Studies on the Mechanism of Zinc Pyrithione Resistance in *Pseudomonas aeruginosa* PAO1

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A thesis submitted in partial fulfillment of the requirements of the

University of Abertay Dundee

for the degree of

Doctor of Philosophy

April 2002

I certify that this is the true and accurate version of the thesis approved by the examiners.

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Qualification	PhD
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Acknowledgements

I would like to express my sincere gratitude to my director of studies Dr. Phillip J. Collier for his supervision and consistent guidance and for both him and his wife Mrs. Sarah for their social entertainment and moral support during my stay in Dundee. I would also like to thank my co-supervisor Dr. Ibrahim Al-Adham at Al-Petra University and Dr. Eldridge Buultjens at Abertay University for their willing advice during certain aspects of this project. I would also like to thank Professor Abdul-Jalil Al-Thuwaini at Al-Petra University for proof reading this thesis. I am greatly indebted to Al-Petra University for offering me this Ph.D. scholarship.

I would like to acknowledge the help of the technical staff in particular Mr. Willie Meldrum at the division of Biology - University of Abertay. I am very much obliged to both Muna Al-Resheq and Reem Jallad for their help in some typing problems. I would like to express my thanks to my colleagues at both the University of Abertay and Al Petra University for their constructive discussions during the work of this project, especially Dr. Janet Naghaway at Al Petra University.

My special appreciation and gratitude goes to my family; in particular my husband Rashad to whom I owe my success, which resulted from his continuous encouragement and help. A big thank you full of love to my children Yousef and Sarah who expressed extreme patience with their mum during her work. I would also like to present my success to my mother who has been very generous in her prayers for me during the time of my stay abroad. My nephew Saleh who is an IT student has been a great help for me with his creative ideas in dealing with my computer. Finally a big thank you full of gratitude for my dear friends in Dundee Ann, Jenny , Rania and Nisreen who have been supportive and generous.

Abstract

Previous workers have suggested that it is possible to passage *Pseudomonas aeruginosa* towards resistance against selected antimicrobial agents (Isothiazolone biocides). These passages in sub-minimal inhibitory concentrations (sub-MIC) of antimicrobial agents also exhibited corresponding shifts in outer membrane protein profiles. The aim of this project was to elucidate whether or not such adaptive resistance was evidenced with consecutive passages in sub-MIC of the Pyrithione group of antimicrobial agents and whether or not such passages yielded comparable outer membrane protein shifts.

Pseudomonas aeruginosa, an opportunistic Gram-negative bacteria known for its resistance to a wide range of antimicrobials was found to develop an adaptive resistance to the biocides Zinc Pyrithione (ZnPT) and Sodium Pyrithione (NaPT) and Cetrimide. Investigation of the mechanism of resistance involved SDS-PAGE analysis of outer membranes of biocide-sensitive and biocide-resistant cells, SDS-PAGE LPS analysis, changes in cell surface hydrophobicity, cross resistance, multiple antibiotic resistance, efflux systems and stress response.

Results indicate that the ZnPT-resistant phenotype exhibited an increase in cell surface hydrophobicity and LPS changes. When outer membrane protein profiles of ZnPT-resistant cells and ZnPT-sensitive cells were compared, a 26.30 kDa protein was observed to be missing from the outer membranes of the former but was expressed in the latter. Furthermore, this protein was observed to reappear when the ZnPT-resistant cells were grown in a ZnPT-free media. The ZnPT-resistant phenotype exhibited resistance towards NaPT and Cetrimide but did not show resistance towards a selection of antibiotics, which indicates that ZnPT is not an inducer of the *mar* regulon in *P. aeruginosa.* Experiments using efflux pump inhibitors suggested the possible involvement of an efflux mechanism in the resistance developed towards ZnPT.

SDS-PAGE of cell lysates of ZnPT-resistant and ZnPT-sensitive cells has shown that resistant cells expresses cytoplasmic proteins that were not expressed in sensitive cells. Using western blot analysis these proteins were found to be dissimilar to *P. aeruginosa* heat shock proteins, suggesting that they could be ZnPT-specific stress proteins or molecular chaperones that assist the cells in recovering from ZnPT-induced cellular damage.

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Chapter One

Introduction

The production of antimicrobial agents to control the detrimental activities of bacteria and other microorganisms has been a great achievement of man. Antibacterial agents comprise a large group of substances, which inhibit bacterial growth (bacteriostatic), cause bacterial death (bactericidal) or destroy spores (sporicidal). Antibacterial agents include disinfectants, preservatives and antibiotics. The term biocide is a general term that denotes non-chemotherapeutic agent that kills microorganisms (Russell and Chopra 1996). Preservatives are frequently added to pharmaceutical, cosmetic and food products to inhibit bacterial contamination and proliferation and hence prevent infectivity or spoilage. Although a large number of biocides are being produced, the use of the appropriate antimicrobial (biocide) is complex. Thus, it will always be the illusion to think that microbial destruction of materials can be prevented just by the use of a mixture of biocides. Preservatives used nowadays should have their active ingredients and formulations "*tailored*" to suit each material. They must be compatible with the large number of processes that materials go through during manufacturing and usage.

Paulus (1993) described four major groups of materials that require the addition of antimicrobials. The first group is the industrial aqueous process fluids, e.g. cooling water, secondary oil recovery systems, and metal working fluids. The second group is the aqueous functional groups e.g. the protection of in-tank/ in-can process such as water based paints, adhesive polishes, rubber latex's, wax emulsions, aqueous cosmetic and some pharmaceutical formulations. The third group includes materials that contain

little or no water in a free state e.g. paper, paint, leather, wood, and textile. The forth group is the inanimate surfaces and equipment.

Selection of the suitable preservative depends upon the type of microbe to be controlled and the environment in which the biocide will be used. For example products that contain sugars are most likely to be attacked by yeast thus, effective anti-fungal should be included in the formulation. However, bacteria usually decompose products that are rich in proteins and have neutral to alkaline pH hence, proteinaceous products should include antibacterials. Relative humidity is a factor that should be taken into consideration when choosing the right preservative. Water is one essential requirement for bacterial growth. A relative humidity of 80% is conductive to bacterial growth (Paulus 1993) thus, formulations containing water would certainly need different preservatives than dry formulations. The correct timing of antimicrobial addition to the product is a crucial factor to achieve the best antimicrobial control. Addition of antimicrobials should be in the early stages, ensuring that bacterial enzyme production doesn't occur. Antimicrobial combinations should be carefully chosen so as not to allow antagonistic reactions to occur thus, resulting in rendering the product unprotected from microbial invasion. It is known that the antimicrobial activity of methyl and propyl *p*-hydroxybenzoates and Quaternary Ammonium Compounds (QAC) is reduced markedly by non-ionic surfactants (Tween) thus, to overcome that effect significant increase in the antimicrobial concentration is required to inhibit the growth of microorganisms.

1.1 Preservation in Cosmetics and Pharmaceuticals

Microbial contamination in cosmetics and pharmaceuticals leads to health hazards and product spoilage. Most cosmetics are intrinsically subject to biodegradation, in that they contain an aqueous media with a variety of natural and synthetic organic materials

that may serve as nutrients for microorganisms (Nelson and Hyde 1981). Microorganisms are introduced in a product through different routes, such as contaminated raw materials or during processing and manufacturing or during usage as a result of consumers inadequate practices (Levy 1987). Microorganisms spoil the products by enzymatically hydrolyzing one or more of their ingredients thus, causing changes in odor, color and viscosity (Brannan 1995). Cosmetics that most need preservatives are those that contain water. Products with low water activity (non-water-based lipsticks, talcs and anti-perspirants) usually need little more than methyl or ethyl parabens to protect against fungi (Brannan 1995). Most products reach consumers in good microbiological condition, but they cannot withstand contamination within use (Brannan 1995; Russell *et al* 1999). In the mid 1970s contaminated mascara caused cases of blindness due to *Pseudomonas* infections of the cornea (Wilson *et al* 1977). In the previous study seven cases of corneal ulcers were investigated, where inadequate preservation was the major factor that contributed to the colonization of the mascara with *P. aeruginosa*. The authors attributed this to the possibility of preservative break-down during usage or that certain consumer practices has contributed to the early break-down of the preservative. Failure to cleanse or sterilize the applicator brush before insertion into the refill may increase probability of infection, furthermore scratching the cornea with the contaminated mascara applicator resulted in the manifested eye infection. The authors suggested that the product should be supplied to the consumer in small quantities that would stand the period of usage and that the applicators should be disposable (Wilson *et al* 1977). *P. aeruginosa* has also been isolated from in-use shampoos and skin lotions (Brannan 1995).

The ideal characteristics of a cosmetic and a pharmaceutical preservative is that it should be safe, stable, compatible with both the product and the container, inexpensive,

readily available, approved by appropriate regulatory agencies and environmentally friendly. Consideration should be given to exposure conditions and how the consumer will use and misuse the product (Brannan 1995).

1.2 Biocide tolerance and resistance

Microbial resistance to biocides is a process that has been known for many years (Chaplin 1951; Hoffmann *et al* 1973; Kolawole 1984; Jones *et al* 1989). The phenomenon of tolerance is often discussed in preservative industry. It describes a situation where a formerly active preservative system no longer controls microbial growth (Chapman *et al* 1998). Inability of a biocide to control microbial growth could result from changes in the formulation of the product that can lead to attenuation of the preservative used (Russell *et al* 1999), development of biofilms in the manufacturing system and or development of microbial resistance (Chapman *et al* 1998). Microbial "*resistance*" (insusceptibility) to biocides is not clearly defined as compared to antibiotic resistance. There are no clear lines drawn between sensitivity and resistance to biocides. Furthermore, antibacterial activity of antibiotics is usually expressed as minimum inhibitory concentrations (MIC), whereas MIC values are often not related to biocide concentration used in practice (Russell 2000). Yet, the term biocide resistance or insusceptibility can be used if the biocide is no longer effective at the in-use concentration or above that concentration. Evolution has provided bacteria with enormous capabilities to survive diverse environments. Such capabilities include mechanisms for resisting various harmful substances (Zgurskaya and Nikaido 2000). Knowledge of resistance mechanisms of a particular isolate can be a powerful tool for regaining control over that population (Chapman *et al* 1998).

1.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic microorganism and a potentially dangerous pathogen. This organism can utilize a broad range of compounds as carbon and energy sources and therefore, it can survive and multiply in diverse environments including nutritionally limited ones, such as mineral water and fuel oil (Favero *et al* 1971; Cooney 1969). Biochemically it represents the most versatile organism encountered through out the cosmetic and pharmaceutical industries. *Pseudomonas aeruginosa* is one of the most antibiotic and biocide resistant, non-sporforming microorganisms. In many cases this resistance is due to its cell envelope (Yoshihara and Nakae 1989). *P. aeruginosa* exhibits higher intrinsic resistance than any other Gram-negative bacteria that share similar envelope structures. As with other Gram-negative bacteria the outer membrane is undoubtedly responsible for conferring much of the intrinsic resistance (Hancock 1997).

1.4 Pseudomonas aeruginosa cell envelope structure

P. aeruginosa is generally as resistant to hydrophobic molecules as other Gram-negative bacteria due to the presence of the LPS molecules (Vaara 1992). However, *Pseudomonas aeruginosa* also exhibits resistance to small hydrophilic antibiotics, like tetracyclines and β-lactams. Hence, the outer membrane of *P. aeruginosa* is suspected of having a lower level of permeability than that of other Gram-negative bacteria. *P. aeruginosa* has 12 to 100 fold less outer membrane permeability than that of *Escherichia coli* (Russell and Chopra 1996; Hancock 1998). Table1.1 demonstrates the differences in exclusion limits between *P. aeruginosa* and some non-fermentative bacteria.

Table 1.1: Outer membrane permeability of some bacterial species (Hancock 1998).

Species	Relative outer membrane permeability (%)	Porins	
		Major	Adjunct
Eschericia coli	100	OprF, OmpC	LamB, PhoE, OmpG, CE1248 porin
Pseudomonas aeruginosa	1-8	OprF	OprB, OprC, OprE, OprD
Burkholderia cepacia	11	OpcPO	OpcS
Stenotrophomonas maltophila	3-5	.....	.....
Acinetobacter baumanii	1-3	45.5-kDa	46.5-kDa

The cell envelope of *P. aeruginosa* is very much like that of other Gram-negative bacteria (Figure 1.1). The cell envelope is made of two concentric bilayer membranes surrounding the peptidoglycan cell wall and confining the periplasmic space (Koebnik *et al* 2000). Both membranes contain proteins that assist in the passage of matter and information, but the two membranes differ markedly with respect to structure and function. The inner cytoplasmic membrane (Figure 1.1) renders the bacteria impermeable to large molecular weight hydrophilic compounds (Russell and Chopra 1996). It is made of inner and outer leaflets. The two leaflets are made exclusively of phospholipids which makes it highly hydrophilic and impermeable to hydrophobic molecules. The cytoplasmic membrane contains proteins that assist in the passage of materials and information in and out of the cell. The major phospholipid in *P. aeruginosa* is phosphatidylethanolamine that constitute 59% of the total phospholipid of the cell (Anderes *et al* 1971). Other phospholipids such as phosphatidylglycerol and cardiolipin are equally distributed among the inner and outer leaflets (Koebnik *et al* 2000). The outer leaflet of the cytoplasmic membrane is bounded by the cell wall (Beveridge and Kadurugamuwa 1996). The cell wall is a rigid highly structured layer that contributes to the mechanical stability of the bacteria. It is composed of peptidoglycan polymer, which is made of two different N-acetylated aminosugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA). A short peptide chain is connected to the carboxyl group of NAMA. Glycan strands are cross linked through direct peptide bonds between the adjacent peptide chains or peptide interbridges to create a covalent network with great mechanical strength (Russell and Chopra 1996). *P. aeruginosa* has an acyl group on the 6^{th} position of the NAMA, which renders the peptidoglycan insensitive to degradation by lysozyme. The presence of an acyl group in that position prevents effective binding of the lysozyme to the β-1, 4 glycosidic bond

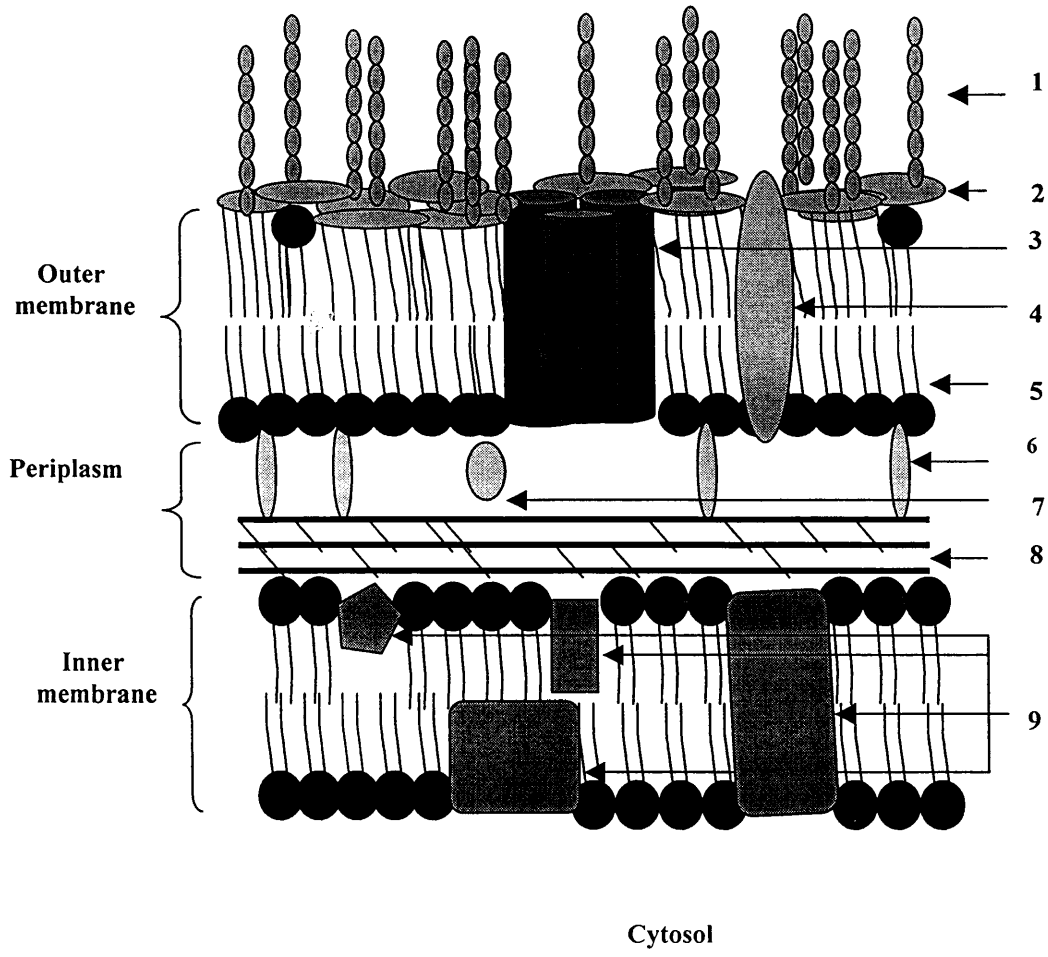
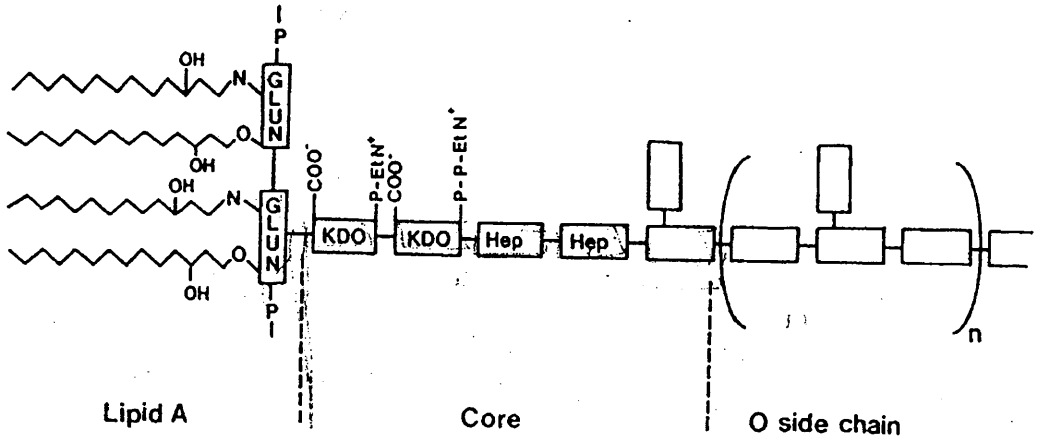


Figure 1.1: Cell envelope of Gram-Negative bacteria. (1) Lipopolysaccharide O side chain (2) Lipid A (3) Porin (4) Outer membrane protein (5) Inner leaflet of the outer membrane (6) bound Lipoproteins (7) free Lipoproteins (8) Peptidoglycan (9) Cytoplasmic membrane proteins.

between the two aminosugars and thus will not be able to break the bond (Hammond *et al* 1984). Nearly all bacteria have an absolute requirement for peptidoglycan, which makes it a good target for antimicrobials (e.g. β-lactams). Peptidoglycan of Gram-negative bacteria is very thin (Figure 1.1). Peptidoglycan is surrounded by the outer membrane and is anchored in its place by covalent bonds with the Braun's lipoproteins. Braun's lipoproteins are found in the periplasmic area between the peptidoglycan and the outer membrane (Beveridge and Kadurugamuwa 1996). Lipoproteins occur in two forms, a bound form and a free form. The N- terminal of the bound lipoprotein contains a cysteine moiety that is linked to the diacylglycerol in the lipid A portion of the outer membrane by a thioester bond. The C-terminal contains L-lysine residue, which is connected to the peptidoglycan through a covalent bond between the L-lysine and the carboxyl group of the L-Diaminopimelic acid. About two thirds of the lipoprotein is freely embedded in the outer membrane (Hammond *et al* 1984). The periplasmic area is entrapped between the two membranes of the Gram-negative bacterial cell envelope (Figure 1.1). The periplasmic area is an essential and highly active component of these cells. It contains high concentrations of a variety of macromolecules that function as hydrolytic enzymes. These enzymes break down relatively large compounds to allow for their uptake by the cytoplasmic membrane into the cell. The periplasmic area also contains waste products and harmful substances waiting to be removed from the cell. A number of detoxifying enzymes that degrade harmful substances preventing them from injuring the cell, and a large number of binding proteins, that facilitate the transport of substrates into the cell through the cytoplasmic membrane, are also found in the periplasmic area. Constituents of the periplasmic area are ever changing in response to cellular and environmental messages (Beveridge and Kadurugamuwa 1996). The outer boundary of the periplasm is the outer membrane, which is a highly asymmetrical

structure with the inner leaflet made totally of phospholipids (primarily phosphatidyl ethanolamine), while the outer leaflet consists largely of a single glycolipid compound, the lipopolysaccharide (LPS). The outer leaflet also contains a few molecules of phospholipids together with a number of functional proteins (Hancock 1997) (Figure 1.1). LPS is a high molecular weight, negatively charged molecule that provides the cell with a polyanionic external surface (Hancock 1997). This anionic feature is partially neutralized by the presence of divalent cations (e.g. $Mg^{2+}$and Ca^{2+}). Divalent cations bridge adjacent LPS molecules resulting in the tight packing of these molecules (Hancock 1997). As shown in figure (1.2), LPS is made of three covalently linked regions, Lipid A, Core lipopolysaccharide and the O side chain. The lipid A region is made of six or seven fatty acid chains connected to a phosphorylated glucosamine disaccharide, unlike phospholipids that only have two fatty acid chains connected to their backbone structure. The amino moieties of the glucosamine are connected to 3-hydroxydodecanoate (C12: 0) through amide linkage. The 3-hydroxydecanoic (C10: 0), dodecanoic (C12: 0) and hexadecanoate (C16:0) of other fatty acids are ester linked to the glucosamine backbone or to the hydroxy groups of other fatty acids (Kropinski *et al* 1985). All of the fatty acids in the lipid A portion are saturated medium length hydroxy fatty acids (Nikaido and Vaara 1985). Lipid A of *P. aeruginosa* contains 3-hydroxy and 2-hydroxy fatty acids; most of these are saturated and even numbered fatty acids. The non-hydroxy fatty acids such as lauric C_{12}, myristic C_{14} and palmitic C_{16} acids are unsaturated. *P. aeruginosa* contains variable amounts of other fatty acids such as 17,19 cyclopropane acids and tetradecanoate (Kropinski *et al* 1987). The core polysaccharide region is connected to the lipid A region by an acid labile linkage between the glucosamine disaccharide backbone and the sugar acid Ketodeoxyoctonate (KDO) (Figure 1.2). The

(a)



(b)

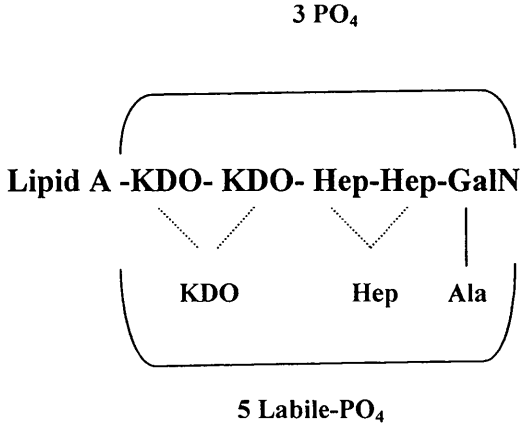


Figure 1.2: The LPS structure of Gram-negative bacteria **(a)**. Structure of the core region in the LPS of *P. aeruginosa* PAO1 **(b)**. KDO: 2-keto3-deoxyoctonic acid; Hep: Heptose sugar; GalN: galactosamine; Ala: alanine (Kropinski *et al* 1985)

core region is made of a short chain of saccharides. The saccharide chain includes heptose, glucose, galactose and N-acetylglucosamine, rhamnose and alanine (Jarrell and Kropinski 1977). This region is highly conserved between species, variations only exist in the degree of substitution by phosphorous residues, *P. aeruginosa* strain PAO1 contain 8 phosphate residues in the core region (Figure 1.2 b) (Kropinski *et al* 1985). The O side chain (Figure 1.1) is a branched or unbranched short polysaccharide chain extending outwards from the core. It has a wide range of variation in the sugars, which accounts for the large number of serotypes. The O side chain is made of a chain of six carbon sugars, e.g. glucose, galactose, rhamnose, fucosamine and mannose. Mutants of *P. aeruginosa* that lack the O-side chain are known as rough mutants (R) while mutants that expresses the side chain are known as smooth mutants (S) (Kropinski *et al* 1985). LPS is important to the Gram-negative bacterial cell, not only in the avoidance of host defense, but it also imparts a negative charge to the bacterial cell, since it contains charged sugars and phosphate. LPS surface has several divalent cations binding sites that stabilize the outer membrane (Hancock 1998). The permeability of the outer membrane can be altered when metal chelators such as EDTA are included in the growth media. EDTA chelates the divalent cations present on the surface of LPS molecules which would result in the disruption of the tight interactions between molecules and hence disruption of the outer membrane. However, the regular permeability is restored by the addition of Mg^{2+} to the growth media (Brown and Melling 1969; Ayers *et al* 1998; Ayers *et al* 1999).

