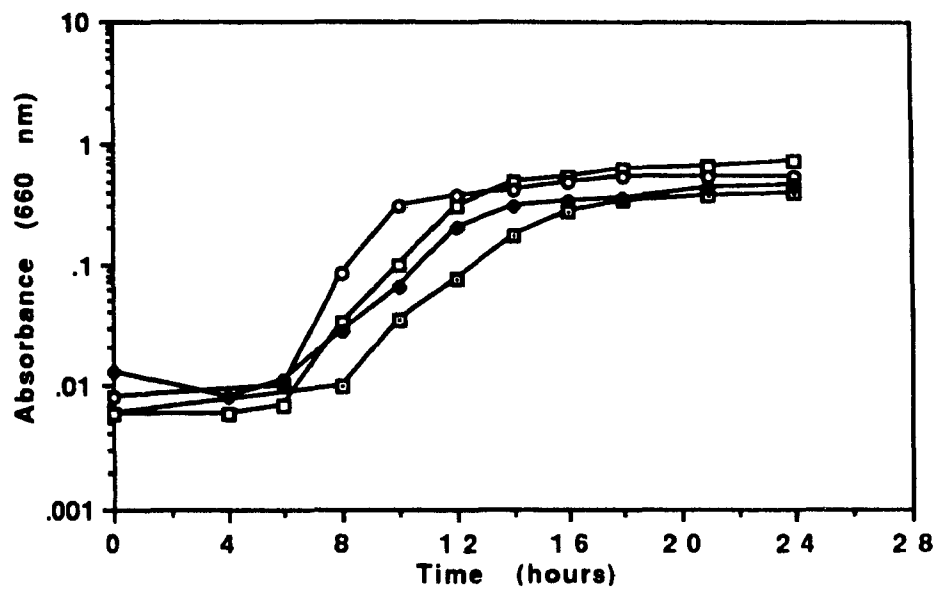
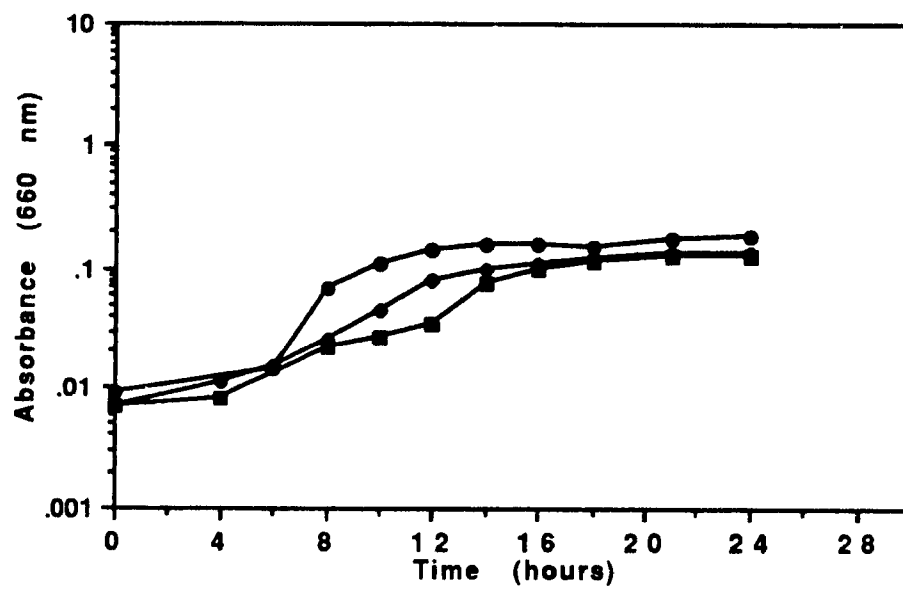


Figure 10

Fig. 10. Elimination of Mg^{++} from medium for revertant cells.

Symbols: (■) no Mg^{++}
(●) 100 μM Ca^{++}
(◆) 100 μM Sr^{++}



In addition to Ca^{++} and Sr^{++} , attempts were made to examine whether other divalent cations could substitute for Mg^{++} in the same way. While a lag of approximately 24 h was observed when cultures were supplemented with 90 μM of Mn^{++} , growth followed an erratic pattern that was not logarithmic. Cultures to which 90 μM of Be^{++} or Zn^{++} had been added showed no appreciable change in absorbance between 24 and 48 h, so work with these cations was aborted.

EFFECT OF EDTA ON *P. aeruginosa*

EDTA is known to cause lysis in *P. aeruginosa* (Repaske, 1958). Lysis can be measured as a decrease in absorbance at 660 nm over time. Initial experiments were monitored for 5 h and no appreciable change in optical density was observed. Subsequent experiments followed the effect of 10 μM and 50 μM of EDTA on PAO1 over 24 h, and results are shown in Figs. 11 and 12 respectively. In these experiments, no lysis is evident in either wild-type or CTC-treated cells; the organisms survive and grow, but the increase is not logarithmic. A slight difference between wild-type and CTC-treated cells can be discerned in Fig. 13: wild-type cells harvested from Mg^{++} -deficient medium failed to grow upon subsequent exposure to 10 $\mu\text{g/ml}$ CTC.

OUTER MEMBRANE PROTEINS

Sucrose-gradient centrifugation, by the method of Hancock and Nikaido (1978), of wild-type, resistant, and revertant strains of PAO1 yielded two distinct bands. The lower of these was collected, as its density corresponds with that of the outer membrane fraction. Electrophoresis in

Figure 11

Fig. 11: Effect of EDTA (10 μ M) on PAO1.

Symbols: (○) CTC-treated cells
 (●) untreated cells

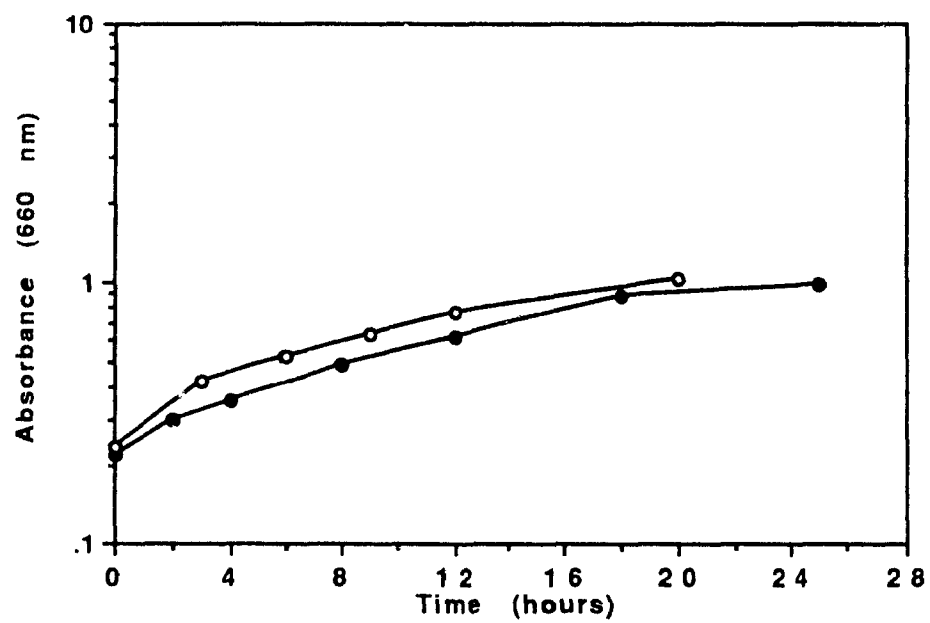


Figure 12

Fig. 12: Effect of EDTA (10 μ M) on PAO1.

Symbols: (□) CTC-treated cells
 (■) untreated cells

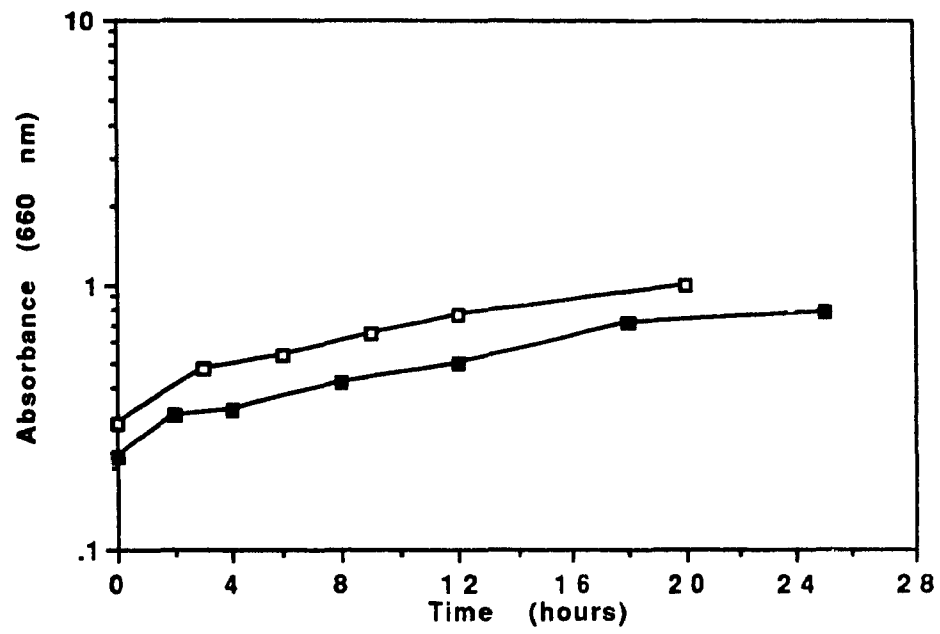


Figure 13

Fig. 13. Effect of CTC on PAO1 (control for EDTA).