LPS is associated with different proteins. These proteins are responsible for the protective nature of the outer membrane. They comprise nearly half the mass of the outer membrane but their number is limited in comparison to the proteins in the cytoplasmic membrane. These proteins prevent or slow down the movement of

antimicrobials or bile salts into the cell (Nikaido and Vaara 1985). Although the presence of the LPS structure in the outer membrane confers very low permeability for hydrophobic solutes and presumably hydrophilic solutes, its outer membrane is considered to be more leaky than the cytoplasmic membrane (Koebnik *et al* 2000), because the permeability of the outer membrane is also controlled by a class of proteins that are associated with the LPS molecules. These proteins produce water-filled channels that function as general or substrate-selective "*conduits*" for diffusion of certain hydrophilic molecules (Hancock 1997). The overall permeability of the outer membrane depends upon the number and properties of these pore-forming proteins that are generally called porins (Figure 1.1) (Trias and Nikaido 1990). Porins are proteins that form holes in the outer membrane by the clustering of three protein molecules (trimeric porins) (Nikaido 1992), by folding into β–pleated sheets to form a closed barrel. The structure of porins differ from other integral membrane proteins that are usually made of transmembrane α-helices (Koebnik *et al* 2000). Porins of the outer membrane form three types of channels (Braun 1995). Type 1 channels allow the non-specific passage of small hydrophilic substrates that are not larger than 600 Da. They have some preference to some anions and cations due to the presence of some charges on the mouth of the channel; thus, the total charge on each porin gives each porin its ion selectivity. This type of porins is called general diffusion porin (also called major outer membrane proteins). They are made of homotrimers that form a barrel like structure, each monomer is made of 16 β-strands that span the outer membrane. The third loop folds back into the barrel forming a constriction zone at half the height of the channel, which keeps it either open or closed (Koebnik *et al* 2000). These porins open and close by means of a process called voltage gating. Voltage gating depends upon the critical voltage (V_C) of the membrane, if the V_C is larger than the naturally occurring Donnan

potential across the outer membrane the general porins like OmpF and PhoE of *E. coli* will close (Koebnik *et al* 2000). However, V_C is affected by several other parameters, such as pH of the media, ionic strength, and the presence of polysaccharides, membrane-derived oligosaccharides or polycations (Delcor 1997), as well as the charges present in the porin channels from specific charged residues (Koebnik *et al* 2000). General diffusion porins discriminate between solutes primarily on their size and charge, they are present in sufficient quantities that can be readily measured by polyacrylamide gel electrophoresis (Nikaido and Vaara 1985). On the other hand, Type II channels e.g OprD (minor outer membrane proteins or specific porins) are usually specific porins that are found in low concentrations. However, when induced the concentration of specific porins might reach above the concentration of normal protein constituents of the outer membrane (Nikaido *et al* 1991). Specific porins allow the diffusion of specific substrates that are larger than 600 Da, but they also allow non specific diffusion of small substrates (Braun 1994). Type II channels are homotrimers, each monomer have 18 antiparallel β-strands that forms a barrel with the third loop folding back inside the barrel forming a constriction. Most of the channel lining residues are conserved between different specific porins and between species but there are three positions at the constriction site that shows differences in amino acid sequence, which was recently found to determine the specificity for each channel (Nikaido and Vaara 1985; Koebnik *et al* 2000). Type III channels (e.g. TonB-dependent receptors), are proteins that are essential for uptake of large molecules (e.g. Siderophore-iron complex) (Braun 1994). The activity of these porins is dependent on the electrochemical potential of the cytoplasmic membrane and the protein complex TonB-ExbBD that is an energy transducer. Ferguson *et al* (1998) and Buchanan *et al* (1999) studied the Fhu-A receptor protein for iron-siderophore uptake in *E. coli*. They found that these receptors

have a plug-and-barrel organization. It is made of 22–stranded β-barrel and an N-terminal plug domain (cork) that is located inside the barrel. The plug domain is arranged in the barrel with the β-sheet plane inclined by ~ 45° to the membrane normal, therefore obstructing the channel interior. The presence of the plug domain suggests that the direct passage of the ferrichrome-iron complex and small molecules is not possible. The cork domain is connected to the barrell inner wall by extensive hydrogen bonding, and it delineates a pair of pockets within the Fhu A, a larger external pocket that opens to the external environment and a smaller periplasmic pocket. Transport of the ferrichrome-iron complex involves the primary binding to the Fhu-A receptor followed by translocation through the channel (Ferguson *et al* 1998). The opening of the channel is influenced by the energy transduced by the TonB system in the cytoplasmic membrane. Outer membrane proteins are of particular interest due to their involvement in transport of harmful substances and in anchoring structures that mediate adhesion and motility. Recently, *P. aeruginosa* genome was published and three large paralogous families of outer membrane proteins have been identified (Stover *et al* 2000). The OprD family of specific porins (19 genes), the TonB-family of gated porins and the OprM family of outer membrane proteins involved in efflux or secretion (18 genes) (Stover *et al* 2000).

1.5 Outer membrane proteins of P. aeruginosa

P. aeruginosa has a reduced permeability to small hydrophilic substances, although it has a relatively large exclusion limit for substrates. In comparison to other Gram-negative bacteria (table 1.1) their saccharide exclusion limits are approximately 3000 to 9000 Dalton (Hancock and Nikaido 1978) while other Gram-negative bacteria studied, e.g. *Salmonella typhimurium* and *E. coli* have exclusion limits of 600 to 700

Daltons. Most of the major outer membrane proteins of *P. aeruginosa* are either closed or non-functional. This in part, can be due to the fact that OprF (38 kDa), which is the major non-specific OMP, has poor efficiency in transport (Hancock 1998); it forms large and small porins. Large porins are less prevalent and only a fraction of them occur in an open conformation (Siehnel *et al* 1990; Nikaido 1998) and consequently, a small area is left for diffusion (Siehnel *et al* 1990) the characteristic that reduces its importance in controlling the permeability characteristics of the outer membrane. However, OprF is a bifunctional protein serving both as a porin and as a structural protein that maintains the integrity of *P. aeruginosa* cell and permits growth of the bacterium in low-osmolarity medium (Gotoh *et al* 1989; Siehnel *et al* 1990).

Iron regulating outer membrane protein (IROMP) (78-87 kDa), was found to be a receptor for iron-siderophores complexes (Hancock *et al* 1990). Siderophores are either produced by the bacteria or supplied exogenously by other sources (Hancock *et al* 1990). *P. aeruginosa* produce at least two of these siderophores, called pyochelins and pyoverdins, as well as two or more IROMPs (Hancock *et al* 1990). Sokol and Woods (1983 and 1986) showed that the ferripyochelin uptake in *P. aeruginosa* involves a unique low molecular weight (9-14 kDa) iron-regulated outer membrane protein, ferripyochelin binding protein (FBP). This protein belongs to the Ton-B dependent receptor group. The outer membrane protein OprC (70 kDa) and OprE (44 kDa) form small pores that allow the passage of solutes less than 250 Da (Yoshihara and Nakea 1989). Outer membrane protein G (OprG) is a specific inducible protein that appears under specific growth conditions, such as iron-sufficiency, high growth temperature, Mg^{2+} deficiency and growth into stationary phase (Hancock *et al* 1990).

OprP (48 kDa) is a phosphate inducible porin detected in strains cultured in media with low concentration of phosphate (0.15 mM or less). Studies on mutants revealed the importance of OprP as a component of the high affinity phosphate starvation- inducible phosphate specific transport system (Pst). It is a constricted (0.6 nm), anion-specific channel with 9 saturable phosphate binding sites that has 100-fold preference to phosphate over other anions (arsenate) (Siehnel *et al* 1990). Outer membrane protein D that has a molecular weight of 46kDais made of two proteins D1 (OprB) and D2 (OprD). OprB (46 kDa) is a specific channel for glucose as well as xylose. It allows the passage of small compounds like amino acids that have structural resemblance to glucose (Trias *et al* 1988). OprD (45 kDa) is a substrate selective porin that facilitates the diffusion of dipeptides that have one basic amino acid in addition to carbapenem antibiotics and their analogues (Nikaido *et al* 1991; Hancock 1998). Both D1 and D2 also allow non-specific diffusion of small solutes (Nikaido *et al* 1991). Outer membrane protein OprH (21 kDa) the smallest outer membrane protein, consists of 8 transmembrane β-strands. This protein was over-expressed when wild type cells were grown in media deficient in divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+} and Sr^{2+}) suggesting that it replaces divalent cations in the outer membrane thus stabilizing the LPS and blocking self promoted uptake of polycationic antibiotics (Rehm and Hancock 1996). OprL and OprI (9 kDa) are lipoproteins that are strongly associated with peptidoglycan (Hancock *et al* 1990). Esterase is a minor outer membrane protein with esterase activity. Esterase deficient mutants lack the ability to grow in Tween 80. The enzyme has specificity for long chain acyl-oxy or thio-esters with hydrophilic head groups (Hancock *et al* 1990). The outer membrane includes other proteins that act as enzymes, (e.g. phospholipase A and proteases) and also includes proteins involved in the biogenesis of flagella and pili (Koebnik *et al* 2000). The outer membrane is permeable to low molecular weight

hydrophilic substances. However, it is impermeable to the protons necessary to create the proton motive force (PMF) and to the hydrolytic enzymes and binding proteins that are found in the periplasm.

1.6 Mechanisms of biocide resistance in bacteria

The term resistance is a well-defined concept when applied to antibiotics. It refers to a bacterial strain that is not killed or inhibited by a concentration of antibiotic that is attainable in vivo (e.g. blood; Russell 2000). It also applies to a strain that is not killed or inhibited by an antibiotic concentration to which most strains of that organism are susceptible, and it applies to a bacterial cell that is not killed or inhibited by an antibiotic concentration that kills or inhibits the majority of cells in that culture (Russell 2000). However, when the term resistance is applied to biocides it is less clearly defined (Russell and Chopra 1996). Therefore, resistance to biocides can be defined as the state when a bacterial strain is not killed by a concentration of a biocide that is used in practice (Russell 2000). Some authors like to restrain the term "*biocide resistance*" to a case where the biochemical or genetic basis of resistance is known. Others would use the term "*tolerance*" where the basis of insusceptibility has not yet been established (Chapman *et al* 1998). In this thesis the term resistance will be used to describe the state of insusceptibility of *P. aeruginosa* to Pyrithione biocides.

1.6.1 Mechanisms of bacterial resistance

Bacteria utilize several ingenious mechanisms to develop resistance. These mechanisms are either specific or general. Specific mechanisms are directed against specific antimicrobials for example alteration of a drug target and degradation or inactivation of a drug by enzymatic modification. However, general mechanisms are

used by bacteria against any harmful substance (e.g. low outer membrane permeability) (Nikaido 1994). Resistance can be either a natural property of an organism that is inherited from generation to generation, thus, it is called intrinsic (Russell and Chopra 1992; Mcdonnell and Russell 1999) or it could be acquired. Acquired resistance can be through mutation or as a result of acquisition of genetic elements (plasmids or transposones) (Mcdonnell and Russell 1999). Adaptation of bacteria to a gradually increasing concentration of a biocide results in an unstable condition of resistance to that biocide, which reverts upon the removal of the inducing agent (Russell 2001).

1.6.2 Intrinsic mechanisms of resistance

1.6.2.a Outer membrane permeability

For a biocide molecule to reach its target in the Gram-negative bacterial cell, it has to cross the outer membrane. It is suggested that the outer membrane contributes to the resistance towards antimicrobials and that porin proteins are responsible for the molecular sieving of hydrophilic solutes into the periplasm (Nikaido and Vaara 1985). Solutes larger than the exclusion limit of *P. aeruginosa* (600 Da) are unable to cross the outer membrane barrier (Nikaido and Vaara 1985; Siehnel *et al* 1990). It is also suggested that development of resistance towards antimicrobials is related to the over-expression or loss of certain outer membrane proteins (Nikaido 1992; Hancock *et al* 1990; Winder *et al* 2000).

Porins of the outer membrane fit into three classes, general porins, that have minimal substrate selectivity, specific porins, that are substrate selective through receptor sites and the gated porins, which are channels that are normally closed but open upon binding with a specific substrate (Hancock 1997). The large porins of the general porin OmpF in *P. aeruginosa* are usually closed while the small porins have a very

reduced exclusion limit with low copy number which potentially explains the reduced permeability of the outer membrane of that species (Hancock 1998). Oxygen stress was found to cause OmpF repression accompanied with increased resistance to other antimicrobial agents (Nikaido 1994). OprD is a substrate specific porin which is specific for the β-lactam imepenem (Trias and Nikaido 1990). Buscher *et al* (1987) and Gotoh and Nishino (1990) reported that OprD (45 kDa) was missing from SDS-PAGE of outer membrane profiles of imepenem resistant *P. aeruginosa*. However, these strains were sensitive to other β-lactams, suggesting the role of OprD in controlling the influx of imepenem into the cell. β-lactam compounds designed to mimic iron chelating compounds (siderophores), were found to select for mutants that lack the siderophore transporter protein (Nikaido and Rosenberg 1990). A protein band of a molecular weight 35kDawas reported to be missing from the SDS-PAGE profiles of the outer membranes of isothiazolone (Kathon^{TM}) resistant *P. aeruginosa* PAO1 (Brozel and Cloete 1994). The authors suggested that outer membrane protein (T) might be the porin responsible for the influx of isothiazolones and that the wild type cells acquire the resistance by becoming adapted to the biocide through the disappearance of the 35kDa OprT (T-OMP) (Brozel and Cloete 1994; Winder *et al* 2000). Shand *et al* (1988) observed that as *P. aeruginosa* PAO1 grew in increasing concentrations of polymyxin B, it became more resistant to that antibiotic. Outer membrane protein profiles of polymyxin B resistant strains showed reduction of some OMPs such as porins D, E, F, and G and the absence of protein H1 which was later suggested to substitute the divalent cations on the LPS molecules thus, preventing self promoted uptake (Rhem and Hancock 1996). Alteration in outer membrane protein structure is an intrinsic mechanism of resistance and has been involved in biocide resistance such as isothiazolones.

1.6.2.b LPS as an intrinsic mechanism of resistance

LPS composition (Figure 1.2) of *P. aeruginosa* differs from other Gram-negative organisms, in the cation content of the outer membrane (Macdonnell and Russell 1999). The high negative charge on the LPS molecules provides a polyanionic external surface, thus rendering the outer membrane more hydrophilic. This increased hydrophilicity prevents the penetration of hydrophobic drugs into the cell (Macdonnell and Russell 1999; Hancock 1997; Nikaido 1990). The polyanionic nature of the outer membrane is partially neutralized by the high Mg^{2+} and Ca^{2+} content. Divalent cations form bridges between adjacent LPS molecules resulting in strong LPS-LPS linkages, which has a strong permeability-decreasing effect (Nikaido 1990). The use of ion chelators such as EDTA or polymyxin B, has been shown to render the bacteria hypersensitive to hydrophobic antibiotics (Nikaido 1990). Chelation of divalent cations causes electrostatic repulsion between the negatively charged LPS molecules. This repulsion results in the loss of some LPS molecules that will be replaced by phospholipid patches. The presence of phospholipid patches on the outer leaflet mediates the penetration of lipophilic molecules (Nikaido 1990). A second factor that contributes to the role of LPS in intrinsic resistance to antimicrobials is the reduced fluidity of the Lipid A region. Lipid A region is dominated by saturated straight chain fatty acids that tend to be less fluid due to close packing of the acyl chains (Nikaido 1988). However, less fluidity has also been attributed to the length of polysaccharide chains and to the LPS concentration. Rottem and Leive (1977) found that lipid A regions of outer membrane preparations of *E. coli* that contained long polysaccharide chains were much less fluid than preparations containing short O-side chains. These authors also reported that the higher the LPS concentration, the more restricted the mobility of the acyl chains in the lipid A region

(Rottem and Leive 1977). This observation could be due to the fact that polysaccharide chains can interact with each other. When the concentration of the chains increases, either through increased LPS concentration or increased polysaccharide length, it is followed by increased side interactions and greater rigidity of the outer leaflet of the outer membrane. Alterations in both the lipid composition and arrangement in LPS leading to a more stable and rigid outer membrane results in alterations in the permeability by preventing the binding or entrance of certain antibiotics (Dunnick *et al* 1970). Conrad and Galanos (1989) have reported that the loss of hydroxy fatty acids from LPS of *P. aeruginosa* perturbed the outer membrane hydrophobicity and was a contributing factor in polymyxin B adaptive resistance.

The presence of an intact LPS is important for offering the membrane stability required to achieve the impermeability state. Mutations that change the LPS structure alter the permeability of the outer membrane. Deep rough mutants (heptoseless mutants), which lack the O-side chain and most of the core polysaccharide, are hypersensitive to hydrophobic antibiotics and disinfectants (Macdonnell and Russell 1999), because of the loss of the high negative charge which was replaced by phospholipid patches (Hancock 1997).

LPS structure by itself composes an intrinsic mechanism used by the bacteria to resist hydrophobic antimicrobials. The polyanionic nature of the layer and the relatively immobile lipid A region makes it a very good permeability barrier.

1.6.3.c The bacterial efflux system

Active Multi-drug efflux systems, are one of the general intrinsic mechanisms of drug resistance (Nikaido 1994). They have recently been recognized as efficient mechanisms of resistance in *P. aeruginosa* (Nikaido 1998; Aires *et al* 1999). The

multi-drug efflux pump (Figure 1.3) is a three component organization: a transporter protein located in the inner membrane, a channel forming outer membrane protein and a periplasmic accessory protein (Zgurskaya and Nikaido 2000). The accessory protein sometimes called the membrane fusion protein (MFP), brings the two other components close together so that efficient efflux can occur. The outer membrane components are sometimes interchangeable between different multidrug efflux systems (Zgurskaya and Nikaido 2000). The simultaneous interaction of substrates with all three components appears to stabilize the complex, whereas, after translocation the complex disintegrates (Zgurskaya and Nikaido 2000). Efflux mechanisms work synergistically with the outer membrane barrier. As a result of the construction of the efflux system the few molecules that succeed in penetrating the outer membrane barrier into the interior of the cell are pumped out into the media without having to go through the impermeable outer membrane again (Nikaido 1998). The slow influx of various substrates through the specifically low permeablility outer membrane of *P. aeruginosa*, makes efflux an especially effective mechanism of resistance in that organism. This observed synergy explains why Gram-negative bacteria become hypersensitive to various drugs either by inactivation of efflux pumps or by permeabilization of the outer membrane (Zgurskaya and Nikaido 2000). This synergy is also apparent in the over-expression of the MexAB-OprM efflux system that is accompanied by the disappearance of OprD from the outer membranes of meropenem resistant *P. aeruginosa* (Kohler 1999).

The *P. aeruginosa* genome, like other bacteria, appears to contain a large number of undescribed drug efflux systems predominantly from the PMF-dependent multidrug efflux systems (resistance-nodulation-cell division family (RND), major facilitator superfamily (MFS) and small multidrug resistance (SMR) types), and ABC-transporters. The *P. aeruginosa* genome contains more of the Mex-type RND

multidrug efflux system than other identified bacteria (Stover *et al* 2000). The RND multidrug efflux systems (Figure 1.3) displays a much wider substrate specificity than other PMF-dependent multidrug efflux systems (Paulsen *et al* 1996), which could explain the observed resistance to a wide range of anti-bacterial agents that are associated with this species. Four RND multi-drug efflux systems have been identified in *P.aeruginosa* (Poole 2001). MexA, MexD, MexF and MexY proteins in *P. aeruginosa* mediate the proton-dependent–export across the cytoplasmic membrane. The genes coding for the RND proteins are frequently found in association with genes encoding proteins that belong to the MFP (membrane fusion proteins) family. MFP proteins (Figure 1.3) span the periplasmic space and interact with various constituents in both the cytoplasmic and the outer membrane. They are involved in allowing substrate transport from the inner membrane to the outer membrane by interacting with a third group of proteins known as the outer membrane factor (OMF). Adjacent to the MFP genes there appear the OMF genes that are found in most RND efflux operones (Stover *et al* 2000). OMF genes code for the outer membrane proteins that act as outer membrane channels and function cooperatively with the RND and MFP proteins (Paulsen *et al* 1996). Clinical isolates of *P. aeruginosa* are characterized by their frequent resistance to antibiotics and cross-resistance to chemically unrelated compounds (Schweizer 1998). It is believed that the major factor contributing to resistance towards hydrophobic and amphipathic compounds is the active efflux of

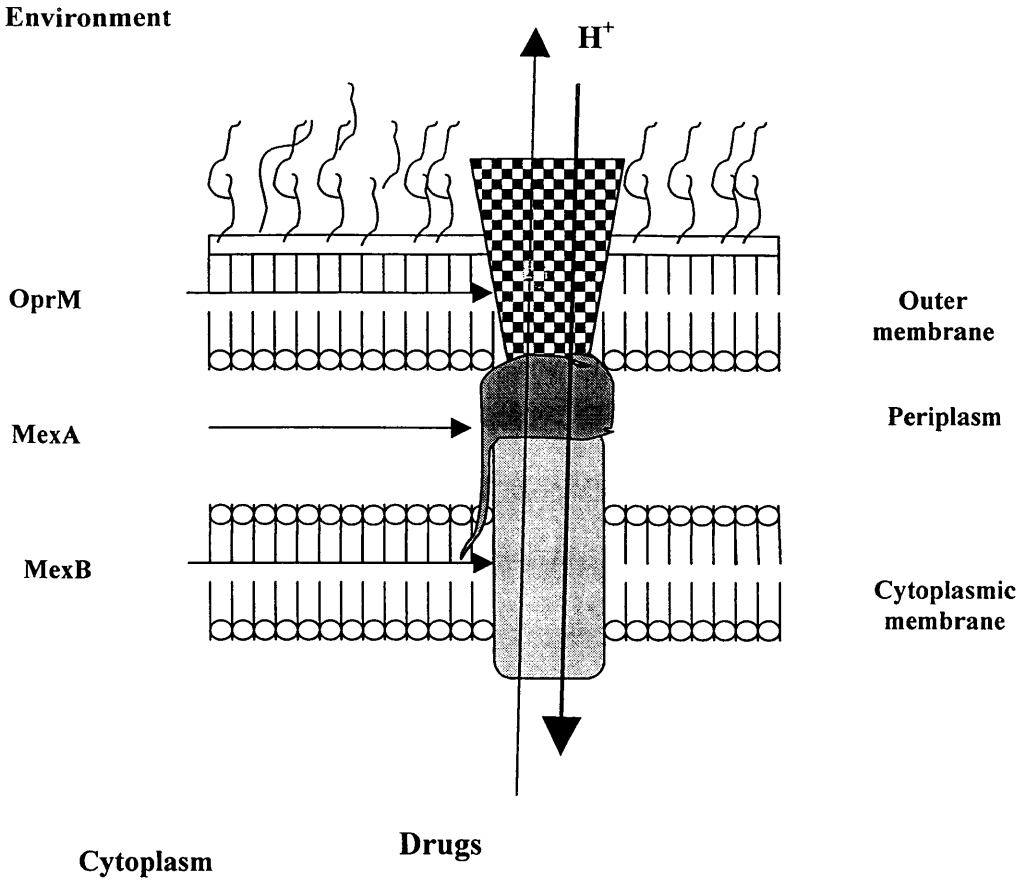


Figure 1.3: The MexAB-OprM multidrug efflux system in *P. aeruginosa.* MexB protein in the cytoplasmic membrane (RND family) utilizes the transmembrane proton gradient to export drugs out of the cell through the auxiliary constituent of the MFP family MexA. An additional outer membrane protein OprM allows the export of the drug to the outside environment. (Paulsen *et al* 1996).

these drugs outside the cells (Hancock 1998). Efflux pumps not only produce the base line level of intrinsic resistance, but can also produce a much higher level of resistance when over-expressed (Nikaido 1998). MexAB-OprM efflux system is constitutive in wild type cells and contributes greatly to the intrinsic resistance of *P. aeruginosa* to a wide range of antimicrobials. The former system confers resistance to floroquinolones, β-lactams (other than Carbapenems) tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim, and sulphonamides (Kohler *eal* 1999b) and to Irgasan and the fatty acid inhibitors thiolactomycin and Cerulinin (Schweizer 1998). MexXY-OprM lacks a link outer membrane gene (Poole 2001), thus it shares the outer membrane component with the former system (Aires *et al* 1999), when expressed it exports aminoglycosides, tetracyclines and erythromycin. MexCD-OprJ is expressed as a result of a mutation in the *nfxB* regulator gene that occurs upstream of the *mexCD-OprJ* operon (Poole 2001). Expression of the former efflux system confers resistance to quinolone antibiotics, macrolides, chloramphenicol, novobiocin, tetracycline, trimethoprim and β-lactams of the 4th generation (Poole 1996). MexCD-OprJ system is distinguished from the MexAB-OprM in the ability of the former to export additional β-lactams such as carbenicillin and aztreonam (Poole 2001). MexEF-OprN is inactive in wild type *P. aeruginosa*, it is expressed by a mutation in the *mexT* gene that is a positive regulator of that system and at the same time is responsible for the decrease in OprD expression in the *nfxC* mutants (Poole 2001). Expression of the former system renders the bacteria resistant to imipenem but slightly enhances susceptibility towards β-lactams and aminoglycosides.

The previously discussed efflux systems described intrinsic mechanisms of resistance that are encoded by chromosomal genes that constitute part of the normal

genome of the cell. These genes are activated by induction or mutation caused by stress of exposure to antimicrobials in clinical or natural environment.

1.6.2.d Periplasmic Enzymes

Low outer membrane permeability will slow down the uptake rate into the periplasm, but will not prevent uptake. A secondary intrinsic defense mechanism such as antimicrobial degrading enzymes is required for that purpose. The periplasmic area in Gram-negative bacteria contains a large number of proteins that serves several functions. Detoxifying enzymes are capable of degrading or modifying antimicrobial agents in such a way to render the agent harmless. *P. aeruginosa* have, in their periplasmic space, several detoxifying enzymes that are capable of degrading toxic antimicrobials, for example, β-lactamase, aminoglycoside-2′-adenylase and alkyl sulphohydrolase. β-lactamase enzymes are required to prevent the build up of β-lactams in the periplasm of *P. aeruginosa*. Thus, resistance to β-lactams in *P. aeruginosa* is a combination of efflux, low outer membrane permeability and hydrolysis of the incoming antimicrobial. Resistance towards Formaldehyde and formaldehyde releasing biocides in *P. aeruginosa* has been attributed to the enzyme formaldehyde dehydrogenase (Chapman *et al* 1998). Isolates of *P. aeruginosa* resistant to formaldehyde displayed cross-resistance to other formaldehyde-releasing biocides (Chapman *et al* 1998). Antimicrobial detoxifying enzymes in the periplasm of *P. aeruginosa* are a specific, as well as an intrinsic, mechanism of resistance exhibited by this pathogen.

1.6.2.e Phenotypic Adaptation as an Intrinsic Mechanism of Resistance

The association of microorganisms with solid surfaces leads to the generation of biofilms. Biofilms are adaptive changes that allow microorganisms to persist despite apparently effective antimicrobial therapy (Bryan 1991). A biofilm is defined as a consortium of organisms organized within an extensive exopolysaccharide (EPS) polymer which provides a strong and a sticky network (Macdonnell and Russell 1999). Within 5 minutes of adhesion of an individual cell to an inert surface the *algC* gene, that produces phosphomannomutase enzyme (alginate synthesis pathway in *P. aeruginosa*), is upregulated, facilitating the process of biofilm formation through the production of alginate (Costerton 1995). Cell adhesion is followed by the formation of microcolonies (Costerton 1995). Microcolonies may be composed of cells of a single species or of different species, but groups of cells of a single species are delineated by their exopolysaccharide matrix, which holds them in stable juxtaposition and regulates their effective contact with the fluid surrounding them (Costerton 1995). Deretic *et al* (1989) found the *algC* gene to be homologous to sequences of a class of environmentally responsive genes, which are upregulated by a special sigma factor that is produced by the *algU* gene. This finding suggested that the biofilm cells are different from planktonic cells. The outer membranes of biofilm cells and planktonic cells differ profoundly in their protein profiles. This finding suggests that the cassette of proteins found in the outer membranes of biofilm cells, are triggered by the special sigma factor indicating that these proteins could be responsible for changes in the cell wall permeability, which could contribute to resistance of biofilms to antimicrobials (Costerton 1995). Slow growing bacteria within a biofilm are less susceptible to the effect of antimicrobials (Nichols *et al* 1989). This reduced susceptibility is due to reduced access of the antimicrobial to the cells within the biofilm as a result of either

reduced cell walls permeability or to the presence of EPS. Production of degradative enzymes by the cells in the biofilm could contribute to reduced susceptibility to antimicrobials. *P. aeruginosa* was found to increase β-lactamase production in biofilms (Brooun *et al* 2000). Another factor that contributes to reduced antimicrobial susceptibility is the genetic exchange between the cells in the biofilm that results in spread of resistance between different species (Macdonnell and Russell 1999). Recently the emergence of super-resistant cells within a biofilm has been considered as a factor that could result in antimicrobial insusceptibility. Super-resistant cells would remain in the biofilm structure protected by the EPS matrix and will be responsible for regrowth in the biofilm after antibiotic treatment (Brooun *et al* 2000). *P. aeruginosa* cells liberated from a biofilm into growth medium were considerably more resistant to antibiotics (tobramycin) than planktonic cells, suggesting that cells become intrinsically more resistant when growing in the biofilm and retain part of this resistance even outside the biofilm (Brooun *et al* 2000).