Symbols: (◇) CTC-treated cells
(◆) untreated cells

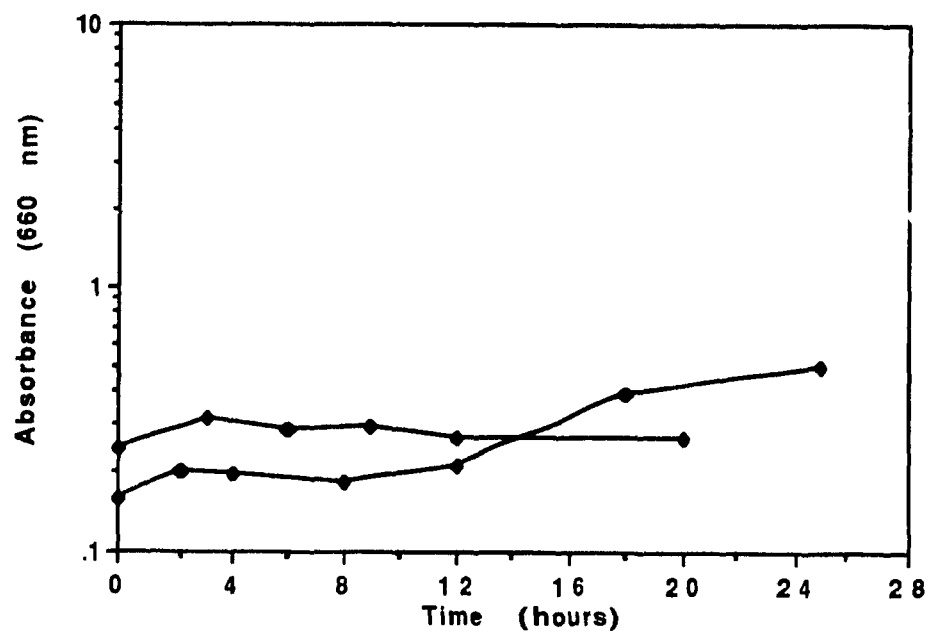


Figure 14

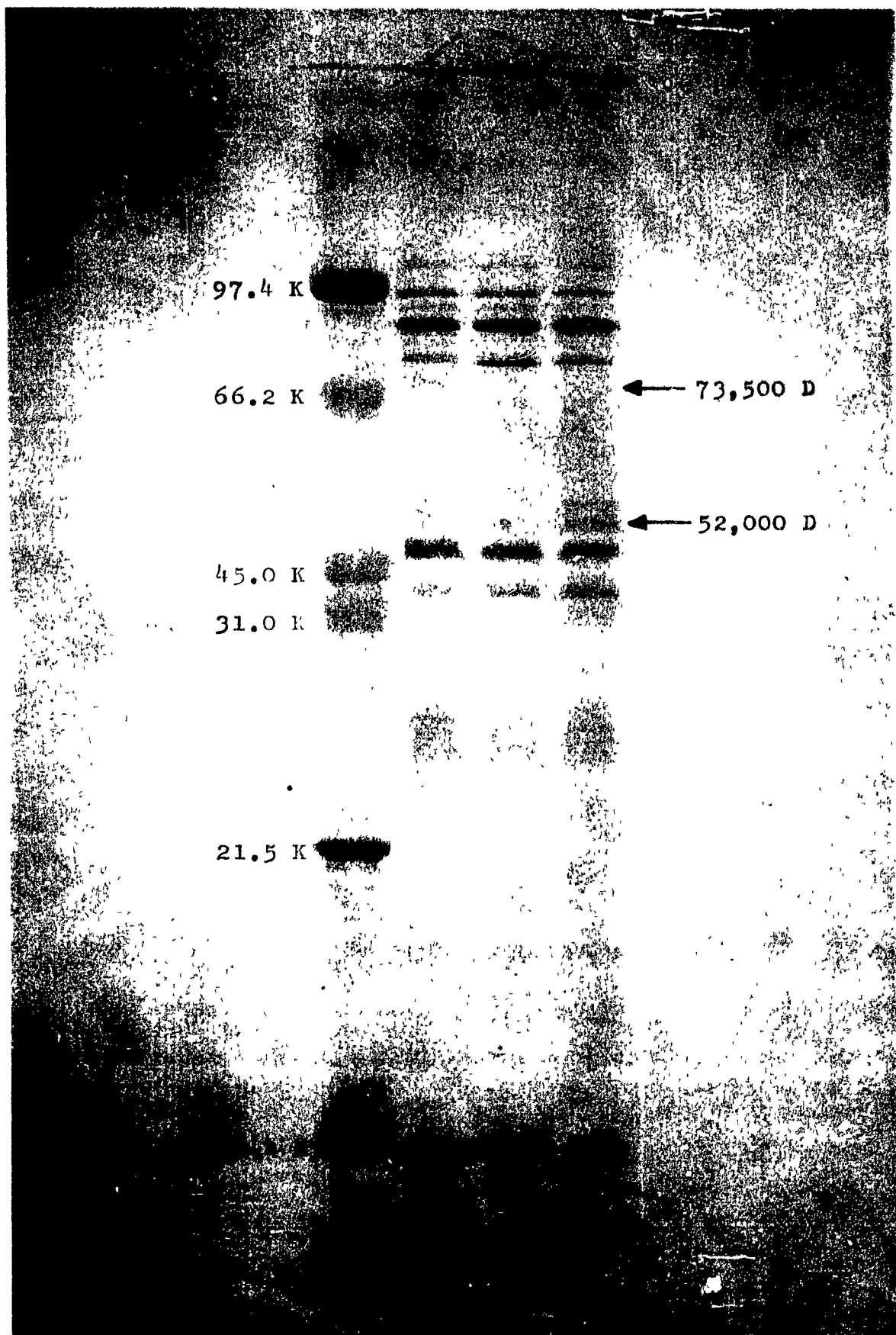
Fig. 14. SDS-PAGE of OM proteins.