The best example of resistance in biofilms to the effect of antimicrobials, is the colonization of *P. aeruginosa* in lungs of cystic fibrosis patients. Initially, the invading *P. aeruginosa* is non-mucoid, but during the course of the disease it changes to the mucoid alginate producing phenotype (Deretic *et al* 1989). The ability to synthesize alginate enhances the colonial and survival properties of the organism by increasing its ability to form a biofilm and thus, protect itself from attack of antibodies (William *et al* 1996).

Many of the genes transcribed during biofilm formation have been found to encode for surface structures (e.g. Pili and flagellae). Flagellae have been associated with the active movement of cells to the substratum which is a critical step before

attachment and for the initial attachment, pili and Fimbriae, on the other hand, are necessary for the irreversible attachment of cells to the surface (Moore *et al* 2001).

Biofilm formation is a powerful intrinsic mechanism adopted by bacteria to withstand harsh environmental conditions through involving multiple mechanisms of resistance as well as the production of the thick layer of EPS that covers different communities of bacterial species and renders it inaccessible to antimicrobials.

1.6.2.f Stress Response as an Intrinsic Mechanism of Resistance

It is well known that bacteria are able to overcome some physical or chemical stresses by qualitative and quantitative adjustments in protein synthesis. Most normal protein bands in SDS-PAGE of cell lysates disappear or fade after a heat shock, while others become dominant or appear as clear bands (Macario 1995). Proteins that become prominent are called heat shock proteins (Hsps). The synthesis or over-expression of stress proteins allows the bacteria to face harsh conditions through assisting in the normalization of cellular functions during recovery (Forreiter and Nover 1998). Some antimicrobials, for example biocides (Allan *et al* 1988; Watson 1990; Vasseur *et al* 1999), amino acid analogues and protein-synthesis inhibitors (Watson 1990) are considered to be stressors. In fact, the chemical stress response mounted by the bacteria as a result of chemical contaminants in the environment have tempted scientists to use this response as a biomarker, due to its sensitivity to the concentration of micropollutants (stressors) (Mason *et al* 1994).

The term heat shock protein (Hsp) is used to describe any anti-stress protein, whether it was heat induced or induced by any other stressor. Most of the stress proteins are constitutively present in the cell, such proteins are referred to as heat shock cognate (Hsc), but they are markedly induced under stress conditions, others are synthesized in

response to a stressor (Forreiter and Nover 1998). Hsps are usually classified on the basis of their approximate molecular weight, thus, stress proteins are referred to as Hsp followed by a number that indicates its molecular weight in kD.

Some of the stress proteins are molecular chaperones. Chaperones are molecules that assist proteins to acquire the correct folding while they are being synthesized in the ribosome or shortly after. They help proteins in regaining the correct folding after being partially denatured due to a cell stressor, and help proteins destined for transport reach their final destination in the cell through maintaining a partially unfolded form, since fully folded proteins cannot be translocated through membranes (Forreiter and Nover 1998). Molecular chaperones also stabilize damaged proteins generated as a result of chemical or physical stress and thus, facilitate renaturation or degradation in the recovery period, they protect cellular peptides from denaturation by preventing aggregation and aid in the disassembly of protein aggregates (Macario 1995; Forreiter and Nover 1998). Members of Hsp families act together in multisubunits complexes called chaperone machines and the activity of such complexes consumes ATP and ADP. In prokaryotic cells Hsps are located in the areas where protein synthesis occurs (cytoplasm) and where the transport of proteins occurs (cytoplasmic membrane and periplasmic area) (Macario 1995).

Heat shock proteins have been extensively studied in *E. coli*. GroEL (Hsp60) and GroES (Hsp10) proteins are the product of the *groEL* and *groES* genes. The two proteins interact functionally in the presence of ATP. Proteins of this group are called chaperonins (Macario 1995). DnaK (Hsp70), DnaJ (Hsp 40) and GrpE (Hsp23) are the second most abundant heat shock family of Hsps in *E. coli*. These proteins are highly conserved between species and they interact together in many cellular functions, such as thermotolerance, processing of newly formed proteins in the cytoplasm and translation

initiation (Forreiter and Nover 1998). Protein 32kDa is a sigma factor that is the product of the *rpoH* gene in *E. coli*. *In vitro* RNA polymerase holoenzyme containing σ^{32} initiates transcription from heat shock promoters, that have a consensus sequence which is different from that utilized by the major σ^{70} in that species (Grossman *et al* 1987). In *P. aeruginosa* three major heat shock proteins of similar size to major Hsps in *E. coli* were identified: GroEL-like protein (61 kDa), DnaK-like protein (76 kDa) and σ^{87} protein (Allan *et al* 1988). A heat shock sigma factor (σ^{40}) was identified in heat shocked cells of *P. aeruginosa* (Allan *et al* 1988).

The bacterial response to stress varies according to the nature and magnitude of the stressor (Allan *et al* 1988; Vasseur *et al* 1999). Heat shock treatments of *E. coli* resulted in a distinct set of 17 proteins that reached the maximum between 5 and 10 minutes after temperature upshift and then declined to a new rate of synthesis. The new rate is characteristic of the higher temperature and greater than the rate at the lower temperature (Grossman *et al* 1987). Stress response mounted in *E. coli* against cadmium chloride induced only six of these proteins and only one of the 17 proteins is induced in response to ethanol (Watson 1990). *Pseudomonas fragi* was found to express a whole array of low molecular weight stress proteins when subjected to various stressors, such as acid shock, osmotic and chemical shocks and biocide shocks (Michel *et al* 1996). Most of these proteins belong to the General stress proteins (Gsp) which are thought to provide a non-specific protective function (Vasseur *et al* 1999).

Stress response is an intrinsic reaction exhibited by the bacterial cell to help it recover and overcome the detrimental effects of various environmental conditions, including antimicrobials. It could contribute to cell survival by rapid adaptation of cell machinery to transient variations in the environment.

1.6.3 *Acquired Biocide Resistance*

Acquired resistance to biocides results from genetic changes in a bacterial cell and occurs either by mutation or by the acquisition of genetic material from another cell either by plasmids or transposons.

1.6.3.a Plasmid mediated resistance in Gram-negative bacteria

There is scant evidence to suggest that plasmid acquisition *per se* is a major factor in conferring insusceptibility to biocidal agents (Russell and Day 1999). However, plasmid mediated resistance to biocides has been mostly studied in metals and especially mercury, although such compounds do not have a major or practical applications as antibacterial agents. Resistance to mercuric compounds has been found to be inducible and may be transferred by conjugation or transduction (Russell 1997). Plasmids conferring resistance to mercurials are either narrow spectrum or broad spectrum. Narrow spectrum plasmids encode resistance to Hg^{2+} and some organomercurials such as merbromin and fluorescein mercuric acetate in *E. coli* and slight resistance to P-hydroxy-mercuribenzoate (PHMB) in *P. aeruginosa*. However, broad-spectrum plasmids encode resistance to the former compounds and phenylmercuric nitrate (PMN) and acetate (PMA), thiomersal and PHMB (Russell *et al* 1999). Plasmids carrying genetic determinants for heavy metal resistance sometimes carry genes specifying antibiotic resistance and in some instances resistance to cobalt, cadmium and arsenate (Macdonnell and Russell 1999; Nies and Silver 1995). The cation efflux system (Figure 1.4) identified in Gram-negative bacteria is plasmid mediated (Nies and Silver 1995). The CzcABC system transports four cations out of the cell Cd^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. The efflux process is catalyzed by the CzcA, CzcB and CzcC proteins. One ion of Zn^{2+} is pumped out of the cell at the expense of two

protons pumped in. The complete system increases the MIC for Zn^{2+} and Co^{2+} by 100-fold (Nies and Silver 1995). Plasmid R773 of *E. coli* was found to encode genes for arsenate efflux system (Nies and Silver 1995). The *arsRDABC* operon confers resistance to arsenate in a clever way. The *ars* determinants that are carried on the plasmid are; *arsB* genes that codes for ArsB protein an arsenic-specific tunnel, *arsC* gene that codes for arsenate reductase protein, which reduces toxic arsenate to arsenite and the *arsA* gene that codes for ArsA protein, which is an arsenite dependant ATPase, that increases the velocity of arsenite efflux. Arsenate is toxic to the cell, but at the same time it has resemblance to phosphate ions. In order to avoid pumping phosphate outside the cell the arsenate is reduced to arsenite (which has no structural analogues in the cell) which will be pumped outside. ArsR protein the product of the *arsR* gene is a transcriptional regulator. The binding of the arsenite to the metal binding site of the regulator will prevent it from binding to DNA thus the gene is suppressed only in the absence of the metal ion. The ArsD protein that is the product of the *arsD* gene is another regulator carried on the same plasmid and function as a feedback inhibitor on the level of transcription initiation (Nies and Silver 1995). Megaplasmids in *Alcaligenes eutrophus* carry the CzcABC efflux system that confers resistance to zinc, cobalt and nickel by active efflux (Nies and Silver 1995).

High levels of disinfectant resistance have been reported in hospital isolates but there has been no clear-cut evidence of plasmid involvement in the transfer of this resistance. In fact it has been shown that transfer of QAC and chlorhexidine resistance under normal conditions is difficult and that plasmid-mediated resistance to these agents in Gram-negative bacteria is an unlikely event (Macdonnell and Russell 1999). On the contrary, it was observed that bacteria harboring antibiotic resistant plasmids exhibited higher sensitivity towards Cetrimide and chlorhexidine (Michel-Brand *et al* 1986).

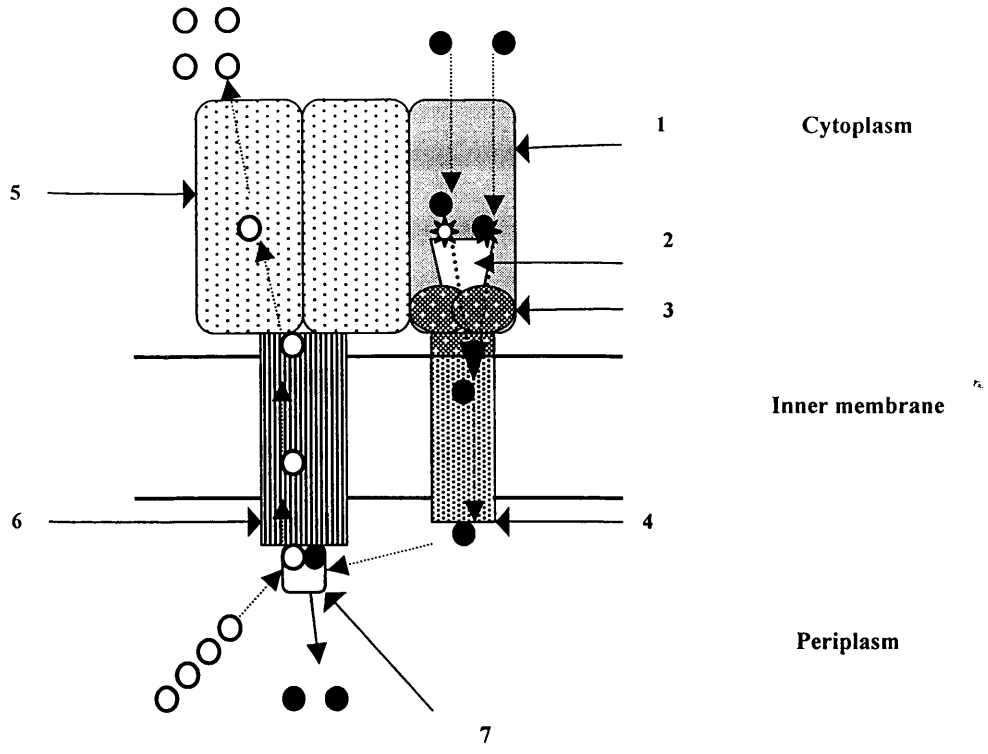


Figure 1.4: Model for Zn^{2+} efflux through the CzcABC efflux system. Zn^{2+} ● is bound to either of two metal binding sites **(2)** in the CzcB **(1)** subunit. It is then released into the cation tunnel **(4)** through the CzcC subunit (4). Two protons O are imported per one Zn^{2+} cation through the proton tunnel **(6)**. The two protons are imported into the cytoplasm via the transmembrane protein CzcA **(5)**. A potential metal-binding site **(7)** in the periplasm, coordinates the proton and cation transport. (Nies and Silver 1995).

However, there is evidence that some plasmids are responsible for producing surface changes in cells, which in turn may modify sensitivity or resistance to certain antimicrobials. Kenward *et al* (1978) reported that plasmid RP1, that is known to confer resistance to carbencillin, neomycin, kanamycin, cephaloridine and tetracycline, induced cell wall changes when transferred to *P. aeruginosa*. The $RP1^+$ strain resisted the chelating action of ethylene-diaminetetraacetic acid (EDTA) suggesting that the cations might have been relocated at a deep site within the cell wall. Cold shock had less effect on $RP1^+$ strains than $RP1^-$. Cold shock exerts its lethal effect through loss of permeability control on the cell envelope and the cytoplasmic membrane. Thus, the reduced cold shock effect indicates a change in the outer membrane permeability that has resulted from the presence of the RP1 plasmid (Kenward *et al* 1978). These results were compatible with the results of Rossouw *et al* (1984), who found that the acquisition of tetracycline resistant plasmid R124 by *E. coli* reduced the level of major OmpF expression along with reduction in KDO (LPS) content. The previous changes resulted in increased resistance to Cetrimide and to increased lysis by lysozyme and EDTA (Rossouw *et al* 1984). The authors suggested that the R124 plasmid could be interfering at the level of transcription or translation of the OmpF or affecting the processing of the *ompF* gene product (Rossouw *et al* 1984). Bacterial isolates derived from toxic chemical waste frequently contained plasmid DNA that encodes resistance to various antibiotics; such strains exhibited resistance to non-antibiotic agents such as dibutylphthalate, nitrobezane, 4-nitroaniline (Russell and Day 1996).

It has been shown that certain detergents exhibits preferential toxicity against plasmid bearing cells or stimulate plasmid curing or inhibit conjugation for example, Phenylalcohol and hexachlorophane inhibit the production of transconjugates while polycationic detergents can inhibit conjugation (Viljanen and Boratynski 1991). The

previous findings could contribute to the unlikeness of plasmids involvement in biocide resistance.

1.6.3.b Chromosomal mutations

Chromosomal mutations leading to biocide resistance are unstable and tend to revert upon removal of the biocide (Russell and Furr 1977). It is known that step-wise exposure of bacterial cells to biocides can lead to biocide adaptation, which can be lost by back mutation upon removal of the biocide (Peckey and James 1974; Russell and Furr 1977; Jones *et al* 1989; Winder *et al* 2000). Triclosan^{TM}, which is a general antibacterial and antifungal agent, was recently found to block lipid biosynthesis in bacteria by specifically inhibiting the enzyme enol-acyl carrier protein reductase (ENR), through mimicking the enzymes natural substrate (Levy *et al* 1999). Triclosan resistance in *E.coli* was investigated at the molecular level by Levy and coworkers, and was found to be the result of a mutation that involved single amino acid substitution in the ENR enzyme. Mutants with the substitution at position 93 (which is the residue that have direct contact with Triclosan) of the enzyme amino acid sequence exhibited the highest level of resistance (100-fold increase in MIC) towards this biocide (Levy *et al* 1999). However, Triclosan resistance has also been attributed to the MexAB-OprM efflux system in *P.aeruginosa.* Strains lacking the MexAB-OprM efflux pump fail to grow on the PIA (*Pseudomonas* isolation agar) that has Triclosan as its selective active ingredient (Shweizer 1998).

Multiple-drug resistance (MDR) in Gram-negative bacteria is a term used to describe resistance mechanisms acquired by chromosomal genes, e.g. the *mar* regulon (George 1996). Products of the multiple antibiotic resistant (*mar* regulon) genes are regulatory proteins that confer multiple antibiotic resistance through decreasing influx

of antimicrobials or increasing their efflux (Alekshun and Levy 1999). These regulatory proteins down-regulate the synthesis of outer membrane proteins and causes over-expression of efflux genes (Alekshun and Levy 1999). Studies on the *mar* regulon of *E. coli* have shown that antibiotics, oxidative stress and chemicals that contain phenolic rings induce it. The *E. coli mar* regulon consists of two transcriptional regions, *marC* and *marRAB*. The expression of *marRAB* operon is regulated by the MarR protein which is a DNA–binding protein. When the *marRAB* is uninduced the MarR protein binds to a site within the *marO* operator and prevent the expression of the *marRAB* regulon. However, when *marRAB* is induced by tetracycline, chloramphenicol, sodium salicylate or other compounds, the MarR protein is prevented from binding to DNA allowing the expression of the *marRAB* operon. MarA protein is a transcriptional activator that controls the level of expression. MarA regulates the expression of many other genes resulting in a variety of phenotypes. Moken *et al* (1997) found that low concentration of the disinfectant pine oil over-expressed the *marA* gene and selected for *E. coli* mutants that demonstrated low level of antibiotic resistance (tetracycline, ampicillin, chloramphenicol and naladixic acid). Antibiotic-selected MAR mutants which over-expressed *marA* are resistant to pine oil (Moken *et al* 1997).

At least three mutational alterations at *nalB*, *nfxB* and *nfxC* cause MAR mutations in *P. aeruginosa.* Mutations in the *nalB* site resulted in derepression of the *mexAmexB-oprM* operon leading to the overproduction of OprM and to cross-resistance to quinolones, meropenem, cephems (Masuda *et al* 1995). On the other hand a knock out mutation in the *mexAmexB-oprM* operon increases the susceptibility to these drugs (Hancock 1998). In *nfxB* type mutation cross-resistance to all quinolones and new cephems e.g. cefpirom and cefozopran is exhibited and the outer membrane protein OprJ is overexpressed. However, in the *nfxC* type mutant the overproduction of outer

membrane protein OprN accompanied by the suppression of OprD, which is a carbapenem-specific porin. Hence, this mutation resulted in cross-resistance to carbapenems and quinolones (Masuda *et al* 1995).

Multiple drug resistance occurs as a result of an inducer, most probably an antibiotic or a biocide. However, it has been shown recently (Clark 2001) that substances other than antimicrobials can act as inducers of *mar* operons and that, in the absence of that specific inducer, bacteria would revert to their susceptible state. In fact there has been a debate about the role of biocides as inducers of the *mar* regulon resulting in the emergence of multiple antibiotic resistance in hospitals. Russell (2000) has recently reviewed studies that correlate biocide resistance with antibiotic resistance. The author found that concentrations of biocides that selected for a low level of multiple antibiotic resistance were lower than biocide concentrations used in practice. Thus, so far, there hasn't been a clear proof for a significant role for biocides in selecting for antibiotic resistant strains.

1.7 Pyrithione Biocides

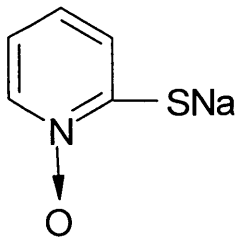
Pyrithione (1-hydroxypyridine-2-thione) is a synthetic analogue of the naturally occurring fungal antibiotic asperigillic acid (Shaw *et al* 1950). It is the parent molecule of Sodium and Zinc salts (NaPT and ZnPT) (Figure 1.5 a and b), also known as Sodium and Zinc Omadine (Nelson and Hyde 1981). Pyrithiones are known to be potent antimicrobials (Pansy *et al* 1953) and excellent chelating agents, the most well known of these chelates is the zinc complex that is used in antidandruff shampoos (Nelson and Hyde 1981).

1.7.1 Chemical and physical properties

Sodium2-pyridinthiol-1-oxide or Sodium Pyrithione (C_5 H_4 NOSNa) (Figure 1-5a) has a molecular weight of 149.15 Da. It is a white to yellow crystalline powder with a mild odor. It has a melting point of 250ºC. NaPT is highly soluble in water (>500g L^{-1}), soluble in ethanol (>100g L^{-1}), polyglcol, polyethylene glycol, DMSO and moderately soluble in isopropanol (Table 1.2). When solubilized it exists as pyrithione anion (PT^-) and sodium cation (Na^+) (Nelson and Hyde 1981). In 2% aqueous solutions it has a pH of 8. It is stable in solutions having a pH ranging between 4.5 and 9.5. Below pH 4.5 it is converted to free pyrithione, which is unstable in the presence of oxygen or light. Above pH 9.5 slow conversion to the sodium salt of pyrithione sulphinic or sulphonic acid occurs. Oxidizing agents convert pyrithione to inactive pyrithione sulphinic or sulphonic acid, while reducing agents attack the N-oxide group and reduce the NaPT to 2-mercaptopyridine, which is less active than NaPT (Paulus 1993). The oral LD_{50} in rats is 875 mg Kg^{-1} and 1172 mg Kg^{-1} in mouse (Nelson and Hyde 1981).

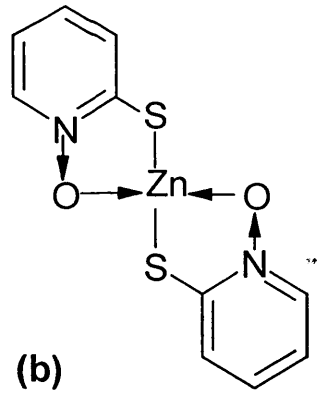
Zinc-bis- (2-pyridine-thiol-1-oxide) or Zinc Pyrithione ($C_{10}H_{18}N_2O_2S_2Zn$) (Figure 1.5b) is a white to yellow crystalline powder of a mild odor having a melting point of 240ºC, with low solubility in water (0.02 g L^{-1}), acetone (0.7 g L^{-1}), ethanol (0.1 g L^{-1}), polyethylene glycol (2.0 g L^{-1}) and good solubility in DMF (80 g L^{-1}) (Table 1.2). In a 10% water solution it has a pH of 6.5 and it is usually stable between pH 4 and 8. Below pH 3.5 it converts to free pyrithione, which is insoluble in water and forms a precipitate. Above pH 8 it is converted to a highly soluble alkali salt (Paulus 1993). ZnPT can undergo complex formation with certain organic amines, which results in its increased solubility as in the case with hair care products (Black and Howes 1978). ZnPT is

Sodium pyrithione

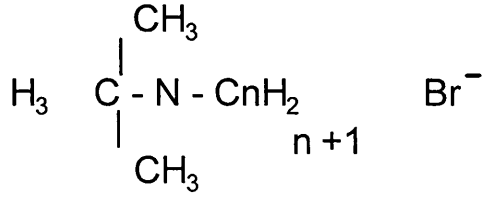


(a)

Zinc pyrithione



(b)



(n=12 , 14 or 16)

(c)

Figure 1.5: The chemical structure of **a**) Sodium Pyrithione **b**) Zinc Pyrithione **c**) Cetrimide.

sensitive to light and to strong oxidizing and reducing agents (Paulus 1993). ZnPT may also exist as a dimer where zinc atoms of the two monomers have valancies of five, allowing two monomers to become adjoined *via* zinc-oxygen bridges (Dinning *et al* 1998a). Toxicity trails have shown that the oral LD_{50} in rats is 180 to 200 mg Kg^{-1} and 300 mg Kg^{-1} in mouse (Nelson and Hyde 1981).

1.7.2 Anti-microbial Effectiveness and Applications

Sodium pyrithione (NaPT) is widely used in preservation. It is known to be a potent antimicrobial agent (Pansy *et al* 1953; Hyde and Nelson 1984). Table1.3 demonstrates its wide range of anti microbial activity (Hyde and Nelson 1981). The zinc salt (ZnPT) is an effective antifungal agent and a strong antidandruff compound (e.g. Head and Shoulders ™ shampoo). Pyrithiones are widely applied in cosmetics and fuel industries as a general preservative. Cooney (1969) reported that zinc pyrithione incorporated in polyurethane foams used for fuel tank coating retarded the rate of bacterial growth, but had little effect on total populations. In the same study, Sodium and Zinc Pyrithione were shown to cause a marked decrease in fungal growth in different fuel systems (Cooney 1969). NaPT and ZnPT have been used in cosmetic and pharmaceutical preparations intended for topical use (Khattar and Salt 1993). These products exhibit high activity against a broad spectrum of microorganisms of interest (Table 1.3), they are compatible with most cosmetic ingredients, safe for topical use and are biodegradable (Nelson and Hyde 1981).

1.7.3 Mode of Action of PyrithionesPyrithiones are known to be strong chelating agents. Thus, they are used in the mining industry for extraction of metals from ore

Table 1.2: Solubilities of Pyrithione biocides Technical-Grade Material (wt/wt % at 25°C) (Nelson and Hyde 1984).