Lane A: Molecular weight marker

Lane B: Wild type PAO1

Lane C: Revertant cells of PAO1

Lane D: PAO1 resistant to 10 $\mu\text{g/ml}$ CTC



12% SDS-PAGE yielded distinctive banding patterns for each of the three strains (Fig. 14). All strains show approximately equal amounts of outer membrane proteins D, E, F, and G, with apparent molecular weights of 49,000 D, 45,000 D, 37,000 D, and 22,000 D, respectively. Proteins H and I appear at the lower end of the gel, and are indistinct. Wild-type strains (Lane B) possess a faint band at 73,500 D that is not visible in either the resistant (Lane C) or the revertant (Lane D) strains. The most significant difference between wild-type and resistant strains is the presence of an extra, distinct protein band with a molecular weight of 52,000 D in cells resistant to 10 µg/ml chlorotetracycline. This protein may be analogous to an additional outer membrane protein with a molecular weight of 54,000 D found in one class of norfloxacin-resistant mutants of *P. aeruginosa* (Hirai et al., 1987), as well as to an extra protein band found in mucoid strains of *P. aeruginosa* (Grabert et al., 1990; Kelly et al., 1990), however, the possible functions of these induced proteins are not known.

GROWTH OF PAO1 IN THE PRESENCE OF CHLOROTETRACYCLINE AND PENICILLIN G

Figure 15 shows the growth response of PAO1 in various combinations of CTC and Penicillin G. Cells treated with 10 µg/ml CTC, then harvested in mid-exponential growth and reinoculated into medium devoid of CTC showed that growth initially continued in an exponential fashion. Stationary phase was reached after approximately six hours. The growth pattern was identical for cultures regardless of whether or not Penicillin G was added (at 2 h after reinoculation).

Growth in cultures reinoculated into medium containing 10 µg/ml CTC

follows a somewhat different pattern. Cells not additionally treated with Penicillin G undergo a steady increase in growth that is arithmetic rather than logarithmic and growth shows no sign of declining even at 24 h. Cells to which Penicillin G was added began to show a decline in growth at 4 h, which continued until 18 h after reinoculation. Cultures transferred to medium containing CTC reached a final density lower than that of cells reinoculated in the absence of CTC whereas those treated with both drugs showed no net increase in optical density.

Figure 15

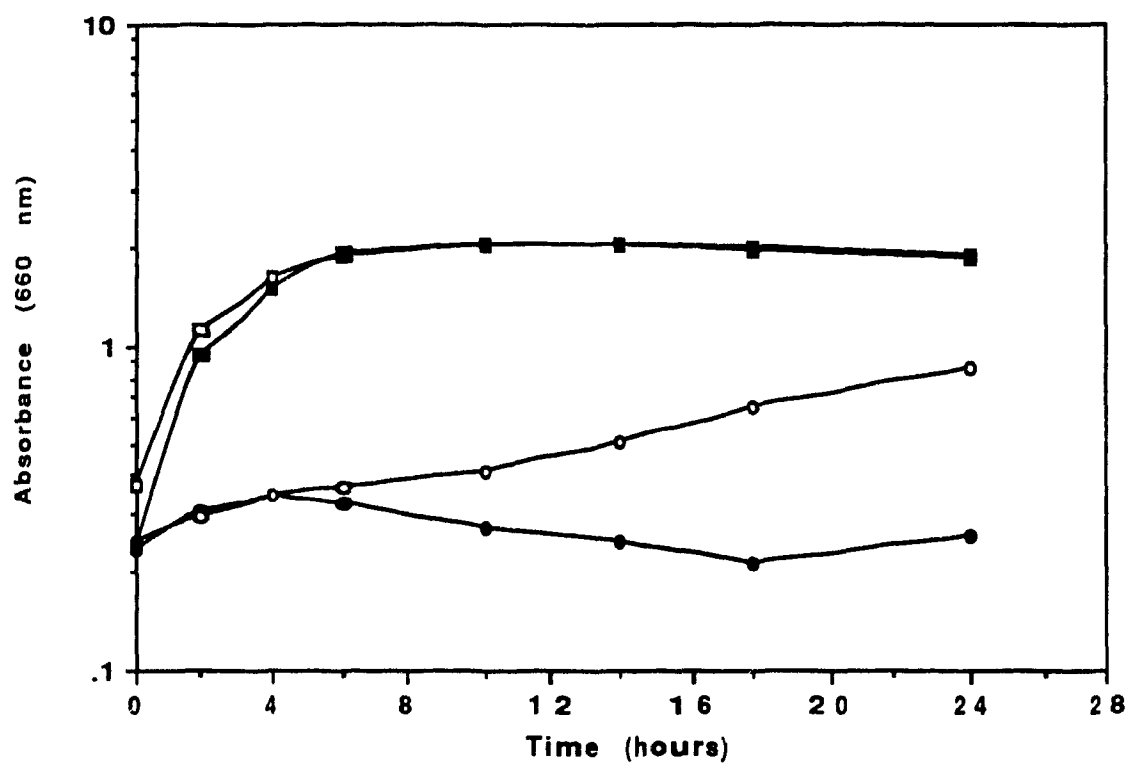
Fig. 15. Synergistic effect of CTC and Penicillin G on PAO1.