Solvent	Zinc Pyrithione Powder	Sodium Pyrithione powder
Water (pH7)	0.0015	53
Ethanol, 40A	0.01	19
Isopropanol	0.008	0.8
Propylene glycol	0.02	13
Polyethylene glycol 400	0.02	12
Poly-Solv EM[b]	0.09	32
Poly-Solv DE[b]	0.01	12
Mineral oil, light	<0.0001	< 0.0001
Solulan 98[c]	0.1	1
Olive oil	<0.0003	< 0.0005
Castor oil	<0.0003	< 0.0005
Isopropyl myristate	<0.0001	< 0.0001
Isopropyl palmitate	<0.0001	<0.0001
Tween 40[d]	0.1	~ 4
Duponol WAQE[e]	-	~ 24
Sipon LLS[f]	-	~ 31
BioSoft EA-8[g]	0.07	~ 9

[a] Approximate solubilities, [b] Olin corporation, [c] Amerchol Unit of CPC International.[d] ICI United States, Inc., [e]E. I.Du Pont de Nemours & Co. [f] Alcolac Inc., [g] Stepan Chemical.

samples (Edrissi *et al* 1971). Several studies have pointed out its membrane activity (Chandler and Segel 1978; Khattar and Salt 1993; Ermolayeva and Sanders 1995; Al-Adham *et al* 1998; Dinning *et al* 1998a; Dinning *et al* 1998b; Dinning *et al* 1998c). Chandler and Segel (1978) and Ermolayeva and Sanders (1995) investigated the effect of pyrithiones on *Penicillium* species and the ascomycete *Neurospora crassa* respectively and found that pyrithione antifungal effect resides in its ability to inhibit transport across the membrane and to decrease cellular ATP. Their results suggested that active pyrithione is the unionized molecule that acts as a proton conductor. The uncharged molecule diffuses across the membrane and ionizes intracellularly, thereby collapsing a transmembrane Δ pH driving force. Thus, the inhibition of protein synthesis by pyrithiones results from diminished supply of ATP. Khattar and Salt (1993) studied the effect of sub-inhibitory concentrations of NaPT on the growth of *Klebsiella pneumoniae* and was found to induce an extended lag phase in the growth cycle of that species. The same concentration inhibited thymidine uptake completely and uridine uptake reversibly which suggested that the cell is probably using endogenous sources for thymidine since the cells resumed controlled log phase growth after the extended lag phase, thus excluding nucleic acid synthesis as a target for pyrithione activity. However, the chelating activity of pyrithiones could be starving intracellular enzymes of important cations like Zn^{2+} and Mg^{2+}, which explains the reduction in uridine uptake and thus, possibly affecting RNA synthesis (Khattar and Salt 1993). The previous theory may explain the reduction in protein synthesis observed by Chandler and Segel (1978). In a study conducted by Dinning *et al* (1998c), a spectrophotometric assay was used to determine the distribution and concentration of pyrithiones in subcellular components of *P. aeruginosa*. The authors found that ZnPT was present in both the cytosol and the outer membrane of *P. aeruginosa,* while NaPT

Table 1.3: Antimicrobial activity of Pyrithiones. Minimal inhibitory concentrations (MIC), micrograms ml^{-1} (ppm) (Nelson and Hyde 1981).

Microorganisms	ATTC No.	Zinc Omadine powder*	Sodium Omadine Powder
Gram-Positive Bacteria			
Bacillus cereus	14579	4	16
Staphylococcus aureus	6538p	4	1
Streptococcus faecalis	19433	16	2
Gram-Negative Bacteria			
Escherichia coli	11229	16	8
Pseudomonas aeruginosa	10145	512	512
Salmonella typhimurium	13311	16	64
Molds			
Aspergillus niger	9642	2	2
Penicillium vermiculatum	1124	1	2
Trichophyton mentagrophytes	9129	≤ 0.25	0.5
Yeast			
Candida albicans	10231	≤ 0.25	4

*Because of the low solubility of ZnPT in water, the starting concentration was prepared in dimethylsulfoxide (DMSO).

was only present in the cytosol. This result suggested that NaPT did not bind the envelope of *P. aeruginosa* while ZnPT did. Thus, it could be that the Pyrithiones interact reversibly with the cell envelope by binding with the phosphatidylethanolamine head groups and simultaneously chelating the metal cations on the cell envelope causing the disruption of the cell envelope. Membrane disruption is followed by the passive diffusion of the free pyrithione molecules into the cell. Pyrithione molecules react with the phosphate head groups in the inner membrane resulting in the chelation of the zinc ions and the release of the perthiolate anions that would in turn chelate important metal ions from the cytosol (Dinning *et al* 1998a and b).

Results from several studies have shown that Pyrithione biocides have an intracellular as well as an outer membrane activity. The membrane activity is similar to that of QACs. It is proposed that the mode of entry into the cells be through chemical structures produced by the cell that would form chelates with pyrithiones facilitating their entry to the cytosol (Dinning *et al* 1998c).

1.8 Cetrimide (Cetavlon^{TM})

Cetrimide (cetyltrimethylammonium bromide, CTAB) (Figure 1.5c) is a cationic surface-active agent. It belongs to the quaternary ammonium compound group(QAC), which is defined as molecules with two different structural groups, one being a hydrophobic water-repellant group and the other is a hydrophilic water- attracting group (Paulus 1993). They may be considered as organically substituted ammonium compounds, in which the nitrogen ion has a valancy of five. Four of the substituted radicals (R^1–R^4) are alkyl or heterocyclic radicals and the fifth (X^-) is a small anion. In the case of Cetrimide, the R chains are long chains (n = 12, 14 or 16) which give it a

good antimicrobial activity and the X is a bromide anion (Figure 1.6c) (Russell *et al* 1999).

1.8.1 Chemical and Physical Properties of Cetrimide

Cetrimide is produced as crystals that are almost colorless and odorless. It has a melting point of 250 to 256°C. It is soluble in water, highly soluble in alcohols, poorly soluble in acetone and virtually insoluble in non-polar solvents. It is stable in acid solutions and incompatible with a wide range of chemical agents (e.g. anionic detergents, peptides, zinc salts, polymeric phosphates, pectins and strong oxidizing and reducing agents) (Paulus 1993). The application of 10 and 20 mg Kg^{-1} day^{-1} with water for one year in rats caused no effect while 45 mg Kg^{-1} day^{-1} reduced their body weights. Intraperitonial application to pregnant mice produced embryotoxic and teratogenic effects (Paulus 1993).

1.8.2 *Antimicrobial Effectiveness and Applications*

Cetrimide (CTAB) is highly effective against Gram-positive bacteria, but it has low activity against Gram-negative bacteria due to the cell wall composition of the latter. *P. aeruginosa* specifically tends to be highly resistant to CTAB (Russell *et al* 1999). *P. aeruginosa* cells survive high concentrations of Cetrimide (160 mg L^{-1}) through formation of cell aggregates which are induced by Cetrimide itself and that would protect the cells inside the conglomerates (Paulus 1993). In fact it has been added to bacteriological media as a selective ingredient for *Pseudomonas* species e.g. *Pseudomonas* selective agar (Legnani *et al* 1999) and Cetrimide agar (Szita *et al* 1998). Cetrimide is mainly used as an active ingredient in disinfectants. It is used as a cosmetic preservative at a concentration not exceeding 0.1% (Paulus 1993).

1.8.3 Mode of Action of Cetrimide

The QAC group of biocides induces leakage of intracellular constituents from exposed bacteria, which is indicative of membrane damage. Potassium ions are released first, followed by PO_4^{3-} and then by 260 nm absorbing material, which is an indication of the leakage of larger molecular weight compounds (Russell and Chopra 1996). Cationic agents also react with phospholipid components in the cytoplasmic membrane, producing distortion in the membrane that is followed by lysis (Russell and Chopra 1996). It has been suggested that exposure to QACs results in reduction in conjugational transfer, perhaps due to its membrane activity, it could be affecting the synthesis of the conjugative apparatus (Pearce *et al* 1999). Pearce *et al* (1999) found that at (0.0002%) concentration, Cetrimide interfered with conjugational transfer of antibiotic-resistance genes between *S. aureus* species. They speculated that this effect could be due to Cetrimide affecting specific target sites such as transduction receptors in the recipient cells. In this study Cetrimide was used as a control to the Pyrithione antimicrobials which have been shown to have a similar mode of action (membrane activity).

1.9 Summary

P. aeruginosa developed resistance towards many antibiotics and biocides. Mechanisms of resistance are mainly intrinsic, involving outer membrane protein alterations (Brozel and Cloete 1994, Winder *et al* 2000), LPS and hydrophobicity changes (Conrad and Galanos 1989; Hasegawa *et al* 1997) and efflux mechanisms (Poole 2001). Chromosomal mutations resulting in multi-drug resistance have been observed (Masuda *et al* 1995; Moken *et al* 1997). As an indication of drug resistance and pathogenicity, *P.aeruginosa* cells express a mucoid phenotype, which in many cases has been associated with biofilm formation and hence, the antibiotic-resistant phenotype. It has been suggested that mucoidy is regulated by an alternative sigma factor (Deretic *et al* 1994), which could be responsible for a stress response mounted by this bacterium against physical and chemical stressors (antimicrobials) (Allan *et al* 1988). Adaptive biocide resistance involves selection of a biocide resistant phenotype as a result of the frequent exposure of bacteria to sub-MIC concentrations of that biocide. It is an acquired resistance that depends on the nature of the organism. Adaptive resistance towards various biocides in different bacterial species has been achieved (Peckey and James 1974; Russell and Furr 1977; Jones *et al* 1989; Brozel and Cloete 1994; Winder *et al* 2000).

Pyrithiones are membrane active group of biocides that are effective against a wide range of microorganisms. They are used as preservatives as well as therapeutic agents. Resistance towards this group of synthetic biocides has not been reported.

Aims of This Study

The Pyrithione biocides are effective and powerful antimicrobials that are used as therapeutic agents as well as preservatives in various cosmetic and fuel products. *P. aeruginosa* exhibits a wide range of antibiotic and biocide resistance. Resistance by this organism against pyrithiones has not been investigated. This study aims to investigate the possible adaptation of *P. aeruginosa* PAO1 to pyrithiones through exposing *Pseudomonas* cultures to increasing sub-inhibitory concentrations (MIC/4) of the biocides (Brozel and Cloete 1994). Adapted "*resistant*" strains will then be used as a model to investigate the mechanism of resistance in that organism. Outer membrane alterations in resistant cells will be studied using SDS-PAGE analysis of both resistant and wild type cells. LPS changes will be checked using LPS SDS-PAGE, and changes in resistant cells hydrophopicity will be investigated using water / organic solvent partitioning of resistant and sensitive cells (Rosenberg *et al* 1980). The involvement of efflux systems will be explored through the use of efflux inhibitors (CCCP and OVN). Multi-drug resistance involvement will also be investigated through the usage of antibiotic susceptibility testing for various groups of antibiotics, cross-resistance between the pyrithione biocides and the QAC Cetrimide will be investigated as well. It is known that bacteria when exposed to biocides mount a stress response. The stress response mounted by *P. aeruginosa* as a result of exposure to pyrithiones will be studied using SDS-PAGE of cell lysates and comparing results with SDS-PAGE of heat shocked *P. aeruginosa* cell lysates. Heat shocked and pyrithione resistant cell lysates will be tested for any immunlogical similarities in their protein profile using Western Blot technique. Results obtained from the previous methods will be analyzed and discussed to conclude the mechanism(s) adopted by *P. aeruginosa* PAO1 to exhibit resistance towards Pyrithiones.

Chapter Two

General Experimental Methods

2.1 Bacterial growth and maintenance of cultures

Pseudomonas aeruginosa PAO1 NCIMB 10548 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK. Cultures were routinely maintained on R2A supplemented with 1% glycerol (Reasoner & Geldrich 1985) and chemically defined media CDM (Dinning, 1995) replacing 0.5% succinic acid with glycerol) slopes in quadruplicate. The four slopes were numbered 1 to 4. Slope number 1 was used to inoculate overnight cultures for experimental purposes, slope number 2 was the backup culture in case of contamination. Slope number 3 was used for further subculturing and slope number 4 was kept as part of the stock culture collection. Inoculated agar slopes were incubated overnight at 37°C. Cultures were then stored at room temperature in the dark. Liquid cultures were grown in R2A and CDM broth for 24 hours at 37°C in an orbital shaker incubator (Gallenkamp, INA-305) at 180rpm.

2.2 Chemicals and reagents

Sigma, Poole, Dorset: Trizma hydrochloride; ethylendiaminetetra-acetic sodium salt (EDTA); pyruvic acid; N-laurysarcosine; protease peptone number 3; soluble starch; sodium carbonate; glycine; TEMED; Trizma base; yeast extract; casein acid hydrolysate; urea.

BDH Chemicals, Poole, Dorset: Potassium dihydrogen phosphate (KH_2PO_4); dipotassium orthophosphate (K_2HPO_4); ferric ammonium citrate; glycerol; ethanol; methanol; sodium chloride (NaCl); D-glucose; calcium chloride ($CaCl_2$); magnesium sulphate heptahydrate ($MgSO_4.7H_2O$); sodium hydroxide (NaOH).

Zinc pyrithione (ZnPT) was the kind gift of Zeneca Specialties (Manchester, UK). Sodium pyrithione (NaPT) was purchased from Sigma (Poole, UK). Cetrimide was supplied by Rhone-Poulenc (Cheshire).

2.3 Sterilisation and preparation of Media

R2A medium was prepared according to the protocol of Reasoner & Geldrich (1985), supplemented with 1% glycerol. The pH was adjusted to pH 7.2 with either crystalline K_2HPO_4 or KH_2PO_4. When used for agar antibiotic sensitivity testing, 1.5% bacteriological agar (Oxoid number1) was added as a gelling agent. The medium was sterilised by autoclaving at 121° C, 15 psi for 15 minutes.

CDM was prepared according to Dinning (1995), replacing succinic acid (0.5% w/v) with glycerol (1M). It is a mixture of four solutions, A, B, C and D. Solution A was prepared by dissolving K_2HPO_4 (2.56g), KH_2PO_4 (2.08g) and NH_4Cl (1.00g) in 900 ml of distilled water. The pH was adjusted to pH 6.8 by the addition of either 0.1M HCl or 0.1M NaOH. The volume made up to 1L with distilled water. The solution was sterilized by autoclaving at 121 ℃, 15 psi for 15 minutes. Solution B was prepared by dissolving ferric ammonium citrate (1.0g) and $CaCl_2$ (0.1g) in 100ml of distilled water. The solution was sterilized by filtration through a 0.22 μm pore size cellulose acetate (Whatman, England) filter under vacuum. Solution C, 1M glycerol solution was prepared by dissolving glycerol (46.45g) in 400ml of distilled water. The pH was adjusted to pH 6.0 by the addition of either 0.1M NaOH or 0.1M HCl. The total volume was made up to 500ml with distilled water. Solution C was sterilized by autoclaving at 121° C, 15 psi, for 15 minutes. Solution D was prepared by dissolving $MgSO_4$.$7H_2O$ (0.5g) in 900ml of distilled water. Solution D was sterilized by autoclaving at 121°C, 15

psi, for 15 minutes. The CDM was completed by the aseptic addition of 5ml of solution B, 15 ml of solution C and 10ml of solution D to 1L of solution A.

2.4 Preparation of cell free extracts

Bacterial cells were fractionated using a homogenizer in order to extract the OMPs for later analysis. This process was validated in order that a suitable time period was applied to disrupt the cells of *P. aeruginosa* (Dinning 1995). The leakage of cytosolic constituents (e.g. free bases, inorganic phosphates) from the cell into the bacterial bathing solution was measured as an increase in absorbance (E_{260nm}).

2.4.1 Determination of Homogenizing time (Ht)

Cells were prepared using a hand-held homogenizer (Ultra Turrax T8, S8N-5G,IKA Labortechnik, Staufer, Germany). The minimum time required for homogenizing in order to yield the maximum absorbency of the supernatant was determined by plotting homogenizing time (Ht) against E_{260nm} (Figure 2.1).

2.4.2 Preparation of cells

An aliquot (5 mL) of an overnight culture of *P. aeruginosa* ($1x10^9$ CFU ml^{-1}) grown in R2A medium was centrifuged at 5000*g* (IEC Centra-4B) for 2.5 minutes. The pellet was washed three times in phosphate buffer (pH 7.2), and then resuspended in phosphate buffer (2ml). Ht was determined by homogenizing prepared cells on ice for 10 seconds, the cell suspension was centrifuged at 10500 *g* for 1 minute (MSE Microcentaur) in order to remove any cell debris from the supernatant (cytosol). The optical density of the supernatant was measured at E_{260nm} using a Jenway 6105 uv/vis spectrophotometer. The spectrophotometer was blanked using fresh phosphate buffer.

This process was repeated at 30 seconds intervals up to 1 minute, and every minute up to 10 minutes, until the optical density readings ceased increasing. The Ht determined for *P. aeruginosa* was 2.5minutes, this was when the E_{260nm} ceased increasing (Figure 2.1).

2.5 Bicinchoninic acid (BCA) assay for protein quantification

The bicinchoninic assay was originally described by Smith *et al* (1985). The method is based on the use of the sodium salt of bicinchoninic acid, which is a stable, water soluble compound capable of detecting the conversion of Cu^{2+} to Cu^{1+} (resulting from the reaction of the protein with alkaline Cu^{2+} (Biuret reaction) by forming an intense purple complex with the cuprous ion (Cu^{1+}). It is similar to the well-known Lowry method. However, it is more stable and can be performed in one step. Additionally, the presence of non-ionic detergents or simple buffer salts does not interfere with the reaction (Smith *et al* 1985). Bicinchoninic acid, sodium salt (BCA-Na) reacts with cuprous ion (Cu^{1+}) in alkaline conditions forming an intense purple complex, this purple coloration can be measured at E_{562nm} in order to determine the protein concentration (Smith *et al* 1985).

2.5.1 Preparation of Solutions for the Standard assay

The standard reagent is made of two solutions. Reagent A consists of (BCA-Na, 1% (w/v); sodium carbonate, 2% (w/v); sodium tartarate, 0.16% (w/v); sodium hydroxide, 0.4% (w/v); sodium bicarbonate, 0.95% (w/v) dissolved in 50ml of distilled water). The pH was adjusted to pH11.25 using 1M NaOH. Reagent B is made of copper sulphate (4% w/v) in $D.H_2O$. Just before carrying the protein estimation assay, the standard working reagent (reagent C) is prepared by mixing 100 volumes of reagent A with 2 volumes of reagent B. Reagent C has an apple green colour.

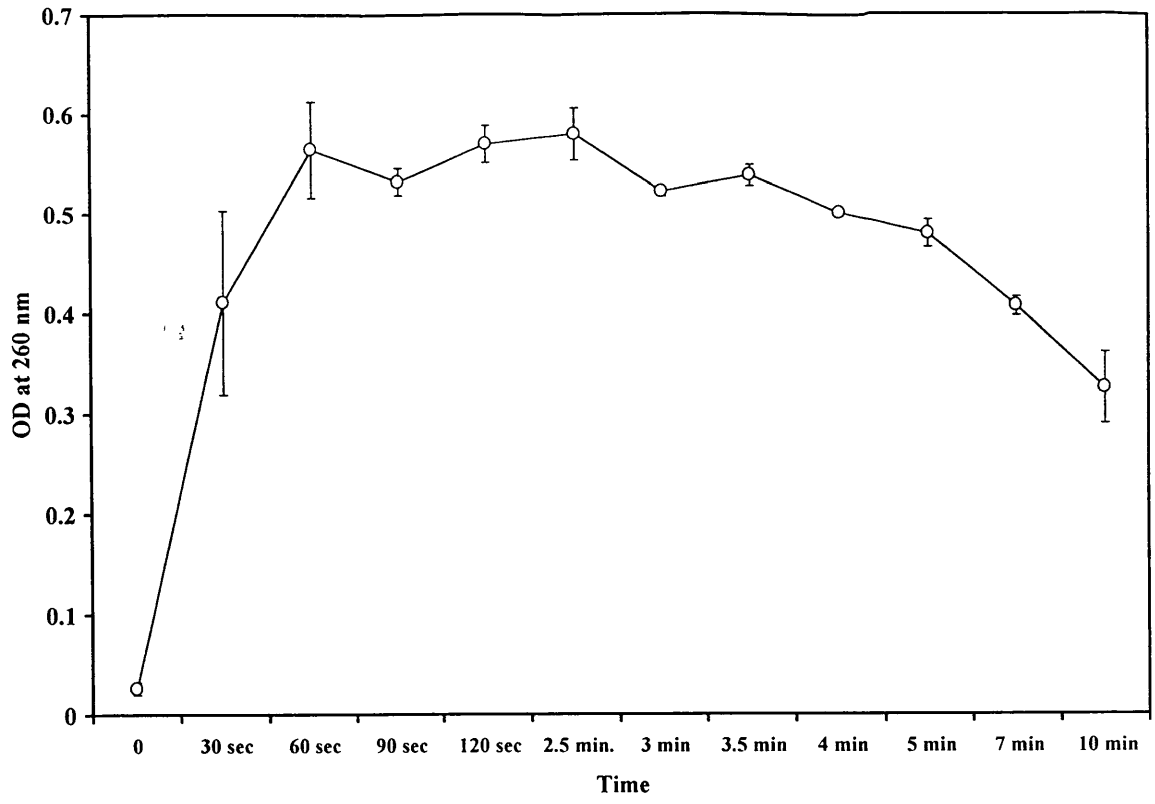


Figure 2.1: Homogenization time standardization curve. Cells were homogenized for different time intervals ranging between 30 sec. to 10 min. The OD at 260_{nm} for the homogenized cell suspension was measured for each time interval to detect the release of the 260 nm substances from the disrupted cells. Error bars represent the standard deviation for each data point.readings represent the results of two experiments.

2.5.2 Preparation of the Standard Curve

Aliquots were withdrawn from a (1mg mL^{-1}) Bovine serum albumin (BSA) stock (Sigma) and diluted with 0.025M Tris buffer pH 7.4 to give a total volume of 50μL. Final concentrations ranged from 0 mg ml^{-1} to 1 mg ml^{-1}. Aliquots (50μl) of each concentration was mixed with 1 mL of reagent C. Tubes were then incubated for 30 minutes at 37°C. The spectrophotometer was blanked using the standard reagent. The absorbency of each tube was measured at 562nm (Novaspec Π, LKB) and plotted against known protein concentrations to produce a standard curve (Figure2.2).

2.5.3 Analysis of the Samples

10 μl of the samples were diluted to a total volume of 50μl in 0.025 M Tris buffer pH 7.4 and 1ml of reagent C was added to it. Samples were incubated for 30 minutes at 37°C. Absorbency was measured at E_{562nm} and compared against the standard curve in order to estimate the protein concentration.

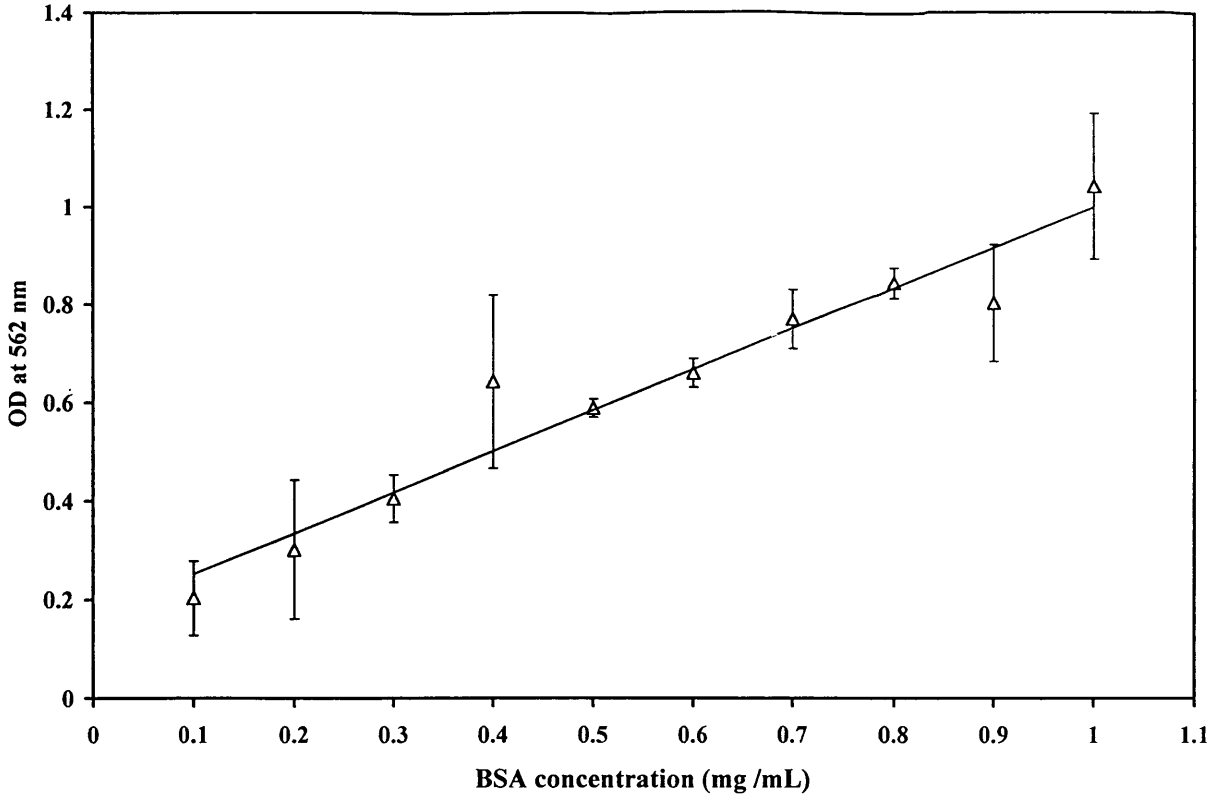


Figure 2.2: Standard curve for estimation of protein concentration using the bicinchoninic acid (BCA) assay. Error bars represent the standard deviation for each data point.Readings represent the results of two experiments.

2.6 Protcols for the preparation of SDS-Polyacrylamide Gels (SDS-PAGE) for Protein Separation

2.6.1 Preparation of the separating gel mixture

Stock acrylamide solution 10mL (acrylamide, 75g; bis-acrylamide, 2g; dissolved in distilled water, 250ml), distilled water, 12ml; and stock separating buffer 7.5 mL (SDS, 1g; Tris buffer, 45.5g; made up to 250mL with distilled water, pH 8.0) were added together and degassed. Stock ammonium persulphate solution 10% w/v (45μL) and N,N,N,N,-tetramethyl-ethylenediamine (TEMED) (15μL) was added to begin the polymerization process. The mixture was mixed gently but thoroughly, and poured into a prepared gel cast (0.5mm thick: mini-PROTEAN Π, Biorad). The gel was overlayed with water-saturated butan-2-ol, and left to set for approximately 1hour at room

2.6.2 Preparation of the Stacking Gel mixture

Stock acrylamide solution, 750μL; distilled water, 3mL; stacking gel buffer 1.25mL (SDS, 1g; and Tris buffer, 15.1g made up to 250mL with distilled water, pH6.8) were mixed together and degassed. Stock ammonium persulphate solution (15μL) and TEMED (5μL) were added and the contents were gently mixed. The butan-2-ol overlay was removed from the polymerized separating gel and the surface was rinsed with distilled water to remove any traces of butan-2-ol. The stacking gel was poured over the separating gel, and allowed to set for approximately 45 minutes with a comb (0.5 mm, 10 wells, Biorad) in place. Once the stacking gel had set, the combs were gently removed. The gel was transferred to a mini-PROTEAN Π system, Biorad. The upper and the lower buffer chambers were filled with reservoir buffer (glycine, 0.192M; Tris, 0.025M; and SDS, 0.1% w/v), ensuring the wells were filled with the buffer.

2.6.3 Preparation of outer membrane Samples for SDS-PAGE analysis

The method of Pugsley *et al* (1986) was adopted. Frozen resistant and sensitive cells were resuspended in 1 mL 25 mM Tris/HCL (pH 7.4) containing 1 mM $MgCl_2$, mixed and centrifuged for 20 minutes at 5000 *g*. The supernatant was discarded and the pellet was resuspended in 2 mL 25mM Tris/HCl buffer. Cells were homogenised for 2.5 minutes at 30 second intervals, between which the homogenizer probe (Ultra Turrax T8, S8N-5G, IKA Labortechnik, Staufer, Germany) was cooled on ice for 10 seconds. After cell disruption, the cells were pelleted by centrifugation (MSE Microcentaur) for one hour at 13000 *g* at 4°C. Supernatants were discarded and pellets were resuspended in 2% N-laurylsarcosine and the mixture was incubated on ice for 20 minutes. The outer membrane was then pelleted by centrifugation for one hour at 13000 *g*. Supernatants were discarded and pellets were washed in distilled water and pelleted by centrifugation for one hour at 13000 *g*. Water was discarded and outer membranes were stored frozen at -20° C until required for SDS-PAGE analysis.

2.6.4 SDS-PAGE Sample buffer preparation

Stock solution of SDS-PAGE sample buffer was prepared by the mixing of 0.5M Tris/HCl pH 6.8 (3.75 mL); SDS, (0.6g); glycerol, (3g); bromophenol blue (stock 0.1% w/v in distilled water) (1 mL), and mercaptoethanol, (1.5 mL) that was added at the end in a fume hood.

2.7 Protocols for staining of Polyacrylamide Gels following protein separation

2.7.1 Coomassie Brilliant Blue R250 Staining

The gels were immersed in Coomassie brilliant blue R250 (0.25g in methanol, 125mL; glacial acetic acid, 25mL; distilled water, 100 mL) and gently agitated using an orbital shaker (Stuart Scientific, UK) for between 1 and 18 hours. The gels were destained by immersing them in destaining solution (methanol, 450 mL; glacial acetic acid, 100 mL; water 450 mL). Gentle agitation was applied using an orbital shaker (Stuart Scientific, UK) for between 3 and 4 hours or until the background colour has disappeared, and a clear banding pattern was observed.

2.7.2 Silver Staining

Silver staining can detect 0.1 to 1 ng of protein per band.Therefore it is a useful technique when the protein concentration is low. The gels were immersed in fixing solution (50% methanol, 100mL; 12% glacial acetic acid, 24 mL; 37% formaldehyde, 100µL; made up to 200 mL with distilled water) for a minimum of 60 minutes, but may be left overnight. Gels were transferred to wash A (50% ethanol, 250 mL; made up to 500mL with distilled water) for 20 minutes; this step was repeated three times. Gels were placed in the pretreat solution (sodium thiosulphate.$5H_2O$, 0.04 g; made up to 200 mL) for one minute, and then washed in distilled water for 20 seconds, this was repeated three times. The gels were transferred to impregnate solution (anhydrous silver nitrate, 0.4; 37% formaldehyde, 150 µL; made up to 200mL with distilled water) and then washed twice in distilled water for 20 seconds. Gels were placed in develop solution (anhydrous sodium carbonate, 12g; 37% formaldehyde, 100µL; sodium thiosulphate. $5H_2O$, 0.0008g; made up to 200mL with distilled water) for as long as required until the bands were sharp and clear. The gels were washed twice in distilled water for 20 seconds

and then transferred to the stop solution (50% methanol, 100ml; 12% glacial acetic acid, 24mL; made up to 200 mL with distilled water) for 10 minutes. The gels were finally placed in wash B (50% methanol, 250mL; made up to 500 mL with distilled water) and stored in the dark.

Chapter Three

Growth Characteristics and Pyrithiones Effect on *Pseudomonas aeruginosa*

P. aeruginosa belongs to the genus *Pseudomonas*. Members of this genus are characterised by their ability to grow in low nutrient environments of neutral pH at the expense of a great variety of simple organic compounds utilized as carbon and energy sources (Oligotrophs). Most *Pseudomonas* species can grow in mineral media with a single organic compound as the sole source of carbon and energy and some species can even grow in distilled water and natural mineral water (Favero *et al* 1971; Legnani *et al* 1999).

In order to establish the effect of any antimicrobial agent on a microorganism, the dynamics of growth of that organism in the absence of the antimicrobial should be considered first. A typical growth curve for a batch culture obtained under optimal growth conditions usually consists of four phases. A lag phase in which the cells are metabolically active but not undergoing cell division, an exponential phase during which cell division occurs and number of cells increase exponentially. The third phase is the stationary phase where there is no obvious growth due to exhaustion of nutrients and accumulation of waste products. The death phase is the last phase in which the cells start to die, total count of the population may remain constant but the viable count is reduced (Bloomfield 1991).

The type of media that supports a bacterial culture largely affects the growth characteristics of that bacterium. Bacteria growing in a nutrient deficient medium

would have different growth kinetics than the same bacteria growing in a nutritionally rich media. Cells growing in nutrient depleted media undergo physiological changes to adapt to their environment (Brown *et al* 1990). These changes may include the phospholipid, porin proteins, LPS and cation composition of the bacterial cell envelope (Brown *et al* 1990).

It is of crucial importance that the environment created *in vitro* closely resembles that *in vivo*, so that obtained results are relevant to real conditions. It is now well established that in many infections, microorganisms are growing slowly in an iron-restricted environment (Gilbert *et al* 1987). Thus, the deployment of homogenous suspensions depleted of a single nutrient, possibly iron, will increase the relevance of the results (Gilbert *et al* 1987).

In this study R2A medium was used. R2A is a low nutrient medium that is deficient in iron (Reasoner & Geldreich 1985). CDM (Dinning 1995) is another medium used in this study. CDM is a chemically defined medium that contains iron in the form of ferric ammonium citrate (Chapter Two). In this chapter the growth kinetics of *P. aeruginosa* on both types of media will be studied using two methods, turbidity measurement and viable count.

3.1 Assay for *P. aeruginosa* Growth

An aliquot (25mL) of R2A medium was inoculated with a pure culture *P. aeruginosa* PAO1 in 100mL Erlenmyer flask. The flask was incubated overnight at 37ºC, 180 rpm in an orbital shaking incubator (Gallenkamp INA-305). Next day, an aliquot (250μL) of that culture was inoculated into a fresh 25mL of R2A medium. This was performed in triplicate. At hourly intervals a sample (1mL) was removed from the freshly inoculated R2A medium, and its optical density was measured at E_{470nm}. The sample was

returned aseptically to its respective flask, so that the volume: surface area of the flask did not alter significantly, and hence, the aeration rate remained constant throughout the duration of the experiment. When the optical density (470_{nm}) readings approached 0.5, aliquots (100μL) were diluted 1 in 10 to avoid inaccuracy of readings (Lawrence & Maier 1977). These samples were not returned to the flask, but were discarded after readings had been taken. The E_{470nm} were observed and multiplied by 10 to give the actual optical density. The Log_{10} average of the three readings at each time interval was obtained and plotted against time to obtain the growth curve. The same protocol was followed when CDM was used. Figures 3.1 and 3.2 show the growth curves of *P. aeruginosa* obtained in R2A and CDM.

3.1.1 Viable Count

Viable counts were performed in parallel with the turbidity studies. At the same time intervals, an aliquot (100μL) from each culture flask was aseptically transferred to a tube containing 9.9 mL of sterile saline (0.9% w/v NaCl). Cultures were returned to the incubators, and the process was repeated every one hour for 24 hours. Ten fold serial dilutions were carried out from this tube to obtain 10^{-2} to 10^{-9} dilutions. Aliquots (100μL) from each dilution tube were transferred to R2A or CDM agar plates in duplicate. The inoculum was spread over the surface of the plates using a sterile glass spreader. The plates were incubated overnight at 37°C. Colonies were counted from the plates of higher dilutions.

3.2 Assessment of antibacterial activity of the Pyrithione biocides

The quantitative assessment of the activity of antimicrobials under appropriate conditions is an essential part of the initial investigation (Broomfield 1991).

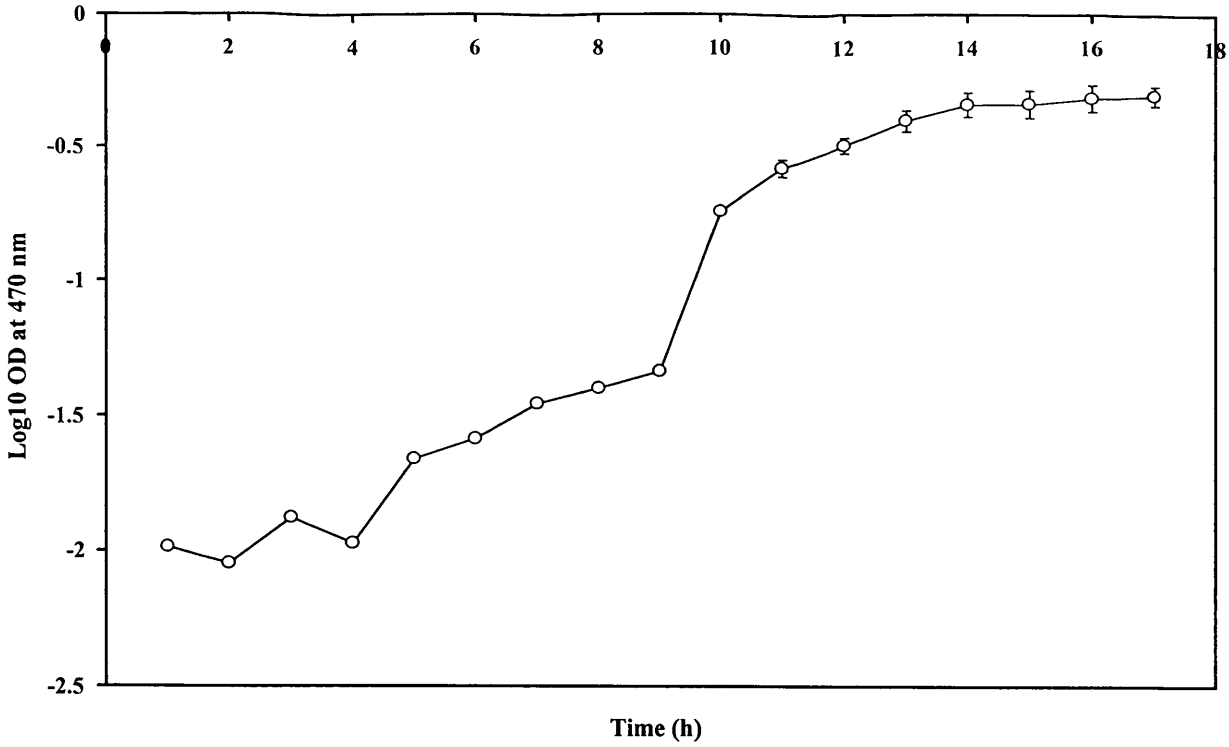


Figure 3.1: Growth curve of *P. aeruginosa* in CDM as determined by the plot of Log_{10} OD_{470nm} as a function of time (h). Error bars are calculated as the standard deviation of the individual data point. Results were obtained from triplicate experiments.

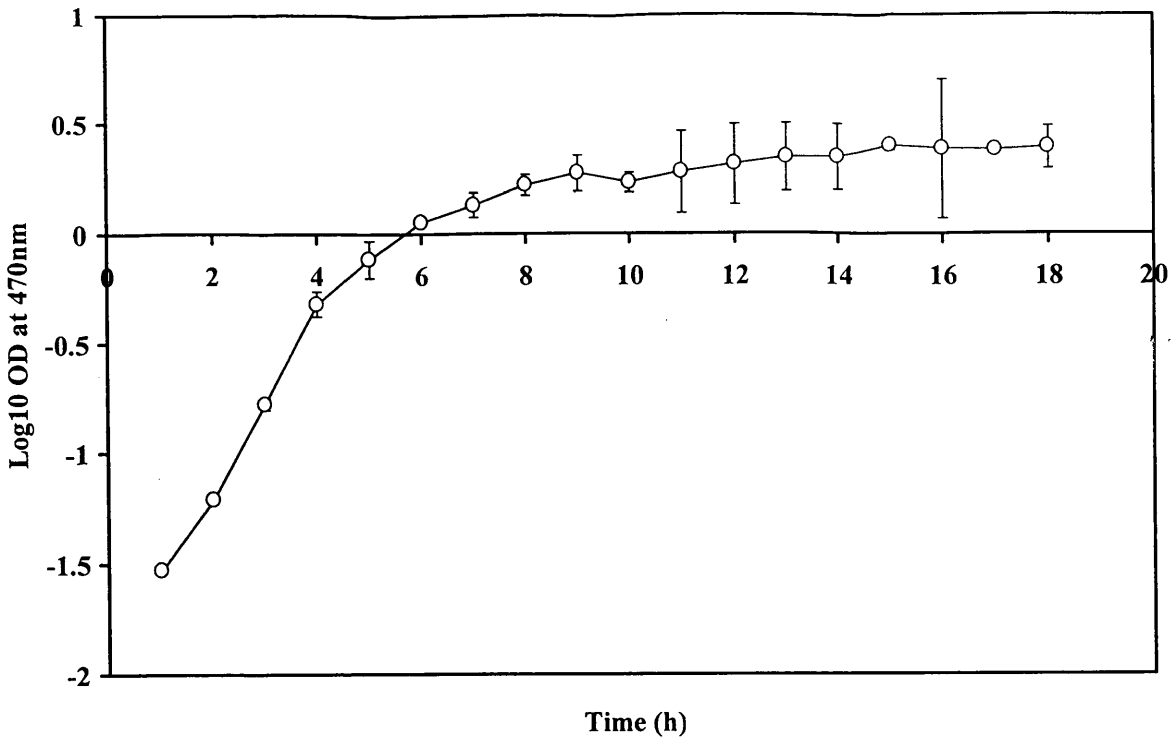


Figure 3.2: Growth curve of *P.aeruginosa PAO1* in R2A medium as determined by the plot of Log_{10} OD_{470nm} as a function of time (h). Error bars are calculated as the standard deviation of the individual data point. Statistics for these data were determined using results of triplicate experiments.

Determination of the Minimal Inhibitory Concentration (MIC) for an antimicrobial is performed by introducing a fixed number of microorganisms into a system that contains various concentrations of the antimicrobial. Following an incubation period of 18 to 24 hours, the cultures are examined for the absence of turbidity. The lowest concentration that inhibits growth of microorganisms assessed by the absence of visual turbidity is considered to be the MIC for that antimicrobial against that microorganism.

This method is subject to criticism due to its low level of reproducibility, that could be due to variability in inoculum size, variation in growth medium and the growth rate of the organism (Gilbert *et al* 1987). Magnesium-depleted *P. aeruginosa* loses susceptibility to EDTA and polymyxin B. This susceptibility is restored after growth for three generations in magnesium plentiful media (Brown *et al* 1990). Thus, mid-logarithmic phase cells should be used for studying population kinetics. At mid-logarithmic phase cells would have undergone all necessary changes for growth in the new media and the cells would exhibit the logarithmic properties. In the present study MIC experiment was performed in R2A and CDM media. The inoculum size was adjusted by obtaining a constant optical density reading from a fresh 16h culture. MIC experiments were performed in triplicate using liquid media (tube dilution method); cultures were incubated at 37°C and examined for absence of visual turbidity after 19, 24 and 48h. The tube dilution method described by Bloomfeild (1991) provides a direct quantitative assessment of the active concentration of the biocide. However, the exact MIC lies between the observed maximum inhibitory concentration and the minimum inhibitory concentration. Thus, the accuracy of the end-point will depend on the range of concentrations used initially, which involves a two-fold serial dilution of the biocide. This should then be followed by an arithmetical series of dilutions (Bloomfield 1991).

Pyrithione biocides (ZnPT & NaPT) were prepared by dissolving the measured powder in N, N-Dimethylformamide (DMF). Cetrimide was prepared by dissolving it in DMF for the sake of unifying the experimental conditions. Drugs were freshly prepared in amber glass sterile bottles and kept refrigerated until required.

3.2.1 Tube Dilution Method for MIC Determination

An aliquot (2mL) of overnight cultures of *P. aeruginosa* in R2A medium was transferred aseptically to a sterile cuvette and the optical density at E_{470nm} was measured. Experiments were undertaken to correlate the observed OD_{470nm} of a growing culture of PAO1 with the viable counts of the same culture. Results (not shown) indicated that an optical density of 0.700 corresponds to 10^8 CFU mL^{-1}. Thus, an inoculum size at OD_{470nm} of 0.700 was chosen as the inoculum for MIC determination. Tubes containing aliquots (9mL) of R2A broth were sterilized and biocides were added in doubling concentrations in 1 mL aliquots starting from 1μg mL^{-1} and ending with 512 μg mL^{-1}. Tubes were then inoculated with 100μL of the culture and incubated at 37ºC.These steps were used to determine the MIC for ZnPT, NaPT and Cetrimide in R2A medium and for ZnPT, NaPT and Cetrimide in CDM medium. A DMF control set was also prepared and included. Tables 3.1& 3.2 demonstrate the initial and exact MIC determination for ZnPT in R2A media.

3.2.2 *Results and Discussion*

Growth curves of *P. aeruginosa* on CDM and R2A media (Figures 3.1& 3.2) demonstrate a clear difference in the growth characteristics of the organisms in these two

Table 3.1: Observation of growth in the dilution tubes of *P. aeruginosa* PAO1 in R2A medium in order to determine the screening MIC of ZnPT.

Biocide concentration $\mu g\ mL^{-1}$	Growth after 19 hours	Growth after 24 hours
0	+	+
1	+	+
2	+	+
4	+	+
8	+	+
16	-	-
32	-	-
64	-	-
128	-	-
256	-	-
512	-	-

+ = presence of growth - = absence of growth

Table 3.2: Observation of growth in dilution tubes of *P. aeruginosa* PAO1 in R2A medium in order to determine the exact MIC of ZnPT.

Biocide concentration $\mu g\ mL^{-1}$	Growth after 19 hours	Growth after 48 hours
0	+	+
1	+	+
2	+	+
3	+	+
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-

+ = presence of growth - = absence of growth

Table 3.3 MIC values of the three biocides against *P. aeruginosa* PAO1 grown in R2A medium. MIC values represent the mean of three experiments

	ZnPT		NaPT		Cetrimide		DMF	
	19 h	48 h	19 h	48 h	19 h	48 h	19 h	48h
Screening MIC (μg mL^{-1})	8/16	8/16	64	64	256	256	256	256
Exact MIC (μg mL^{-1})	4	4	50	50	nd	nd	nd	nd

nd = not done

Table 3.4: MIC values of the three biocides against *P. aeruginosa* PAO1 grown in CDM medium. MIC values represent the mean of three experiments

	ZnPT		NaPT		Cetrimide		DMF	
	19 h	48 h	19 h	48 h	19 h	48 h	19h	48h
Screening MIC (μg mL^{-1})	8	8	8	32	16	16	256	256
Exact MIC ((μg mL^{-1})	3.4	3.4	25	30	10	10	nd	nd

different nutritional environments. R2A is a relatively rich medium that is iron depleted. Although nutrient concentrations are low (Reasoner & Geldreich, 1985), it supported a rapid growth of *P. aeruginosa* with a very short lag phase (1hour) that was followed by a long (15 hour) exponential phase. In an iron deficient medium a low growth rate is expected (Brown *et al* 1990). On the contrary the growth curve showed a rapid growth that resembled growth in normal enriched media with a doubling time of approximately 1 hour. This suggests that the bacteria might have switched to another metabolic machinery to adapt to the absence of iron. This alternative machinery could be the use of alternative substrates or modification of cellular components and/or reduction of synthesis of some cellular components that require iron for its synthesis (Brown *et al* 1990). The cultures were observed to be highly pigmented (fluorescent green) which is typical of growth of *P. aeruginosa* in iron deficient media. Bacteria are known to synthesize iron scavengers (chelators) termed siderophores. Siderophores are secreted into the medium in order to bind iron. The chelated iron is then transferred into the cell through specific outer membrane proteins that bind the siderophore-iron complex. *Pseudomonas* species have three reported iron siderophores: (1) Pyochelin, which is a phenolic iron binding compound produced by *P. aeruginosa*; (2) ferripyochelin and (3) pyoveridine (Sokol& Woods 1983). Pyoveridine is a fluorescent siderophore that has higher affinity for Fe^{3+} than pyochelin, which explains the strong fluorescent green colour of the *P. aeruginosa* culture on R2A medium.

Growth in CDM expressed a long lag phase (9h) and a short (6h) exponential phase. This type of growth is expected since CDM is considered a nutritionally limited media that would result in slow growth rate (Brown& Williams 1985; Gilbert *et al* 1987; Brown *et al* 1990).

MIC results were reproducible between the triplicate cultures. ZnPT appears to be more effective in low concentrations in comparison with the NaPT. However, NaPT is a stronger chelating agent. The chelating activity of NaPT was clearly observed upon the addition of the biocide to the CDM tubes, the colour of the medium changed from transparent to violet-blue colour as the biocide concentration increased. Complexes that have formed settled at the bottom of the tubes, leaving the cells partially deprived of iron. In fact the MIC results for NaPT on CDM could not be read before 48h, due to low growth rates. When comparing the MIC values for NaPT obtained from cultures of *P. aeruginosa* grown in CDM with the MIC values of the same biocide obtained from growth in R2A medium, it was clear that *P. aeruginosa* exhibited an increased susceptibility to the biocide on CDM. *P. aeruginosa* cells grown on CDM struggled to survive the presence of a powerful biocide and poor conditions of growth.

P. aeruginosa has a known resistance to Cetrimide. The sharp decrease in MIC from 256μg mL^{-1} on R2A medium to 10 μg mL^{-1} on CDM for that particular biocide suggests that *P. aeruginosa* grown under conditions of nutrient depletion becomes more susceptible to the effects of Cetrimide.

There was no pronounced difference between the MIC of ZnPT obtained from growth of *P. aeruginosa* in R2A or CDM. This suggests that the biocide was not neutralized by any of the ingredients of the two media nor does the growth rate of *P. aeruginosa* have any effect on the mechanism of action of ZnPT. However, the MIC for NaPT and Cetrimide dropped when *P. aeruginosa* was cultured on CDM. It is possible that the bacteria became more susceptible to the effect of these biocides when grown in CDM. As discussed earlier, cells in nutrient depleted media undergo cell envelope alterations that increase the affinity of surface components for the growth limiting substances making the uptake into cytosol more competitive (Brown *et al* 1990).

Envelope alterations could on the other hand explain the reduction in MIC for biocides on CDM medium by the possible enhancement of biocide uptake by the cell.

3.3 Outer membrane studies

The composition and structure of the cell envelope reflects the nature of the growth environment (Brown and Williams 1985). It has been suggested earlier that when bacteria are transferred to a nutrient limited media, it undergo some alterations in their surface properties that participate in their survival. Nutrient depleted dividing cells in batch cultures will manufacture envelopes with characteristics that suits the particular depletion encountered (Brown 1979). Different nutrient depletions give rise to cultures with different envelopes. *P. aeruginosa* cells grown in Mg^{2+} deficient CDM were found to induce the production of an outer membrane protein H_1 (Shand *et al* 1988). Protein H_1 was suggested to substitute Mg^{2+} in the cross linkage between LPS molecules in the outer membrane and thus maintaining the integrity of the cells (Shand *et al* 1988). In order to investigate the possibility of outer membrane protein alterations, outer membranes of wild type *P. aeruginosa* PAO1 strains grown on both R2A and CDM media were obtained as explained in Chapter two. Outer membrane preparations were analyzed by SDS-PAGE (Chapter two).

3.4.1 Results and Discussion

Figure 3.3 demonstrates the outer membrane profiles of *P.aeruginosa* PAO1 grown in R2A and CDM media. The two profiles matched with no observable variations in the protein bands. The results shown in figure 3.3 demonstrate that there is no change in the protein structure of the cell envelope as a result of media type employed for growth. However, this does not rule out any other alterations in the envelope

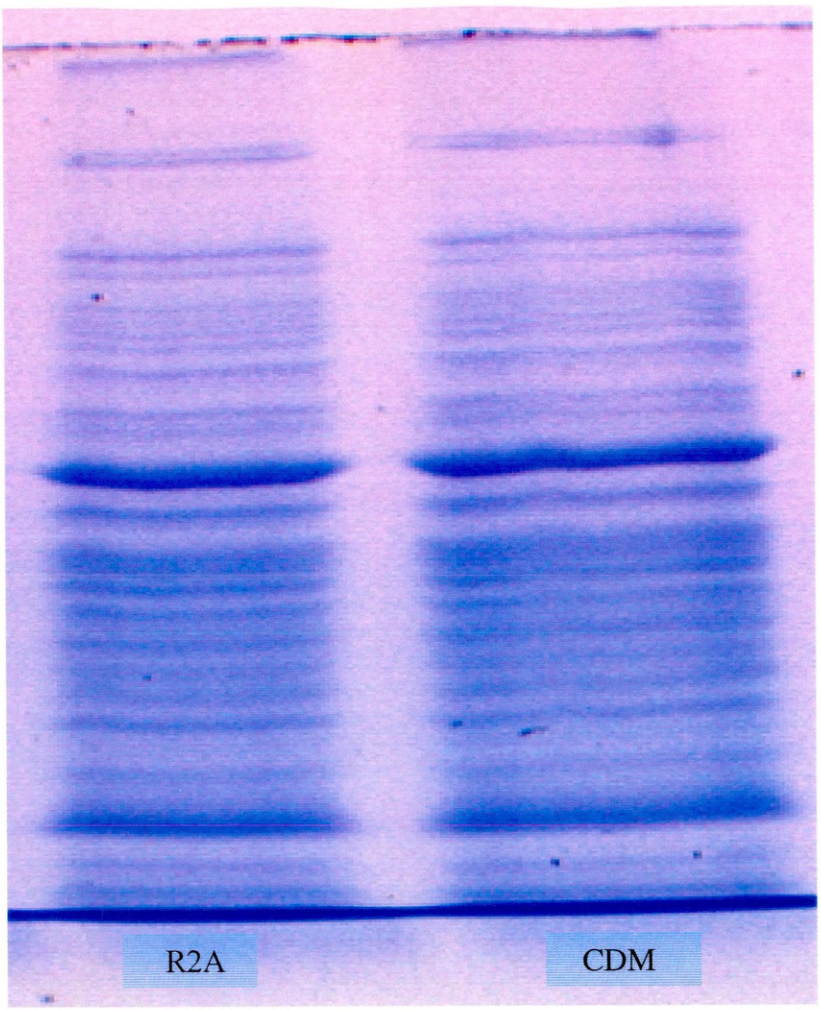


Figure 3.3: Coomassie blue stained SDS-PAGE gel demonstrating comparison between the outer membrane profiles of *P. aeruginosa* grown in R2A and CDM.

components such as fatty acids, phospholipids, polysaccharides and extracellular enzymes (Gilbert *et al* 1987) that were not investigated in this study.