Symbols: (□), (■) no CTC

(○), (●) CTC in medium

Open symbols: no Pen G

Closed symbols: Pen G added at 2 h



DISCUSSION

Phenotypic changes in *P. aeruginosa* strains 9027 and PAO1 were most easily observed by examining growth characteristics. A minimal medium (Eagon and Phibbs, 1971) was used in order to provide a buffered environment in which to more easily manipulate antibiotic and divalent cation concentrations. Both strains of *P. aeruginosa* were able to grow upon exposure to 10 µg/ml TC or CTC. In solid cultures, adaptive resistance was observed as the result of successive "training" steps, each of which took a maximum of 48 hours under the conditions described. Since liquid cultures using TC were not standardized, these results cannot be compared with those for CTC. As observed in liquid cultures, resistance was a stable characteristic only in the sense that it persisted in the presence of CTC, but cells reverted to sensitivity as soon as the drug was removed (Figs. 1 and 2). This would imply that development of strains resistant to TC and CTC was not due to mutations, which are stable, heritable characteristics, but more likely resistance acquired in response to the presence of tetracycline as a selection pressure in the environment (Cairns et al., 1988).

The fact that resistant substrains grown on solid media appeared more mucoid than the wild-type deserves further consideration. The predominant microorganism responsible for pulmonary infections in cystic fibrosis patients is likely to be *P. aeruginosa*. The emergence of persistent antibiotic-resistant strains in this disease is due, in part, to widespread use of many antibiotics against other bacteria which produce complications in these patients. It has been shown that the majority of

strains of *P. aeruginosa* isolated from chronically-infected patients overproduces a mucoid substance found to be an extracellular alginate capsule (Pennington et al., 1979). Whether there is any relationship between mucoid substances produced *in vivo* and those demonstrated in our study would be an interesting area for further study

Drug inactivation would appear to be a consideration in experiments of this type. As shown in Table 1, CTC did lose some activity at incubation temperatures. This was likely due, at least in part, to chelation of CTC by Mg^{++} in the medium. However, as growth of CTC-resistant *P. aeruginosa* was delayed by as long as 24 hours under these conditions, the degree of inactivation of the drug was not so significant as to prevent resistance phenomena from being consistently observed. The disadvantage of using TC and oxytetracycline in growth experiments is that these derivatives are light- and heat-sensitive, making their potential for inactivation at incubation temperatures an additional consideration.

Both strains of *P. aeruginosa* exhibited an extended lag phase in CTC, after which a normal pattern of growth ensued. This response was also observed in this laboratory when *P. aeruginosa* 9027 was grown on hexadecane as the sole carbon source. While growth was delayed, the final turbidity of the culture was comparable to that of glucose-grown cultures (Miguez et al., 1986). Extended lag phases most likely correspond with the extra time required for *P. aeruginosa* to adjust its metabolic processes allowing it to survive under adverse conditions. Development of tolerance to a normally toxic agent may require that the cell divert some of its functions to providing extra protection against the agent interfering with more vital cellular processes. Thus, it is not

surprising that cells exposed to TC and CTC rapidly lost the ability to produce their normal pigments. This was observed previously in this laboratory in *P. aeruginosa* strain 9027 resistant to chloramphenicol (Ingram and Hassan; 1975), and is not uncommon among strains isolated from hospitals and many other sources (Haynes, 1951).

Subjecting the organisms to higher concentrations of tetracycline resulted in more erratic growth patterns. Lengths of the lag phases in these different cultures were highly variable, making measurement and replication difficult. Because both strains 9027 and PAO1 exhibited a significantly altered growth pattern in the presence of 10 µg/ml of both TC and CTC, it was felt that observing changes in these substrains would provide adequate phenotypic information. It was also felt that substrains resulting from exposure to higher concentrations of the tetracyclines would be more fragile and that gross changes which cells must make under highly stressful conditions would be difficult to quantify.

Changes in growth patterns of both 9027 and PAO1 upon exposure to TC and CTC illustrates the importance of taking into consideration strain differences when discussing an organism's resistance to antibiotics. While the lag phase of both cultures was approximately 24 hours, growth of 9027 was slightly more inhibited, and logarithmic growth was not as pronounced as that of PAO1. Cells of PAO1 grew to a final optical density that was higher than that of cells unexposed to the drug. In contrast, cultures of 9027 did not reach the same level of turbidity at stationary phase as their corresponding wild type cells. Much of the work reviewed in this study has involved one or the other of these two strains of *P. aeruginosa*. Therefore, caution must be taken when comparing results from these studies, so that broad conclusions are not drawn when

different strains exhibit variations in response and resistance to antibiotics.

The effects of substitution of CTC-resistant cells with divalent cations other than Mg^{++} were quite dramatically different than the response of wild-type cells. It is understandable that Ca^{++} is able to compensate for a deficiency of Mg^{++} in the medium, since Ca^{++} is known to be an essential component in the *P. aeruginosa* cell wall and occurs in approximately the same concentration as Mg^{++} (Leive, 1974). However, the ability of Sr^{++} to substitute as effectively as Ca^{++} is surprising, at both 90 μM and 100 μM concentrations. While Sr^{++} is not essential to biological membranes, a possible explanation for its analagous action to Ca^{++} may be its size alone. MacLeod et al. (1978) noted that the ability of an ion to pass through a membrane is related to its hydrated ionic radius. The value for Mg^{++} is quite considerably larger than those of Ca^{++} and Sr^{++} , which are almost identical (Robinson and Stokes, 1955). Thus, the fact that a "non-biological" ion such as Sr^{++} is able, *in vitro*, to take the place of Ca^{++} , an ion native to bacterial membranes, is simply a result of similar physical properties of the two ions. The absence of conclusive results with other ions such as Mn^{++} , Zn^{++} , or Be^{++} may also be due to their size (Mn^{++} has a hydrated radius similar to that of Mg^{++}) or to other properties which make them toxic to *P. aeruginosa*. Finally, the similarity of results in revertant cells to the wild type in divalent cation substitution experiments confirms the observations of the similarity in their growth characteristics (Fig. 1), indicating that resistance to CTC is transient and is almost completely lost after one transfer into antibiotic-free medium.