CHAPTER FOUR

Induction of Resistance in *Pseudomonas aeruginosa* PAO1 against Zinc and Sodium Pyrithiones

Tolerance is defined as a situation in which a formerly active biocide no longer controls microbial growth (Chapman *et al* (1998). Chapman *et al* (1998) has defined three routes in industry that can lead towards a state of tolerance to a biocide: destabilization of biocide, establishment of biofilms and development of resistance. There are several factors that affect the stability of biocides in formulations, for example: temperature, pH, organic matter, and biocide concentration. Minor changes in formulation, such as change in raw materials, may destabilize the biocide rendering it ineffective. Such changes could be spotted and rectified. Singer (1976) has proposed a series of procedures, which reproduce, as closely as possible, certain critical features of the industrial situation, so that the effectiveness of the biocide can be adequately predicted. He outlined the possible reasons for inactivity of antimicrobials in preparations for each group of materials. For example, in metal-working fluid biocides, destabilization could be due to: (1) low bactericidal activity in aqueous solutions or (2) high oil phase solubility, thus there will be no activity in the aqueous phase or (3) the emulsifying agent used in the formulation is rendering the biocide inactive. Thus, following a series of tests in the lab the exact problem can be identified and treated.

Tolerance to biocides is detected clearly in bacteria present in biofilms. Biofilm formation is a condition that occurs frequently in industry as a result of inadequate hygiene. Correction of this problem is often difficult and expensive and may require extreme measures. There are always new agents for treatments of biofilms and new

methods of attack. Flushing of the dental unit water systems (DUW) with Tegodor and Spor-klenz (Walker *et al* 2001) could eliminate biofilms accumulating on the tubing of the dental unit water systems. A novel mode of attack is the photodynamic antimicrobial chemotherapy (PACT) which uses exogenous biologically active compounds and light energy (photoenergy) to selectively target and eradicate microbes. Matchett *et al* (2001) used phenothiazinium compounds (blue dyes), new methylene blue (NMB) and toluidine blue O (TBO) and light to reduce the viable counts of *P. aeruginosa* biofilms by as much as 99.7% CFU cm^{-2}.

The frequent exposure of a microbial population to low concentrations of a biocide induces biocide resistance. This has been shown by Chaplin (1951) who reported that *Serratia marcescens* acquired resistance against alkyldimethylbenzyl ammonium chloride (QAC) and that the bacteria was adapted to grow in 10000 ppm of the biocide after being suppressed by less than 100 ppm. This adaptation resulted from growing the bacteria in a medium that contained a gradually increasing concentration of the biocide starting from as low as 1.64 ppm to 1000ppm. Soprey & Maxcy (1968) managed to adapt *E. coli* and *P. fluorescens* to 28 μg mL^{-1} and 120 μg mL^{-1} respectively of QAC after being sensitive to 4 to 5μg mL^{-1} and 15μg mL^{-1} respectively. On the other hand the possession of some bacteria to biocide detoxifying enzymes leads to a state of resistance towards these biocides. *P. aeruginosa* exhibited resistance to formaldehyde through possessing a formaldehyde dehydrogenase enzyme that conferred resistance not only to formaldehyde, but also to formaldehyde releasing biocides (Chapman *et al* 1998). Chapman *et al* (1998) isolated a *P. aeruginosa* strain from matrices preserved with chloromethyl isothiazolone (CMIT). This strain exhibited both resistance to CMIT and cross-resistance to dimethoxy dimethyl hydantoin and chloroallyltriazine-

azonidamantane (CTAA) as well as formaldehyde and other structurally unrelated preservatives (Chapman *et al* 1998).

Several authors have explained methods for induction of resistance. Shand, *et al* (1988) adapted *P. aeruginosa* to 6000 Units mL^{-1} of polymyxin B in eight successive steps. They sub-cultured fixed inoculum of 10^7 CFU mL^{-1} into a series of tubes containing increasing concentrations of polymyxin B in a fixed volume of chemically defined medium. Cultures were incubated for one week at 37ºC and the tube containing the highest concentration of antibiotic was sub-cultured in another set of tubes of ascending concentration of polymyxin. This method resulted in a progressive increase in resistance towards polymyxin B in eight steps or passages. Peckey and James (1974) grew sensitive *P. aeruginosa* on nutrient agar plates containing sub-inhibitory concentrations of gentamicin (MIC/2). They obtained small, slow growing cultures from which colonies were sub-cultured on agar plates containing the same concentrations of antibiotics until larger and faster growing colonies were obtained. These steps were repeated until highly resistant bacteria were obtained. Brozel & Cloete (1994) gradually adapted *P. aeruginosa* to tolerate an increasing concentration of isothiazolone (Kathon^{TM}). *P. aeruginosa* was trained to grow in the presence of isothiazolone biocides by subjecting it to increasing low concentrations of the biocide (MIC/4) in successive passages. The MIC increased from an initial value of 300 µl L^{-1} to 607 µl L^{-1} by passage ten. The increase in MIC was gradual during an exposure period of 15 days (Brozel & Cloete 1994).

Pyrithione biocides are used in industry as preservatives as well as topical therapeutic agents (Nelson & Hyde 1981). To the limit of our knowledge, the possibility of bacterial tolerance towards this group of biocides has not been investigated. In this

chapter the development of resistance against this group of biocides will be studied. The method of Brozel and Cloete (1994) will be employed with some variations.

4.1 Induction of resistance

A fresh 24h culture of *P. aeruginosa* PAO1 on R2A broth was prepared. The culture was diluted to achieve an inoculum of 10^8 CFU ml^{-1}. Aliquots (250µL) from the culture were inoculated into 25mL of fresh R2A medium containing ZnPT or NaPT at one-quarter the concentration of the determined MIC (MIC/4). The new cultures were incubated overnight at 37ºC in an orbital shaker incubator (Gallenkamp INA-305) at 180 rpm. All cultures were prepared in triplicate. The MIC values were determined for the new cultures and the inoculum size was adjusted to 10^8 CFU ml^{-1}. A triplicate set of fresh (25mL) R2A media that contained quarter the newly determined MIC were inoculated with the previous culture and incubated as mentioned above, with the exception of CDM cultures containing NaPT incubation was for 48h instead of 24h. The previous steps were repeated until ten successive passages had been performed in the presence of increasing concentration (MIC/4) of the biocide. Cultures of the 10^{th} passage were deemed sufficiently resistant for our purposes. At this point aliquots from the final cultures in the presence of biocide were inoculated into fresh biocide-free media and MIC values were redetermined. The previous step was repeated until three successive passages had been performed in the absence of that biocide.

These steps were repeated in triplicate for NaPT and ZnPT in R2A media and for Cetrimide, NaPT and ZnPT in CDM. It was not possible to perform the passage experiment for Cetrimide in R2A media, because the MIC of Cetrimide to *P. aeruginosa* PAO1 grown in R2A medium was $\geq$ 256 μg L^{-1}, which means that *P. aeruginosa* is almost resistant to Cetrimide on this media.

4.1.1 Results and Discussion

Pseudomonas aeruginosa adapted to growth in R2A or CDM media containing ZnPT, NaPT and Cetrimide. Figures 4.1 to 4.5 show that triplicate cultures containing each biocide adapted gradually and in the same pattern.

4.1.1.a Adaptation to Zinc Pyrithione (ZnPT)

Induction of resistance to ZnPT was gradual and involved small increases in the biocide concentration. In R2A medium there was a steady state period that started from passage three and lasted until passage five. During this period no increase in MIC was observed. However, following the steady state period the MIC increased with large concentrations until passage number 11 in which the MIC of ZnPT reached its highest values (19.3 $\mu g\ mL^{-1}$) (Figure 4.1). On CDM adaptation of *P. aeruginosa* to ZnPT was gradual but slow in the first passages, and was followed by rapid increase in MIC from passage 6 to passage 11 (Figure 4.3). The initial MIC values for ZnPT on R2A and CDM were 4 $\mu g\ mL^{-1}$ and 3.4 $\mu g\ mL^{-1}$ respectively. As the cells reached passage 11, the MIC values became 19.3 $\mu g\ mL^{-1}$ and 12.5 $\mu g\ mL^{-1}$ respectively (Table 4.1).

4.1.1.a Adaptation to Sodium Pyrithione (NaPT)

The adaptation of *P. aeruginosa* to NaPT on R2A medium was gradual and required larger concentrations of the biocide than those required to induce resistance for ZnPT on R2A medium. However, in CDM, there was a steady state period between passages 4 and 8, after which the MIC increased until it reached its highest values in passage ten (105 $\mu g\ mL^{-1}$) (Figure 4.2 and 4.4). The initial MIC values for NaPT on R2A and CDM media were 40 $\mu g\ mL^{-1}$ and 30 $\mu g\ mL^{-1}$ respectively. The MIC values became 170 $\mu g\ mL^{-1}$ and 105 $\mu g\ mL^{-1}$ respectively (Table 4.1).

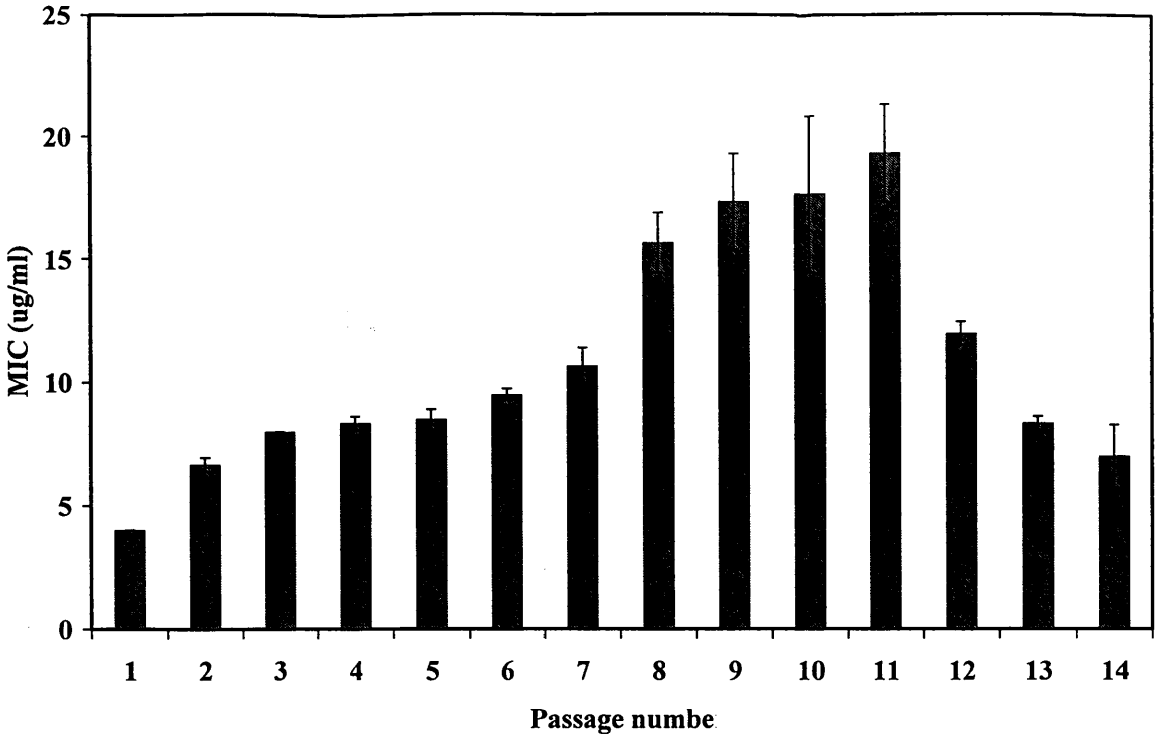


Figure 4.1: Adaptation of *P. aeruginosa* PAO1 to ZnPT in R2A medium. Column number 1 demonstrates the initial MIC of the sensitive strain. The columns from 2 to 11 demonstrate MICs from passages 1to10 in the presence of increasing concentrations of ZnPT. Columns 12 to14 represent MICs for biocide passaged *P. aeruginosa* in biocide-free R2A medium. Error bars are calculated as the standard error of each data point.

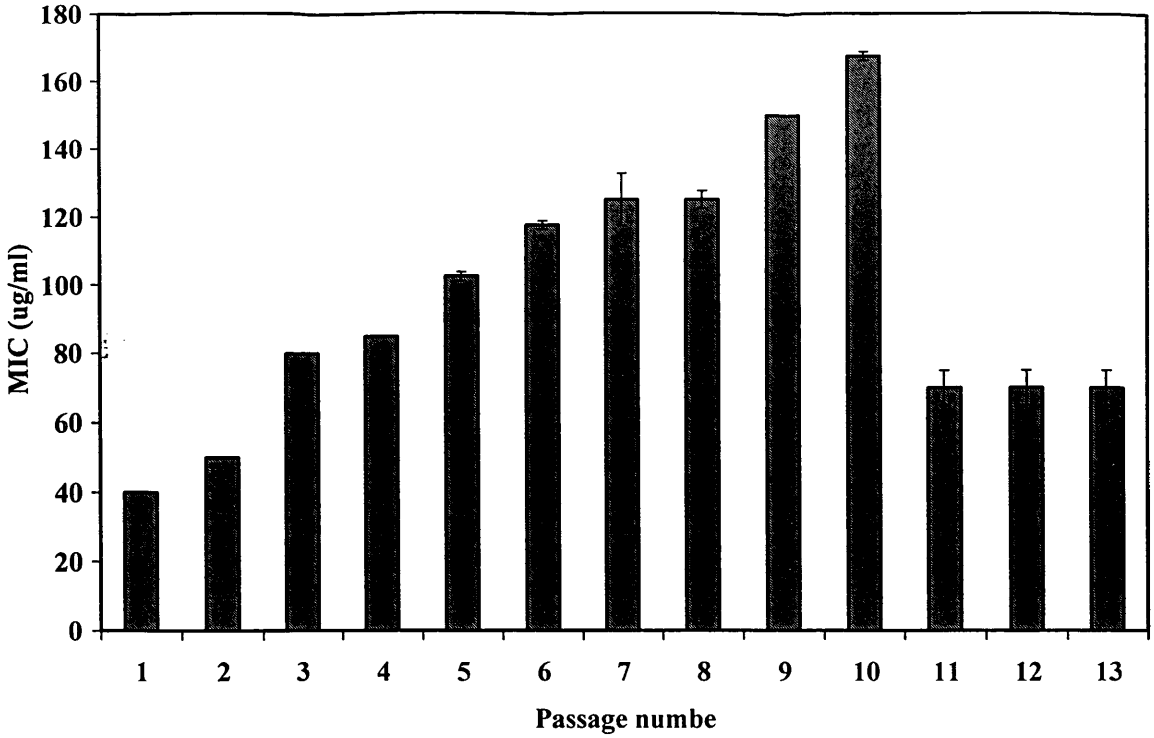


Figure 4.2: Adaptation of *P. aeruginosa* PAO1 to NaPT in R2A medium. Column number 1 demonstrates the initial MIC of the sensitive strain. The columns from 2 to 11 demonstrate MICs from passages 1 to 10 in the presence of increasing concentrations of ZnPT. Columns 12 to14 represent MICs for biocide passaged *P. aeruginosa* in biocide-free R2A medium. Error bars are calculated as the standard error of each data point.

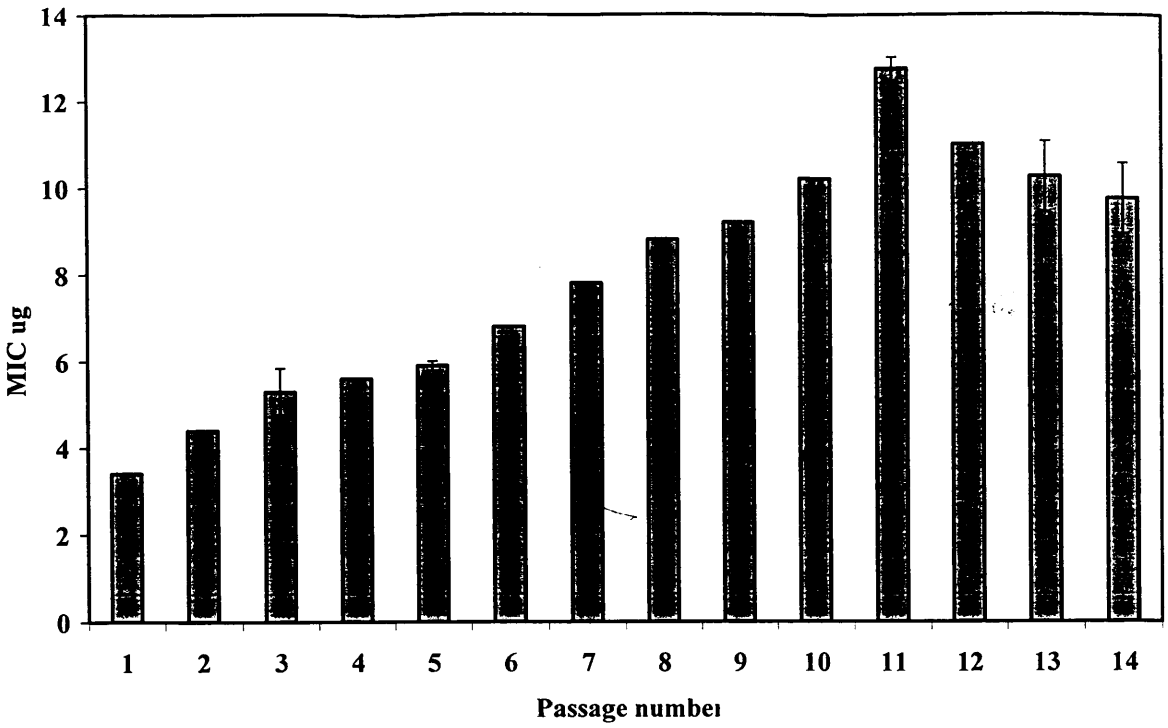


Figure 4.3: Adaptation of *P.aeruginosa* to ZnPT in CDM. Column number 1 demonstrates the initial MIC of the sensitive strain. The columns from 2 to 11 demonstrate MICs from passages 1 to 10 in the presence of increasing concentrations of ZnPT. Columns 12 to 14 represent MICs for biocide passaged *P.aeruginosa* in biocide-free CDM medium. Error bars are calculated as the standard error of each data point

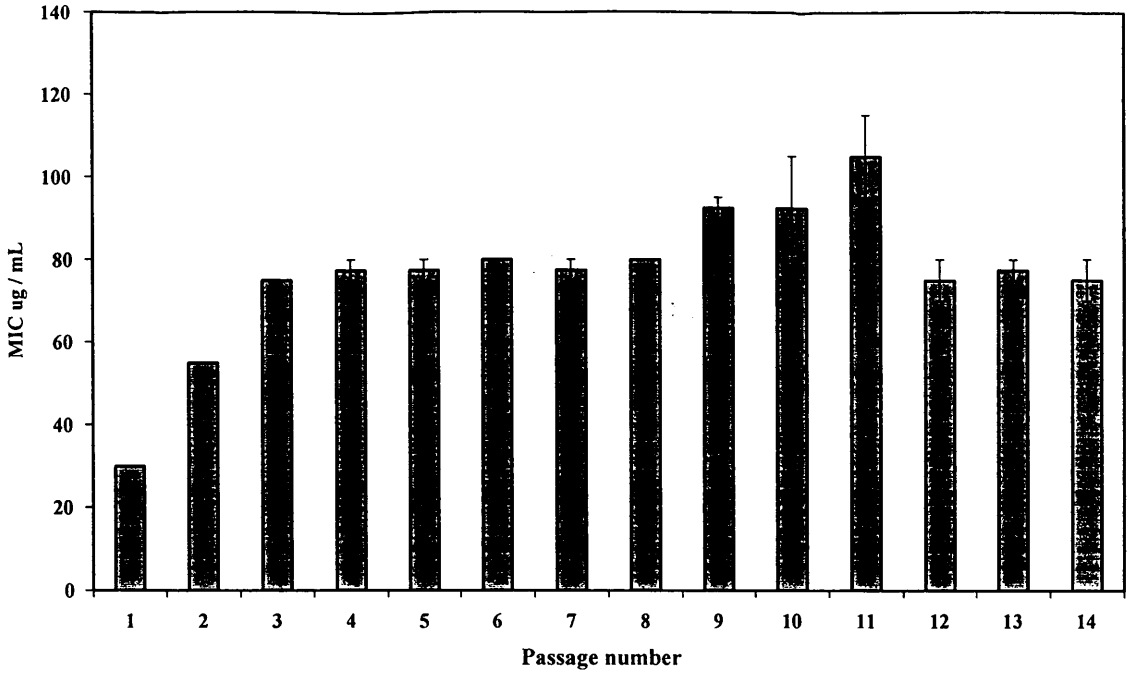


Figure 4.4: Adaptation of *P. aeruginosa* PAO1 to NaPT in CDM. Column number 1 demonstrates the initial MIC of the sensitive strain. The columns from 2 to 11 demonstrate MICs from passages 1to10 in the presence of increasing concentrations of NaPT. Columns 12 to14 represent MICs for biocide passaged *P. aeruginosa* in biocide-free CDM medium. Error bars are calculated as the standard error of each data point.

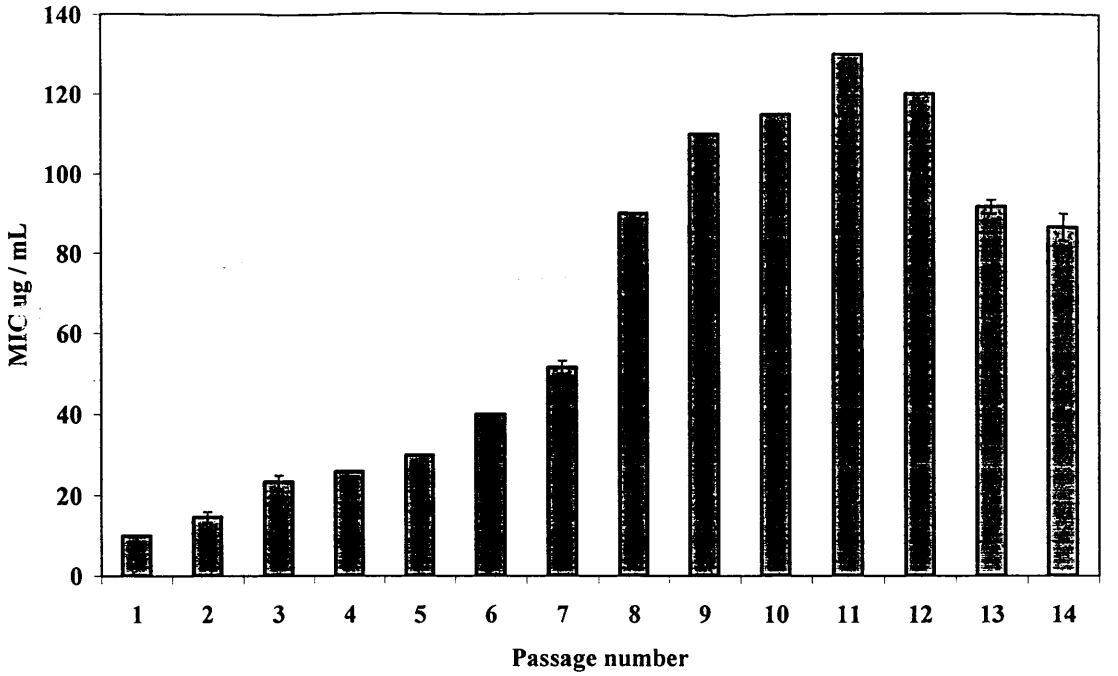


Figure 4.5: Adaptation of *P. aeruginosa* PAO1 to Cetrimide in CDM. Column number 1 demonstrates the initial MIC of the sensitive strain. The columns from 2 to 11 demonstrate MICs from passages 1 to10 in the presence of increasing concentrations of Cetrimide. Columns 12 to14 represent MICs for biocide passaged *P. aeruginosa* in biocide-free CDM medium. Error bars are calculated as the standard error of each data point.

4.1.1.c Adaptation to Cetrimide

Adaptation of *P. aeruginosa* to Cetrimide followed the same pattern of ZnPT on CDM, with the MIC changing from 10 μg mL^{-1} in passage 2 to 130 μg mL^{-1} at passage 11 (Figure 4.5).

In order to examine the stability of acquired resistance to ZnPT, NaPT and Cetrimide, resistant bacteria (passage 11) were sub-cultured in biocide-free media for three successive passages (passages 12 to 14). The MIC for each passage was determined. Zinc pyrithione adapted cells grown in R2A medium exhibited a sharp decrease in MIC from 19.3 μg mL^{-1} to 12 μg mL^{-1} in passage 11 (Table 4.1). On CDM the MIC decreased from 12.5 μg mL^{-1} to 11 μg mL^{-1} in passage 11 (Table 4.1). The MIC for ZnPT on R2A further decreased to reach 7 μg mL^{-1} in passage 14 while on CDM it dropped to 10 μg mL^{-1} in passage 14. However, NaPT adapted cells on R2A and CDM, showed a sharp decrease in the MIC from 170 μg mL^{-1} and 105 μg mL^{-1} respectively for passage 11 to 75 μg mL^{-1} for passage 12 on both media. The MIC then leveled until passage 14 (Table 4.1). Figure 4.5 showed that Cetrimide MIC started decreasing gradually as the cells were transferred to the Cetrimide-free media.

The previous results indicate that *P. aeruginosa* resistance to zinc pyrithione and sodium pyrithione can be acquired by successive passaging in MIC/4 of the biocides. The resistant phenotype was acquired slowly and gradualy, but didn't persist for many generations after removal of the biocide. Due to time limits, the biocide-free passages were not extended. There was a 67% decrease in ZnPT MIC and 55% decrease in NaPT MIC from the MIC in passage 10 as determined from *Pseudomonas* cultures in R2A medium. However, a much lower percentage of MIC reduction was observed when cultures were prepared in CDM (Table 4.1). These results indicates that the acquired

Table 4.1: Summary of the average *P. aeruginosa* PAO1 MICs for ZnPT, NaPT and Cetrimide in R2A and CDM media. The figures in brackets indicate the percentage increase in MIC of Passage 11 over Passage 1 (bold black) and the percentage decrease in the MIC from Passage 14 to Passage 11 (bold red).

Antimicrobial	MIC in CDM ($\mu g\ mL^{-1}$)					MIC in R2A Medium ($\mu g\ mL^{-1}$)				
	P1	P11	P12	P13	P14	P1	P11	P12	P13	P 14
ZnPT	3.4	12.5 **(268)**	11	11	10 **(20)**	4	19.3 **(382)**	12	8	7 **(63.7)**
NaPT	30	105 **(250)**	75	75	75 **(29)**	40	170 **(325)**	75	75	75 **(55)**
Cetrimide	10	130 **(120)**	120	90	90 **(31)**	nd	nd	nd	nd	nd

P1 = Passage 1 (initial MIC); P11 = 10 th Passage in media containing the biocide; P12 = Passage No. 11 in biocide-free media; P 13 = Passage 12; P 14 = Passage 13; nd = not done.

resistance on CDM is relatively more stable than the resistance acquired on R2A medium. Returning to Figures 4.1 and 4.3, it can be predicted that there is a tendency for ZnPT-adapted cells to revert to their original MIC, whilst, for NaPT exposed cells (Figures 4.2 & 4.4) the leveling of the MIC in the three biocide-free passages, suggests that the MIC might not revert to the original value. This type of change in the MIC, excludes the possibility of a plasmid mediated acquired resistance, since the process of MIC reduction occurred gradually. The triplicate cultures for each biocide followed the same pattern in adaptation to that biocide. Plasmid acquired resistance could occur in one culture but it would not happen in three cultures and follow the same pattern. Temporary acquired resistance resulting from training the bacteria to grow in gradually increasing concentrations of a biocide is known to be non-plasmid-encoded and to be unstable (Russell 2001).

During the process of adaptation, MIC levels paused for two or three passages and then resumed their increase. This suggests that challenged bacteria are switching from one stage (susceptibility) to another (insusceptibility or resistance), either by decreasing their outer membrane permeability, through switching off the synthesis of certain proteins, or through inducing the synthesis of other proteins (e.g. efflux systems) or by using new enzymatic pathways.

4.2 Investigation of cross-resistance

Cross-resistance is when an organism develops resistance against one drug and simultaneously develops resistance against other related or unrelated drugs. However, multi-drug resistance, is a term used to describe mechanisms of resistance by chromosomal genes that are activated by induction or mutation caused by the stress of exposure to antibiotics in natural and clinical environments (George, 1996). Exposure to

a single drug may lead to cross-resistance to many other structurally and functionally unrelated drugs. *P. aeruginosa* that was trained to grow in dodecyldimethyl ammonium chloride (Baradac 22) exhibited increased resistance to other QACs (Barquat MB50, Bardac LF) and to biguanide compounds (chlohexidine, Vantocil and Hibitane) (Jones, *et al* 1989). *P. aeruginosa* PAO1 cultures adapted to Zinc pyrithione, Sodium pyrithione and Cetrimide, were tested to assess whether resistance to one biocide had conferred any increased resistance to the other two biocides. An aliquot (250 µL) of the resistant culture (passage 11) was inoculated in duplicate into fresh medium containing MIC/4 concentration of one biocide (MIC determined from passage 11). Cultures were incubated for 24h at 37° C in an orbital shaker incubator at 180 rpm. The MIC was determined against the other two biocides by the tube dilution method. The results were expressed as the average of MIC obtained from duplicate test culture (Table 4.2 and Figure 4.6).

4.2.1 Results and Discussion

The MIC for the other two biocides (average of duplicate test cultures in CDM) of the previously induced resistant cultures was obtained in order to determine whether cross-resistance occurs between the three biocides. The results indicate that once resistance has been induced in a culture towards one of the biocides, resistance is exhibited towards the other two biocides in varying degrees (Table 4.2 and Figure 4.6). ZnPT-resistant cells exhibited resistance towards NaPT. The MIC for NaPT increased from 50 $\mu g\ mL^{-1}$ for the wild type strain to 72.5 $\mu g\ mL^{-1}$ for the ZnPT-resistant strain. ZnPT-resistant cells also had an increased resistance to Cetrimide with the MIC increasing from 12$\mu g\ mL^{-1}$ for the wild type strain to 75 $\mu g\ mL^{-1}$ in the ZnPT-resistant *P. aeruginosa* PAO1 strain. NaPT-resistant cells exhibited an increase in ZnPT MIC

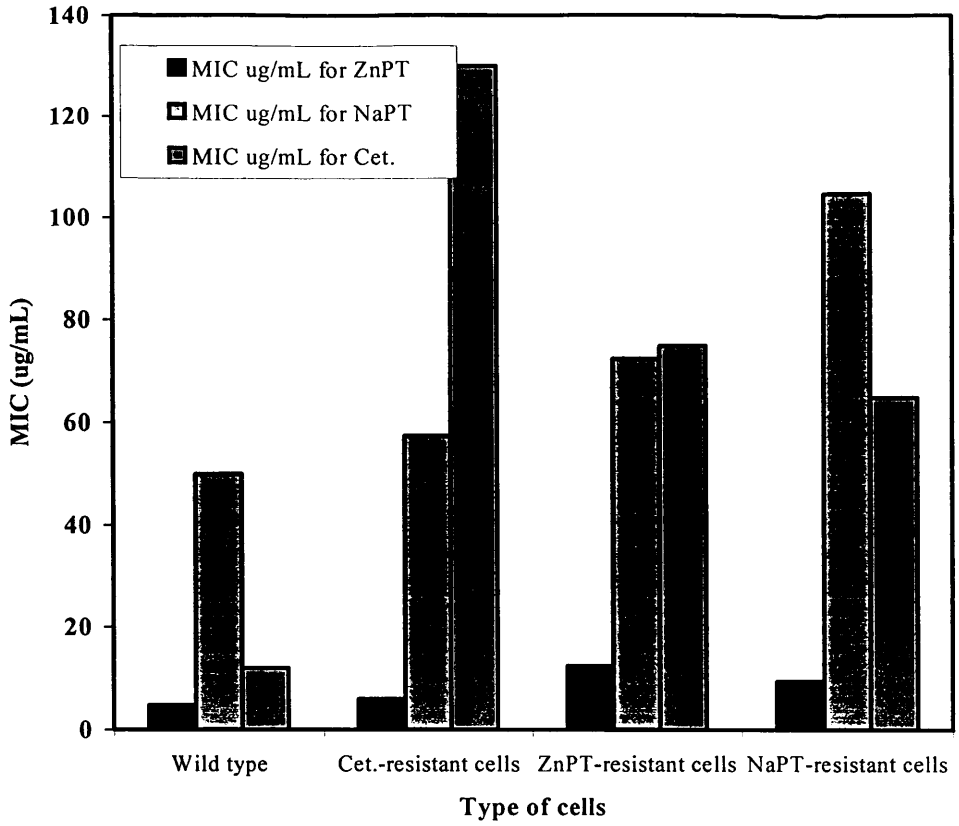


Figure 4.6: Cross resistance exhibited by the three resistant phenotypes of *P. aeruginosa* PAO1: ZnPT-resistant cells, NaPT-resistant cells and Cetrimide- resistant cells as compared to the MIC for the three biocides in the wild type *P. aeruginosa* PAO1. The MIC for the other two biocides was measured for each phenotype.

Table 4.2: Average MIC of the resistant cultures of *P. aeruginosa* PAO1 grown in CDM when examined against the other two biocides. MIC values are the mean of triplicate experiments. Figures in bracket represents the standard error for each value.

Tested cultures	Biocides MIC (μg mL^{-1})		
	ZnPT	**NaPT**	**Cetrimide**
Sensitive strain	4.8	50	12
ZnPT resistant	12.5	72.5 (+/- 8.1)	75 (+/- 5.4)
NaPT resistant	9.5 (+/-1.6)	105	65 (+/- 5.4)
Cetrimide resistant	6 (0)	57.5 (+/- 2.7)	130

(4.8 μg mL^{-1} to 9.5 μg mL^{-1}) and Cetrimide MIC (12 μg mL^{-1} to 65 μg mL^{-1}). Cetrimide resistant cells also expressed minor increases in resistance against the pyrithione biocides, with the MIC changing from 4 μg mL^{-1} to 6 μg mL^{-1} for ZnPT and from 50 μg mL^{-1} to 57.5 μg mL^{-1} for NaPT. Although the degree of resistance exhibited by the three types of resistant strains was not equal, it suggests that the mechanism of resistance towards pyrithione biocides is multifactorial and confers resistance towards the QAC Cetrimide. The mechanism of resistance adopted by *P. aeruginosa* towards NaPT did confer low level of resistance towards ZnPT and a high level of resistance towards Cetrimide. However, Cetrimide-resistant cells expressed low level resistance towards the pyrithione biocides which suggests that the mechanism of resistance towards Cetrimide has a little role in conferring high level of resistance towards pyrithiones and that other mechanisms are involved in Pyrithione resistance. ZnPT-resistant cells grown in R2A medium exhibited resistance to NaPT with the MIC changing from 50 μg mL^{-1} to 68 μg mL^{-1}. MIC for Cetrimide on R2A medium was not done.

Pyrithiones are membrane active compounds that induce leakage of intracellular material (potassium ions and O.D $_{260nm}$ absorbing material) and reduce intracellular ATP levels in *P. aeruginosa* due to their action on the bacterial membrane (Al-Adham *et al* 1998; Dinning, *et al* 1998; Dinning, *et al* 1998a). Cetrimide is also a membrane active compound that induces leakage of intracellular constituents, thus the cross-resistance results indicate that the bacterial response (mechanism of resistance) towards the attack of these two groups of membrane active biocides may be similar in some aspects.

4.3 Investigation of multi-drug resistance

P. aeruginosa exhibits resistance to a wide range of commonly used antibiotics and antimicrobials. Only a few antimicrobial agents, such as carbapenems and

quinolones, exhibit potent antibacterial activity against *P. aeruginosa* (Masuda *et al* 1995). Recently, resistant strains to these drugs have been isolated. These mutants exhibited cross-resistance to structurally unrelated antimicrobial agents as well (Masuda *et al* 1995). Three types of multi-drug-resistant mutants have been identified. (1) The *nalB* type mutant, which confers resistance towards meropenem, cephems and quinolones with the overexpression of the outer membrane protein OprM. (2) the *nfxB*-type mutant, which show cross-resistance to quinolones and new cephems (e.g. cefpirome and cefozopran), with the overproduction of the outer membrane protein OprJ. (3) The *nfxC*-type mutants, that show cross resistance to carbapenems and quinolones accompanied by the increased production of the outer membrane protein OprN and the decreased production of OprD (Masuda *et al* 1995). It has been suggested that the induction of the multidrug-resistance (MDR) genes in Gram-negative bacteria could be through exposure to low concentrations of biocides and that the derepression of multi-drug resistance genes could lead to resistance towards a group of unrelated antimicrobials. Moken *et al* (1997) have shown that mutants of *E. coli,* that developed resistance against the disinfectant pine oil, exhibited resistance to multiple antibiotics namely tetracycline, ampicillin, chloramphenicol and naladixic acid. Similarly, in another study McMurry *et al* (1998) proposed that low concentrations of Triclosan in the environment could select for *E. coli* multiple-antibiotic-resistant mutants. Clarke (2001) reported that a group of scientists performed a study on inducers of the *mar* (multiple antibiotic resistance) genes in bacteria by using randomly chosen groceries from a super-market. They found that mustard, cinnamon and shower gel triggered the *mar* operon and as a result, the bacteria exhibited resistance to a variety of antibiotics.

In the present study *P. aeruginosa* was adapted to Pyrithione biocides and Cetrimide, which are structurally dissimilar compounds. The resistant cells to one

biocide exhibited cross-resistance to the other biocides. Hence, there is a possibility of *mar* gene derepression. The possibility of resistance emerging from the derepression of *mar* gene was investigated. The antibiotic susceptibility pattern was measured at 37°C using antibiotic impregnated discs. Pyrithione-Sensitive and -resistant cultures were challenged with a variety of antibiotics that included Cephems and Penems. The disc diffusion test for antibiotic sensitivity was used (Bloomfield 1991).

An aliquot (100µl) of an overnight R2A culture of ZnPT-resistant cells (passage 11) was transferred to the surface of R2A agar plate. The inoculum was spread using a sterile swab to allow for the complete coverage of the plate with bacteria. Antibiotic sensitivity discs were applied on the surface of the seeded plate. The plates were left to stand for a period of one hour to allow diffusion of the antibiotic from the disc into the medium and then incubated overnight at 37°C. This experiment was performed in triplicate for both ZnPT-resistant and -sensitive strains. Results were obtained by measuring the diameter of the zone of inhibition created around the disc.

Antibiotics

The following antibiotics were supplied from Mast diagnostics, tetracycline (T) (10 μg disc^{-1}), Sulphatriad (ST) (300 μg disc^{-1}), Polymyxin B (PB) (100 units disc^{-1}), Streptomycin (S) (25 μg disc^{-1}). Chloramphenicol (C) (10 μg disc^{-1}) was purchased from OXOID. Ceftriaxone (30 μg disc^{-1}), Meropenem (10 μg disc^{-1}) and Ceftazidime (30 μg disc^{-1}) were the kind gift of Dr. Gabby Phillips, Ninewells Hospital, Dundee, Scotland, UK.

Table 4.3: The average diameters of zones of inhibition (cm) for ZnPT-sensitive and -resistant *P. aeruginosa* PAO1 when exposed to various antibiotics. Figures in bracket represent the standard deviation of each reading (+/-). Results are obtained from triplicate experiments

Antibiotics	Sensitive strain*	Resistant strain•
Meropenem	4.13 (0.208)	4.13 (0.208)
Ampicillin	R	R
Streptomycin	1.73 (0.152)	1.76 (0.152)
Sulphatriad	1.8 (0.378)	2.6 (0.378)
Ceftriaxone	1.9 (0.057)	1.93 (0.057)
Ceftazidime	2.36 (0.1)	2.3 (0.1)
Chloramphenicol	1.03 (0.115)	1.26 (0.115)
Tetracycline	R	R

R = No zone of inhibition (*) ZnPT sensitive *P.aeruginosa* PAO1 (•) P11 Cells

4.3.1 Results and Discussion

From the results shown in Table (4.3) there is no significant difference between the degree of susceptibility of ZnPT-sensitive *P. aeruginosa* and the ZnPT-resistant strains towards the tested antibiotics. Although there is cross-resistance between the biocides, there doesn't seem to be an involvement of the multi-drug resistance regulon (*mar*). Reports about biocide involvement in triggering antibiotic resistance in Gram-negative bacteria indicate that development of resistance to biocides may also result in insusceptibility to some antibiotics, possibly, as a result of outer membrane changes. However, such linkage exists under laboratory conditions and with specific types of bacteria (Moken, *et al* 1997; McMurry *et al* 1998; Russell 2001). In this study the possibility of ZnPT being an inducer of the *mar* regulon was investigated. Results have shown that ZnPT is not an inducer of the *mar* genes. Thus it can be concluded that development of resistance to ZnPT in *P. aeruginosa* is not associated with resistance to clinically relevant antibiotics.

4.4 Maintenance of Resistance Induced Cultures on Gradient Plates

During passage experiments, MICs for cultures were measured by two methods: the tube dilution method and a modified agar diffusion assay known as the gradient plate method (Bloomfield 1991). The gradient plate assay allows direct determination of MIC and maintenance of the resistant cultures on solid media. Square petri dishes were used (BDH: 10 cm x 10 cm). Molten R2A agar (10 mL) that contained 2x MIC of the culture to be inoculated was poured into the plates. The plates were allowed to set at an angle in the form of a wedge, and were then laid flat. A second layer of molten R2A (10mL) was added and allowed to set (Figure 4.7). Plates were then stored at room temperature for

24 h to allow for diffusion of the biocide to the upper layer forming a concentration gradient across the plate. The MIC for different biocides in the passaged cultures was first measured by the tube dilution method and the same cultures were streaked on the surface of the gradient plates. Plates were incubated overnight (or until growth appears) at 37°C. Colonies will appear growing on the line of streak to a certain point at which growth stops. Above this point the concentration of the biocide will be inhibitory to the bacteria, and thus, colonies growing at the edge must be highly resistant. The MIC from the inoculated gradient plate could be calculated using the following equation:

$$MIC\ (\mu g\ /\ mL\ ) \equiv \frac{Y - X}{Y \times C\ (\mu g\ /\ mL\ )}$$

Where, **Y** is the length of the plate, **X** is the zone of inhibition and **C** is the concentration of the biocide added to the media of the lower layer of the plate (Figure 4.6).

During training of *P. aeruginosa* to grow in the presence of ZnPT and NaPT on R2A media, it was noticed that colonies with a mucoid phenotype were frequently obtained. These colonies would tend to grow in the middle of the zone of inhibition or at the end of it, but most of the time at the edge of the growth. These colonies were highly resistant to the biocide incorporated in the media. When cultured on biocide-free nutrient agar plates the colonies lost their mucoid phenotype. It was thought that this phenotypic change was related to alterations in the lipopolysaccharide of the outer membrane. The mucoid colonies were harvested and LPS gel analysis was performed.

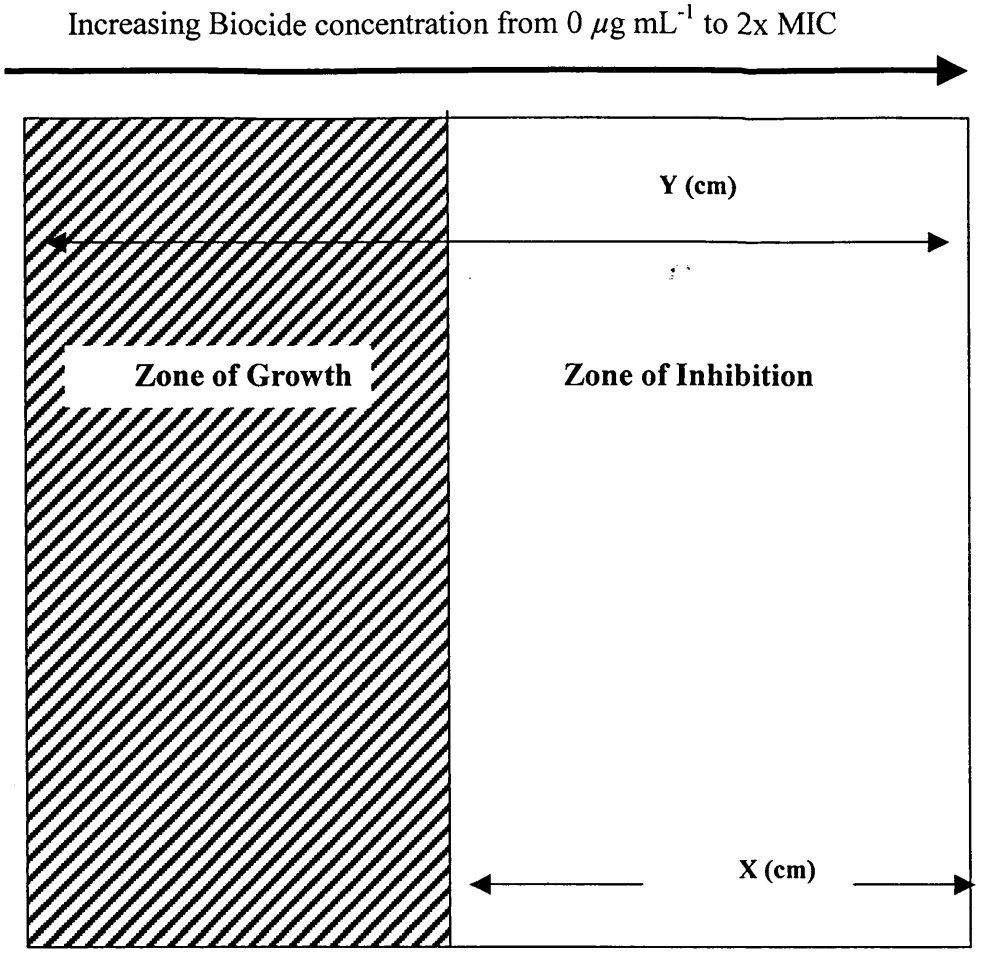


Figure 4.7: Illustration of the gradient plate used to determine the minimum inhibitory concentration (MIC). (**Y**) is the length of the plate (cm) (**X**) is the length of the zone of inhibition (cm).

4.5 Lipopolysaccharide analysis

The pathogenicity of *P. aeruginosa* is attributed to the production of diverse virulence factors. Alginate and lipopolysaccharides (LPS) are two of these factors. LPS molecules have a structural role in the cell envelope of *P. aeruginosa*. It is the main constituent of the outer leaflet of the outer membrane. LPS is made of three regions: (1) the lipid A region, which anchors the LPS molecule to the outer membrane, (2) a core oligosaccharide region, whose side chain sugars appear as a ladder pattern in silver stained LPS gels; and (3) the O antigen (O polysaccharide). The core oligosaccharide region in *P. aeruginosa* contains KDO, heptose, glucose, rhamnose, galactosamine, phosphate and alanine (Figure 1.2b). The O-polysaccharide chain was found to contain fucosamine, rhamnose, glucose and possibly mannose (Jarrell and Kropinski 1977). *P. aeruginosa* has been found to co-express two distinct types of LPS, the A-band (short polysaccharide chain) and the B-band (long polysaccharide chain) (Rocchetta and Lam 1997). The A-band is antigenically conserved with an O-polysaccharide region composed of short-chained polymers of D-rhamnose arranged in trisaccharide repeat units. The B-band LPS is serospecific, with variations in the O-antigen structure that differentiate *P. aeruginosa* into 20 distinct serotypes (Rocchetta and Lam 1997). *P. aeruginosa* isolates from patients of cystic fibrosis become non-typable because of the loss of the B-band, while the A-band and alginate become the surface structural components of the isolates (Rocchetta and Lam 1997). *P. aeruginosa* strains expressing only short-LPS (A-band) were found to exhibit resistance to polycationic antibiotics including gentamicin (Hasegawa *et al* 1997). Bryan *et al* (1984) analyzed the LPS of several strains of *P. aeruginosa* that show resistance to aminoglycosides. He suggested that changes in the LPS banding pattern in SDS-PAGE were responsible for aminoglycoside resistance. The authors argued that the repeating side chains of LPS

that changes in the LPS banding pattern in SDS-PAGE were responsible for aminoglycoside resistance. The authors argued that the repeating side chains of LPS were necessary, either for the direct binding of the aminoglycoside or for enhancement of the access of aminoglycoside through the outer membrane. Alterations and modifications in the chemical structure of the LPS layer, that plays a role as a regulator of the porin channels in the outer membrane can be related to observed increases in resistance to antimicrobial agents (Giordano *et al* 1993).

In the present study, large mucoid colonies were obtained on gradient plates during the adaptation of *P. aeruginosa* to ZnPT and NaPT on R2A medium. In order to establish whether any alterations in LPS structure were associated with the resistant phenotype, SDS-PAGE for the extracted LPS from the mucoid and the wild type colonies was performed.

4.5.1 Extraction of LPS

LPS was extracted from mucoid and wild type cells according to the method of Preston and Penner (1987). Mucoid colonies were grown in fresh R2A agar medium containing the respective biocide (ZnPT or NaPT) concentration that is equal to the measured MIC in that passage divided by 4. Non-mucoid colonies were sub-cultured onto fresh R2A agar. Mucoid and non-mucoid colonies were harvested in 5 mL of cold phosphate buffer saline (PBS) using a curved glass rod. Cultures were collected in tubes and cell density was measured at E_{600nm}. Cell suspensions were diluted in PBS to an E_{600nm} of 0.6. An aliquot (1.5 mL) of the diluted cell suspension was centrifuged for 1.5 minutes at 5000 *g*. The supernatant was discarded. The cell pellets were solubilised in 200 μL of the Lysis buffer (Glycerol, 20%; 2-β-mercaptoethanol, 5%; SDS, 4.6%; 0.125M Tris HCl (pH 6.8), bromophenol blue, 0.004%) and heated for 10 min at 100°C.

The heated lysates were allowed to cool at room temperature. An aliquot (40 μL) of lysis buffer containing 2.5mg mL^{-1} of Proteinase K was added to the cooled lysates. The samples were incubated at 60°C for one hour, followed by incubation in a boiling water-bath for 5 minutes. Samples were cooled and used for SDS-PAGE.

4.5.2 Determination of LPS profile

LPS profiles were prepared using 12% separator and 5% stacker gel as described earlier (Chapter Two). Electrophoresis was performed with a constant current at 35 mA for 1.5 hours or until the blue front reached the end of the gel.

4.5.3 Staining of the LPS gels

Lipopolysaccharide gels were stained according to Preston and Penner (1987). Gels were immersed in fixing solution (ethanol, 400 mL; glacial acetic acid, 50 mL; made up to 1L with distilled water) for 16 hours. The gels were transferred to oxidizing solution (periodic acid, 3.5 g; ethanol, 200 mL; glacial acetic acid, 25mL; made up to 500mL with distilled water) for five minutes with mild agitation and then washed twice with distilled water for 15 minutes. The gels were immersed in freshly prepared staining reagent (concentrated NH_4OH, 2 mL; 0.1M NaOH, 28 mL; 20% w/v $AgNO_3$, 5 mL; distilled water, 115 mL). Gels were agitated vigorously for 10 minutes, and then washed three times each for 10 minutes in distilled water. Gels were transferred to the develop solution (citric acid, 0.05 g; 37% formaldehyde, 0.5 mL; made up to 1 L with distilled water) for 2 to 5 minutes or until bands were seen clearly. The develop solution was decanted from the gels. The gels were washed twice in distilled water, and then stored in distilled water at 4°C in the dark for up to one week.