Outer membrane protein electrophoresis of PAO1 indicated the presence of an extra protein band of approximate molecular weight of 52,000 D in the CTC-resistant substrain. This protein does not correspond with any normally found in this organism (Hancock and Carey, 1979). Proteins of almost identical molecular weights were induced in three other systems, one of which was a norfloxacin-resistant mutant of PAO4009 (Hirai et al., 1987). The other two proteins were found in mucoid isolates obtained from cystic fibrosis patients (Kelly et al, 1990; Grabert et al., 1990). These were examined further in order to determine their possible role in the production of alginate, but whether this pertained to biosynthesis or excretion remains to be determined.

In the present study, the disappearance of a protein band at approximately 73,500 D cannot be explained, although strains resistant to some of the β -lactams and quinolones have been known to lose certain proteins (see Literature Review). This has been explained as a decrease in permeability resulting from a loss of pores in the outer membrane in response to polymyxin (Gilleland and Lyle, 1979). Electron microscopy examining the effects of the polymyxins and aminoglycosides on cell structure would seem to support this view, as cells are altered and become less permeable, thus excluding antibiotic entry (Gilleland and Farley, 1982).

It is not unusual for *P. aeruginosa* to overproduce certain proteins in response to growth in some antibiotics. These organisms are easily able to adapt cellular functions in order to grow on many different nutrient sources (Haynes, 1951). The actual function of induced proteins, however, is somewhat more difficult to define. It is plausible that the outer membrane of *P. aeruginosa* is able to rearrange itself somewhat in

response to the presence of a toxic agent in the growth medium, as may be the case with carbenicillin (Pataryas et al., 1982). The appearance of an extra protein band in the outer membrane of gentamicin-resistant *P. aeruginosa* was postulated to be a degradation product of outer membrane disruption. This is in keeping with the action of the aminoglycosides, which are large cationic molecules which most likely displace metal cations from the outer membrane thus causing cell lysis (Martin and Beveridge, 1986). Whether the protein produced in this study is a structural protein, or one produced in order to somehow participate in exclusion or inactivation of antibiotics, remains to be further studied.

It should be noted that resolution of protein bands observed on SDS-PAGE can vary considerably according to experimental conditions. Hancock and Carey (1979) describe a total of eight proteins, including D1-D2 and H1-H2, separable in 14% acrylamide. The use of 12% acrylamide in this study is responsible, then, for the less well-defined number of bands observed in Figure 14. Other conditions such as the purity of SDS used must also be taken into consideration (Hancock and Carey, 1979). This may account for variations in the literature of the molecular weights of proteins lost or induced upon treatment of *P. aeruginosa* with different antibiotics.

The addition of EDTA to *P. aeruginosa* in this study system had no effect on either wild-type or CTC-treated cells. This cannot be explained in the current context. Experimentation with higher concentrations of EDTA are called for, but proved to be difficult in this test system. It is interesting to postulate, however, that cells resistant to CTC may somehow be stronger, with higher concentrations of divalent cations

holding the outer membrane together. It is also conceivable that alterations made by the cells to the outer membrane render the divalent cation sites more difficult to reach by chelators. Keeping in mind that CTC is also a chelator of divalent metals (Dockter and Magnusson, 1974), this may play a part in the resistance of *P. aeruginosa* to this antibiotic. Further work should therefore include atomic absorption spectrophotometry of outer membrane preparations to examine any changes in quantity of divalent cations in resistant cells. Cells grown in different concentrations of the divalent cations used in this study should also be examined for any changes in outer membrane proteins. Lastly, fluorescence assays could be performed in order to examine the time course of CTC uptake in resistant cells. The work presented in this study indicates the importance of such studies in elucidating CTC-resistance mechanisms beyond primary interactions with cells of *P. aeruginosa*.

Finally, work presented here regarding the synergistic effect of the combination of CTC with penicillin G reiterates the importance of such phenomena in the search for improved antimicrobials against persistent bacterial infections such as those caused by *P. aeruginosa*. Work with new combinations of antibiotics and chemical agents is only just beginning, but caution must be exercised when extrapolating these results for use in clinical settings. Many combinations of antibiotics result in an antagonistic effect of one on the other, and the toxic effects of both can be additive (Sande et al., 1990). A significant number of the chemicals used *in vitro* are toxic to human cells, making the search for non-toxic antibiotic analogs or some cellularly-derived biological antimicrobials look promising. In spite of the efforts of researchers to improve antimicrobial agents to keep up with the development of newly-resistant

strains of bacteria, we must keep in mind that bacteria possess intricate ways of eluding these developments. This study is but one illustration of the versatility and adaptability of *P. aeruginosa* and other bacteria in order to survive under adverse conditions.

APPENDIX I: GROWTH OF *P. aeruginosa* IN TETRACYCLINE

Figure 1A

Fig. 1A. Growth of PAO1 in TC.

Symbols: (●) no TC
(□) 10 µg/ml TC

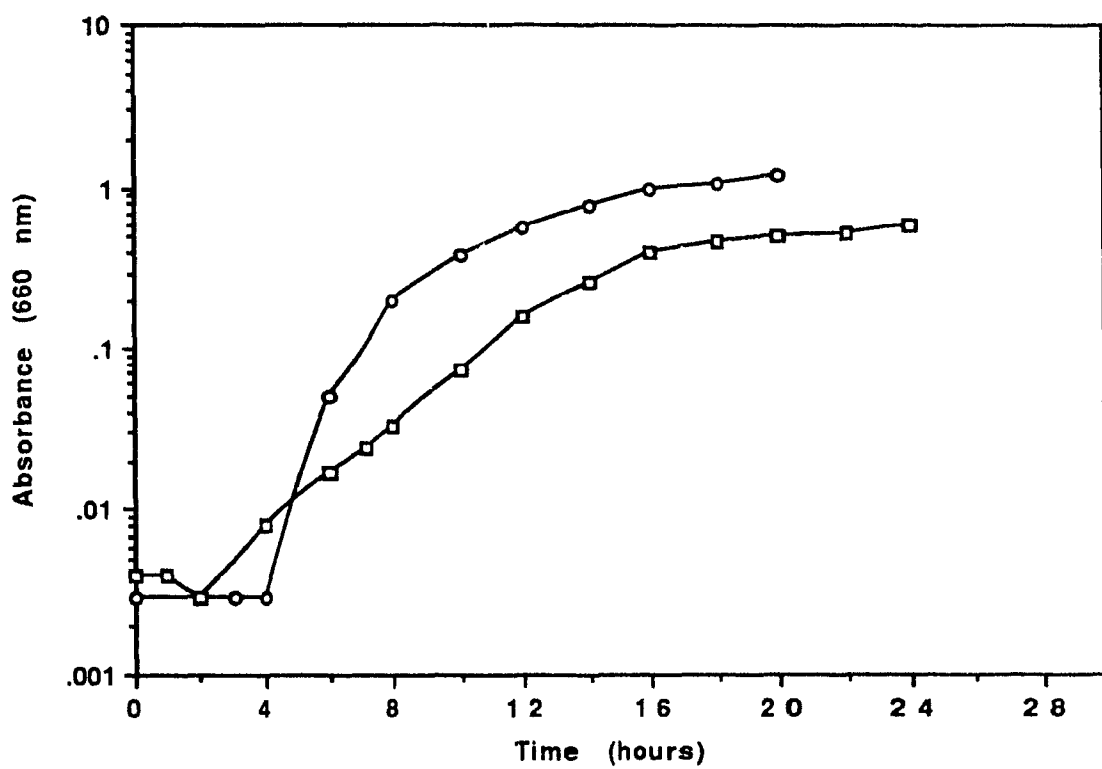


Figure 2A

Fig. 2A. Growth of PAO1 in 50 $\mu\text{g/ml}$ TC.

Symbols: ($\blacklozenge$) 50 $\mu\text{g/ml}$ TC

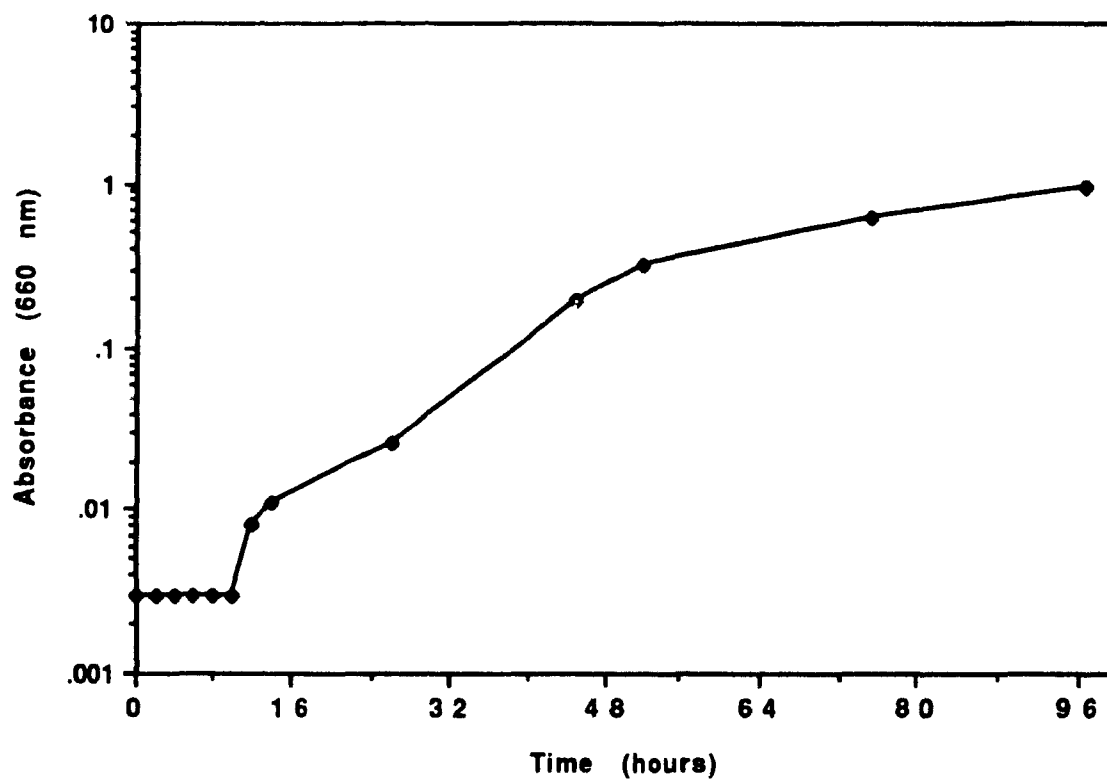


Figure 3A

Fig. 3A Growth of 9027 in TC.

Symbols: (●) no TC
(□) 10 $\mu\text{g/ml}$ TC

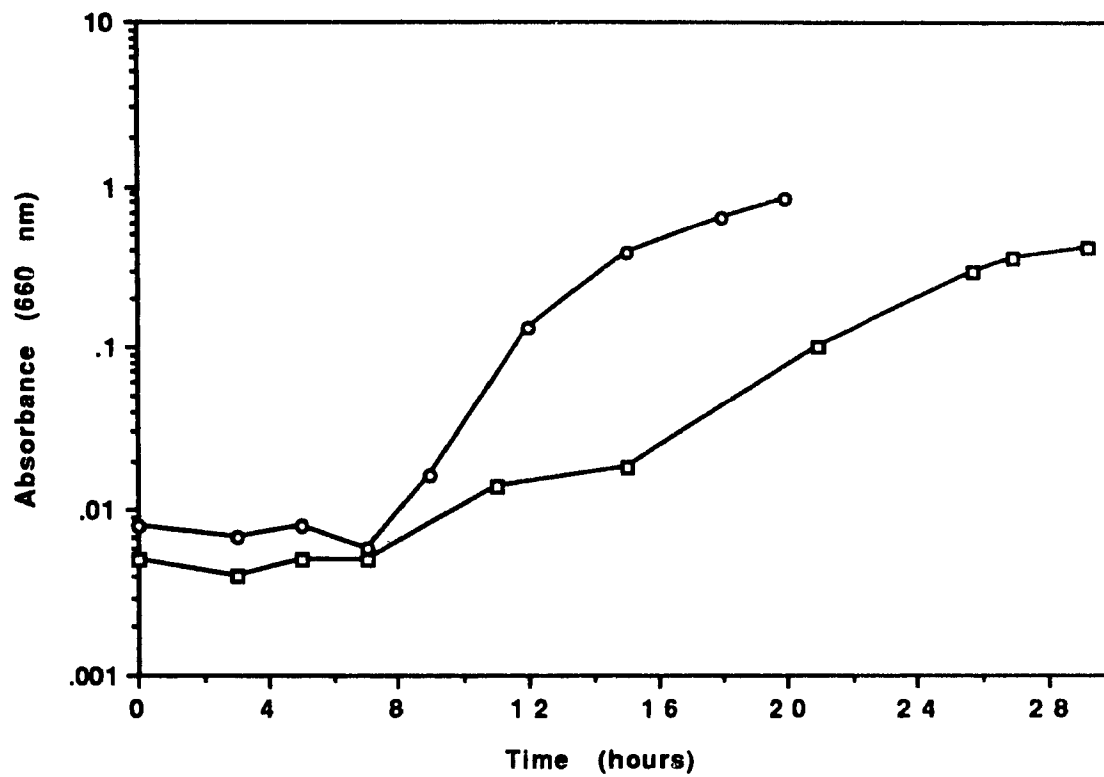
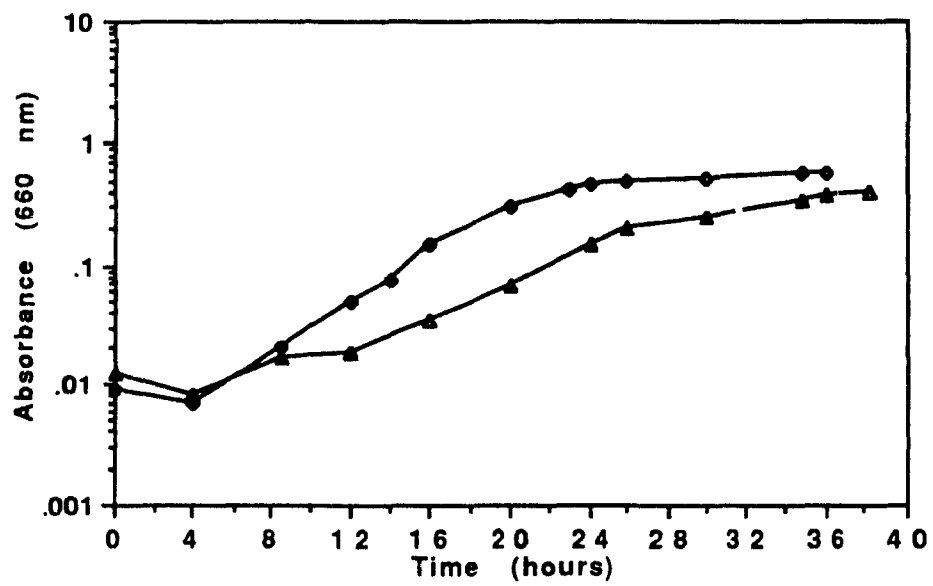


Figure 4A

Fig. 4A. Growth of 9027 in 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ TC.

Symbols: ($\diamond$) 50 $\mu\text{g/ml}$ TC

($\triangle$) 100 $\mu\text{g/ml}$ TC



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