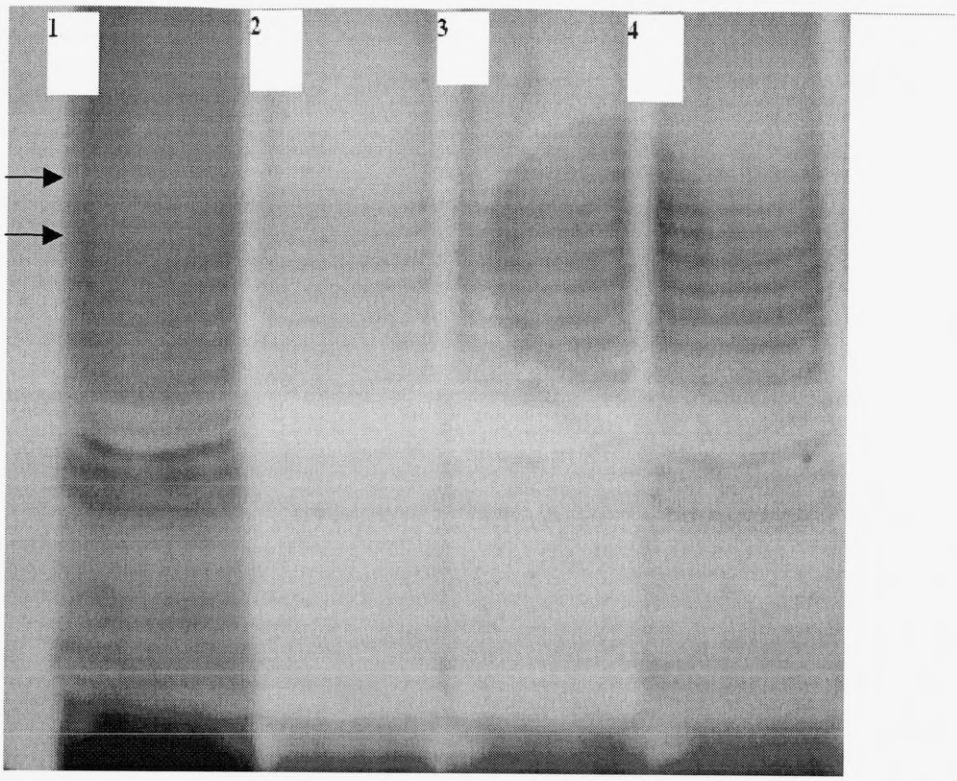


Figure 4.8: Silver-stained SDS-PAGE for LPS of mucoid and wild type non-mucoid *P. aeruginosa* PAO1 . Lane (1) corresponds to LPS from *P. aeruginosa* PAO1 sensitive strain. Lane (2) corresponds to LPS obtained from mucoid colonies from P5, Lane (3) mucoid colonies from passage 4, Lane (4) mucoid colonies from passage 6.The area between the two arrows represent the missing bands.

4.5.4 Results and Discussion

Mucoid colonies were found to grow in the middle or at the far end of the zone of inhibition on R2A gradient plates, where the biocide was at its highest concentration. The colonies were observed clearly after 48 h of incubation. These colonies were highly chromogenic and resistant to both ZnPT and NaPT. Upon LPS gel electrophoresis the ladder pattern consistent with LPS side chains was observed (Figure 4.8). The two types of O-polysaccharide chains can be seen with the A-band being the short chain with low molecular weight and the B-band with the higher molecular weight (top part). *P. aeruginosa* isolates from chronic Cystic Fibrosis (CF) patients showed loss in the B-band, yet they were known to express the mucoid phenotype. Such colonies were shown to have only A-band chains, with the excessive production of alginate (Rocchetta and Lam 1997). Thus, mucoidy in *P. aeruginosa* is determined by the production of the exopolysaccharide slime. Wild type *P. aeruginosa* PAO1 on LPS gel analysis show all three components of LPS. Any variation or loss of any part of the LPS molecule will be apparent in the LPS gel. Thus, after examining the gel image (Figure 4.8) of the mucoid ZnPT-resistant colonies and the non-mucoid ZnPT-sensitive cells, it is apparent that there is an increase in the number of high molecular weight bands (B-band) in the mucoid strains in comparison with the non-mucoid sensitive strains. It has been found by Rottem and Leive (1977) that the higher the LPS concentration in the outer membrane the more restricted the mobility of the acyl chains in the Lipid A region, and consequently reduced permeability of the outer membrane. The former explanation could partially explain the increase in ZnPT-resistance exhibited by the mucoid colonies. However, such a change in morphological appearance could be due to production of exopolysaccharide alginate, that is known to be produced when bacteria are subjected to

sub-inhibitory concentrations of antimicrobials (Govan & Fyfe 1978; Pechey & James 1974; Jones *et al* 1989).

4.6 Investigation of Cell Surface Hydrophobicity

ZnPT-resistant and wild type colonies on R2A agar were further investigated for changes in their surface hydrophobicity. It has been reported that alterations in colony morphology were associated with changes in the cell surface hydrophobicity (Pechey & James 1974; Jones *et al* 1989). *P. aeruginosa* trained to grow in increasing concentrations of gentamicin exhibited changes in cell surface properties, which had resulted in an overall increase in the negative charge carried by the cell and in amounts of surface lipids (Pechey & James 1974). Jones *et al* (1989) found that QAC and amphoteric compounds-adapted *P. aeruginosa* cells were more hydrophobic than the unadapted organisms. Sub-inhibitory concentrations of benzalkonium chloride and chlorhexidine diacetate caused an increase in cell surface hydrophobicity of *P. aeruginosa* (El-Falaha *et al* 1985).

This experiment was performed to detect any differences in the surface hydrophobicity of ZnPT-resistant and ZnPT-sensitive cells, this experiment was also used to investigate changes in hydrophobicity during the process of adaptation. Wild type *P. aeruginosa* PAO1 cells and ZnPT-resistant cells from passages 2, 6 and 11 were tested. Resistant cells grown in biocide-free media were also tested and these were cells from passages 12, 13 and 14.

4.6.1 Preparation of bacteria

Cells were inoculated in 25 mL of biocide-free R2A medium, and incubated in a shaking incubator at 37°C, 180 rpm for 48h. At the end of the incubation period cells

were sub-cultured in fresh R2A media and harvested at mid-exponential phase (6 hours). Cells were pelleted by centrifugation at 3000 *g* for ten minutes. The pellet was washed twice in PUM buffer ($K_2HPO_4.3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; urea, 1.8 g; $MgSO_4.7H_2O$, 0.2 g dissolved in 900 mL of distilled water, the pH was adjusted to 7.1 and the volume was made up to 1 L), centrifuged for 15 minutes at 10000*g*. Bacterial pellets were then resuspended in PUM buffer to an absorbency of 1.0 at E_{470nm}.

4.6.2 Assay procedure

An aliquot (5mL) of the bacterial suspension was mixed with 2.5 mL of xylene in a 20 mL capped glass bottle. The mixture was vortexed for 2 minutes. The solutions were allowed to settle for 15 minutes at room temperature to allow for phase separation to take place. The lower aqueous phase was collected carefully and the absorbance was measured at E_{400nm} (Novaspec, LKB).

4.6.3 Results and Discussion

Following separation of the two layers, the upper layer is composed of a xylene in water emulsion and the lower layer is the aqueous part. A decrease in the absorbency of the aqueous part from the absorbency of the original cell suspension indicates the removal of cells from the aqueous phase to the aqueous: hydrocarbon phase, thus, indicating an increased hydrophobicity. Figure 4.9 demonstrates the change in hydrophobicity between ZnPT-sensitive *P. aeruginosa* (PAO1) and ZnPT exposed and resistant strains (P11) of *P. aeruginosa*. ZnPT-resistant strain exhibited an increase in hydrophobicity in the form of a decrease in OD_{400nm} as compared with the OD_{400nm} before separation. However, ZnPT-sensitive strain exhibited an increase in the OD_{400nm}

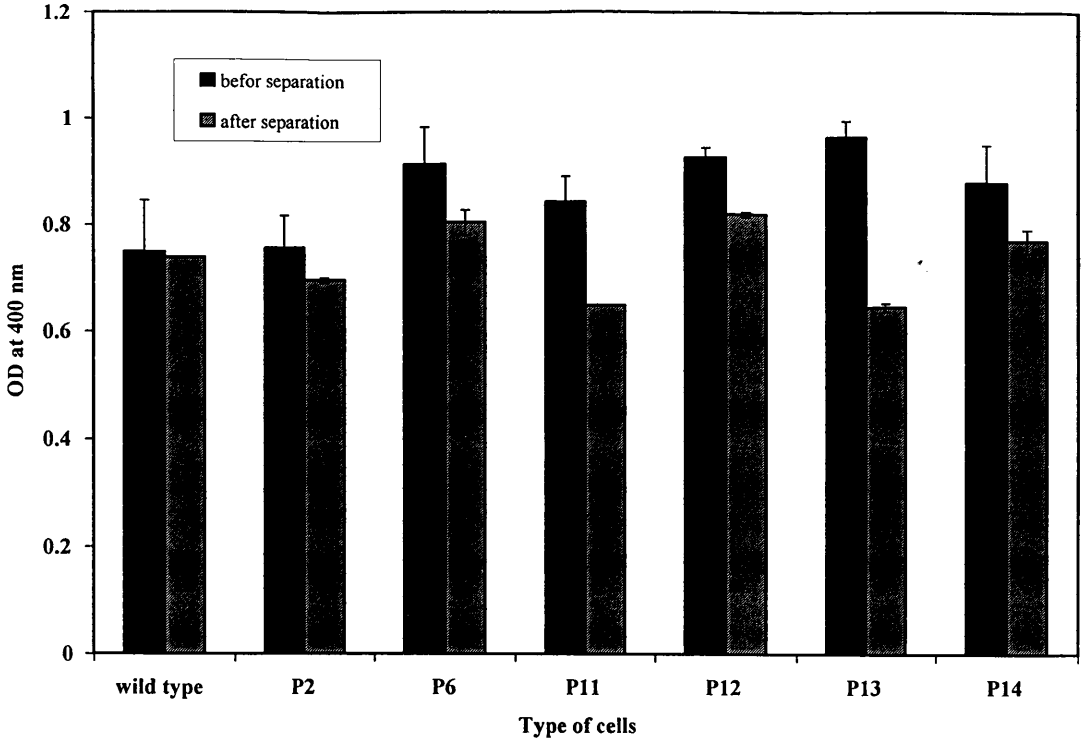


Figure 4.9: Changes in optical density at 400nm, before and after partitioning of *P. aeruginosa* (ZnPT-sensitive (wild type) and -adapted strains grown in R2A medium) in Xylene as an indication of altered hydrophobicity. P2, P6 and P11 are ZnPT adapted cells. P12, P13 and P14 are adapted cells grown in ZnPT-free R2A medium.

readings after separation, indicating no adherence to the hydrocarbon phase. Instead, the cells were hydrophilic. This change in hydrophobicity was compatible with the increase in the MIC. Interestingly, this hydrophobic phenotype persisted even when the resistant cells were transferred to a ZnPT-free medium, which is demonstrated in the biocide-free passages P12, P13, P14 (Figure 4.9) although the MIC dropped from 19.3 μg mL^{-1} (P11) to 7 μg mL^{-1} in these passages. Although the resistant bacteria started growing in a stressless environment (i.e. absence of ZnPT), the hydrophobic phenotype persisted. Such results are confusing since ZnPT is a hydrophobic biocide and as the cell become more hydrophobic during adaptation, ZnPT penetration into the cell should become easier. However, this also suggests that when the cell becomes more hydrophobic it change the outer leaflet structure by expressing more phospholipids on the surface of the cell, thus altering the structure of the outer leaflet. This alteration could mask or remove target sites necessary for the initial binding or penetration of the biocide.

4.7 General Discussion

Previous studies (Br.zel and Cloete 1994, Winder *et al* 2000) have reported the possible induction of resistance to Isothiazolone biocides in the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. In the present study the adaptation of *Pseudomonas aeruginosa* PAO1 to the Pyrithione biocides has been observed. Adaptation has been the result of gradual exposure of *P. aeruginosa* to sub-MIC concentrations of the two Pyrithione salts, Zinc Pyrithione (ZnPT) and Sodium Pyrithione (NaPT). The MIC for ZnPT has increased from 3.4 $\mu g\ mL^{-1}$ to 12.5 $\mu g\ mL^{-1}$ when *P. aeruginosa* was grown on CDM medium, and from 4 $\mu g\ mL^{-1}$ to 19.3 $\mu g\ mL^{-1}$ when grown in R2A medium. The MIC for NaPT increased from 30 $\mu g\ mL^{-1}$ to 105 $\mu g\ mL^{-1}$ when grown on CDM while it increased from 40 $\mu g\ mL^{-1}$ to 170 $\mu g\ mL^{-1}$ when grown on R2A medium. Results in Table 4.1 have shown that the percentage increase for MIC in R2A media (382%, 325% for ZnPT and NaPT respectively) is higher than the percentage increase in CDM (267.6%, 250% for ZnPT and NaPT respectively). The percent reduction in MIC after the removal of the biocide from the media was higher in R2A medium (63.7%, 55% for ZnPT and NaPT respectively) in comparison to the percent reduction in CDM medium (20%, 28.5% for ZnPT and NaPT respectively). These results indicate that R2A medium, which is relatively a nutritionally rich medium in which the bacteria grow easily, allows the rapid selection of the resistant phenotype. As the biocide is removed from the media the bacteria reverts rapidly to its normal phenotype. However, obtaining the resistant phenotype in CDM was relatively slow in comparison to obtaining the resistant phenotype in R2A medium and the reduction in MIC observed after the removal of the biocide from the media was also slow. This indicates that the process of ZnPT adaptation and de-adaptation in CDM was affected by the growth conditions. During the process of biocide adaptation, mucoid colonies, that expressed higher MIC

values than the MIC value of the rest of the cells in the cultures, appeared. When investigated for their LPS structure using LPS SDS-PAGE analysis, these mucoid colonies were found to exhibit some variations in the banding profile in the form of increased B-band which is the core area. It is known that heptoseless mutants are more sensitive to antimicrobials. Thus the increase in the saccharides of the core or the B-band area could explain the increase in resistance of these mucoid cells. Brooun *et al* (2000) has reported the emergence of super-resistant cells in biofilms after antibiotic treatment. These super-resistant cells were suggested to be responsible for the regrowth of bacteria in the biofilm after the antibiotic treatment. Could these planktonic ZnPT highly resistant (super-ZnPT- resistant cells) mucoid cells found in the present study be similar to the super-resistant cells in biofilms, is a matter that remains to be elucidated.

ZnPT-resistant *P. aeruginosa* cells from P11 were found to be more hydrophobic in comparison with the ZnPT sensitive cells. This increase in hydrophobicity could be as a result of outer membrane changes that resulted in exposing phospholipid patches on the surface of the cell. Pyrithione drugs are excellent metal chelators. The introduction of these drugs in the growth media of *P. aeruginosa* could be causing the chelation of divalent cations from the LPS molecules, which would lead to disruption of the outer membrane. This effect is expected when the biocide is used in the "in use" concentrations (250-1000 ppm) (Nelson and Hyde1981). However, low concentrations (MIC/4) are expected to induce little change in LPS resulting in the exposure of fewer phospholipid patches, which would explain the increased hydrophobicity. On the other hand, the bacteria are compensating for that damage in LPS by the increase in the long chain polysaccharides, which seemingly could stabilize the structure.

The former response of *P. aeruginosa* to the presence of Pyrithione biocides explains its cross-resistance towards Cetrimide, which is a membrane active biocide. It is

known that Cetrimide resistance is intrinsic and is due to the tight interactions between the LPS molecules. Thus, changes in the LPS structure in response to Pyrithiones could be conferring resistance towards Cetrimide as well. This suggestion is true since resistance to Cetrimide does not confer high resistance towards Pyrithione biocides (Table 4.2). On the other hand, resistance to ZnPT did not confer resistance to a number of antibiotics (Table 4.3) suggesting that Zinc Pyrithione is not an inducer of the *mar* regulon in *P. aeruginosa.*

Chapter Five

Investigation of mechanisms of ZnPT resistance in *P. aeruginosa*

Resistance to antimicrobials can be either a natural property of the organism (intrinsic) or acquired by mutation or acquisition of plasmids or transposons (McDonnell & Russell 1999). Gram-negative bacterial resistance to antimicrobials is mainly intrinsic owing to their outer membrane structure. *P. aeruginosa* exhibits high levels of resistance to a wide range of antimicrobials including antibiotics and biocides. This resistance is mainly intrinsic. The low outer membrane permeability is the property that distinguishes *P. aeruginosa* from other Gram-negative bacteria. Although porins of *P. aeruginosa* have a large exclusion limit in comparison to other Gram-negatives e.g. *E. coli* (Table 1.1) its outer membrane permeability is 12 to100 fold less than that of other Gram-negatives.

P. aeruginosa adopts three main mechanisms of intrinsic resistance; outer membrane permeability, drug efflux mechanisms and biocide degrading enzymes. Development of resistance involves one or more of these mechanisms. For bactericides to be effective they must be able to penetrate the cell envelope and attain a sufficiently high concentration at the target site in order to exert their antibacterial action (Bryan *et al* 1984). Mutations producing reduced expression or loss of nonspecific or specific porin genes can make Gram-negative bacteria significantly more resistant to certain agents (Nikaido 1994). For example, an outer membrane protein (T) of a molecular weight 35kD was found to disappear as *P. aeruginosa* adapted to growth in the presence of increasing concentrations of the biocide (Kathon^{TM}), suggesting that this protein could be the route of entry of these biocides (Brozel & Cloete 1994). Winder *et al* (2000) reported the absence of the same protein (T-OMP) from the outer membrane of a *P.*

aeruginosa PAO1 strain adapted to growth in the presence of 5-chloro-N-methylisothiazolone (CMIT), Benzisothiazolone (BIT) and N-methylisothiazolone (MIT). T-OMP re-appeared when the biocides were removed from the media, but the re-appearance was not accompanied by a complete reversal of the induced resistance (Winder *et al* 2000). In another study, MIT resistant isolates of *Pseudomonas* had an altered outer membrane profile in the form of a missing protein (42kD) believed to be D-OMP which is a water-filled trans-membrane diffusion channel (Chapman *et al* 1998). The MIT sensitivity was restored when the outer membrane was permeabilized by treatment with the chelating agent EDTA. These results confirm the role of the outer membrane as a permeability barrier in MIT resistance.

Over-expression of some existing outer membrane proteins contributes to the development of resistance. The MIT resistant *Pseudomonas* isolates exhibited cross-resistance towards structurally unrelated preservatives (Chapman *et al* 1998). Outer membrane analysis of *Pseudomonas* (003) strain that is resistant to a mixture of preservatives (Dimethoxydimethyl hydantoin (DMDMH), iodopropargyl butylcarbamate (IPBC), chloroisothiazolone (CMIT) and methylisothiazolone (MIT)), revealed the over-expression of a 72 kD protein which has been related to an efflux system (Chapman *et al* 1998).

In this study, outer membranes of ZnPT-, NaPT- and Cetrimide-sensitive and -resistant cells will be analyzed using SDS-PAGE, to look for alterations in their protein profile that could contribute to the development of induced resistance towards these biocides.

5.1 Outer Membrane Alterations in Pyrithione-Resistant *P. aeruginosa* PAO1

5.1.1 Outer Membrane Protein (OMP) Preparations

Passaged cultures of *P. aeruginosa* PAO1 were centrifuged (IEC Centra-4B) at 5000 *g* for 15 minutes. Outer membranes were prepared according to the method of Pugsley *et al* (1986) as described in section 2.6 (Chapter Two)

5.1.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

5.1.2.a Preparation of the separating gel mixture

A 10% stock acrylamide solution was prepared as described in section *2.6.1* (Chapter Two).The gel was overlayed with water-saturated butan-2-ol, and left to set for approximately 1hour at room temperature.

5.1.2.b Preparation of the stacking gel mixture

Stacking gel mixture was prepared as described in section *2.6.2* (Chapter Two).

5.1.2.c Preparation of outer membrane protein profiles

Molecular weight marker (Low molecular weight calibration kit for SDS electrophoresis, Amersham Pharmacia Biotech; 10μL) and test samples (of an equal protein concentration) were loaded into the wells. A constant current (35mA) was applied to the gels by a Power Pack 300 (Biorad) for approximately 1.5h, or until the bromophenol blue front reached the bottom of the gel. The gel was removed from its cast and immersed in either Coomassie blue R250 stain or in the silver stain fixative

(Chapter Two). Gels were analysed using Phoretix imaging analysis software (non-Linear Dynamics Ltd., Newcastle upon Tyne, England).

5.1.3 Gel image analysis

The Phoretix imaging system was used to detect the bands of all the outer membrane proteins in the samples and analyse them to allow for comparison between profiles of resistant and sensitive strains.

In order for the Phoretix system to analyse the gel it first has to identify the lanes and individual proteins. Initially the system was instructed as to the position of lanes on the gel. The background of the gel was determined to allow the system to recognise the distinguished protein bands. Once the protein bands were detected, protein peaks are displayed. The edges of the protein band could then be identified, such that the system knew when the protein band began and ended on the image. These were the preliminary steps that had to be established before the system starts to make the matching between the different lanes and the reference lane (outer membrane of the sensitive strain) and display their peak density profiles. A molecular weight calibration curve was constructed manually, and specific molecular weights were propagated on the bands (Figure 5.1).

5.1.4 Results and Discussion

SDS-PAGE analysis was performed on the outer membranes of ZnPT-, NaPT- and Cetrimide-sensitive and -resistant cells. Electrophoretic profiles of the outer membranes showed alterations in the protein structure, which manifested clearly in the ZnPT-resistant cells (Figure 5.2). Phoretix analysis of ZnPT-sensitive and -resistant outer membrane gel images revealed the absence and the over-expression of proteins. Figures 5.2 and 5.6 demonstrate the gel image of outer membranes of ZnPT-sensitive *P.*

aeruginosa and the ZnPT-exposed and -resistant cells grown in R2A and CDM media respectively. A protein having a molecular weight of 26.30 kDa was observed to have disappeared from the outer membranes of P2 and P11 cells of *P. aeruginosa* grown in both R2A and CDM media. The same protein was observed to reappear in the outer membranes of passages P12, P13 and P14. Figure 5.3 demonstrates comparison between the peak intensities of each protein band in the resistant strain (P11) represented by the green line and sensitive strain represented by the red line. It can be observed that there are some variations in the intensity of peaks between samples. Due to variable protein concentrations applied. Equal loading of the samples was achieved, but with minor variations in protein concentration that are stated in the caption of Figure 5.2.

Upon examining the peak intensity profiles, a protein band of a molecular weight 26.30 kDa that lies between proteins number 9 and 8 appeared to be missing. When examining the P14 peak intensity profile (Figure 5.4) and comparing it with the profile of P11 (Figure 5.3), the above mentioned protein band reappears in the peak intensity profile of P14. This protein was suppressed within 24 h of exposure to ZnPT. The disappearance of this protein coincided with the increase in MIC for ZnPT, which suggests the direct involvement of the 26.302 kD protein in the development of resistance against ZnPT. ZnPT-resistant cells exhibited cross-resistance towards Cetrimide and NaPT on CDM medium. However, Cetrimide resistant cells did not express a significant cross-resistance towards ZnPT. Cetrimide resistance raised the MIC towards ZnPT from4 μgmL^{-1} to 6 μgmL^{-1} (Table 4.1). Furthermore, Cetrimide-resistant

cells grown on CDM did not show absence of the 26.30 kDa protein from its outer membranes (Figure5.5). The previous result suggests two points: The first point is that this protein is not a ferripyochelin receptor, because it was observed to be missing from outer membranes of ZnPT-resistant cells grown on CDM , which is rich in iron when compared to R2A media (Figure 5.7). It is known that under conditions of iron limitation *P. aeruginosa* produces siderophores and that these siderophores complex or chelate iron as well as other ions like Zinc (Visca *et al.* 1992). Therefore, in the present study a ferripyochellin binding protein receptor is not the identity of the missing protein, but it could be another ligand binding protein receptor for Zn^{2+} complexes. The second point suggests the involvement of multiple mechanisms in ZnPT-resistance. Cetrimide is a membrane active compound that exerts its antimicrobial action by inducing leakage from the cell. Therefore, resistance towards Cetrimide could be due to the unique structure of *P. aeruginosa* cell envelope that involves the tight interactions between the LPS molecules and the excess Mg^{2+} content (Russell & Chopra 1996). Thus it seems that *P. aeruginosa* adopts multiple mechanisms to express resistance towards ZnPT, one of which is alteration in the LPS structure. These LPS alterations conferred high level of resistance towards Cetrimide, but such alterations in Cetrimide-resistant cells didn't confer high level of resistance towards ZnPT suggesting the involvement of other mechanisms in ZnPT resistance. The absence of a protein from the outer membranes of ZnPT-resistant cells (P11) was accompanied by expression of other proteins. Figure 5.3 demonstrates the over-expression of a protein that is designated number 6 by the Phoretix software. This protein (35.48 kDa) was observed to be over-expressed in the outer membranes of P14 (Figures 5.4). The peak intensity of that protein was not altered when the cells were passaged in biocide-free media. Another two proteins (43.65 kDa and 47.80 kDa) were over-expressed in the ZnPT-resistant strain.

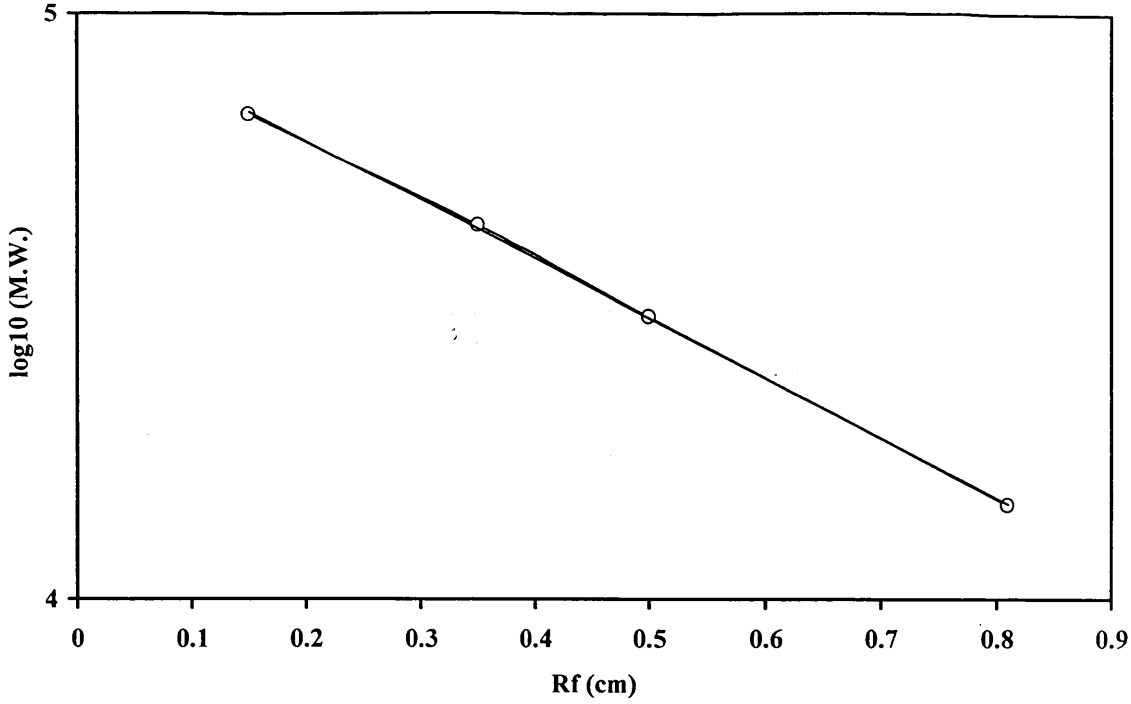


Figure 5.1 Molecular weight calibration curve. R_f values calculated for the low molecular weight standards by dividing migration distance of the protein by the migration distance of the dye (bromophenol blue) marker after migration in SDS-PAGE. The gels were stained with Coomassie Brilliant Blue.

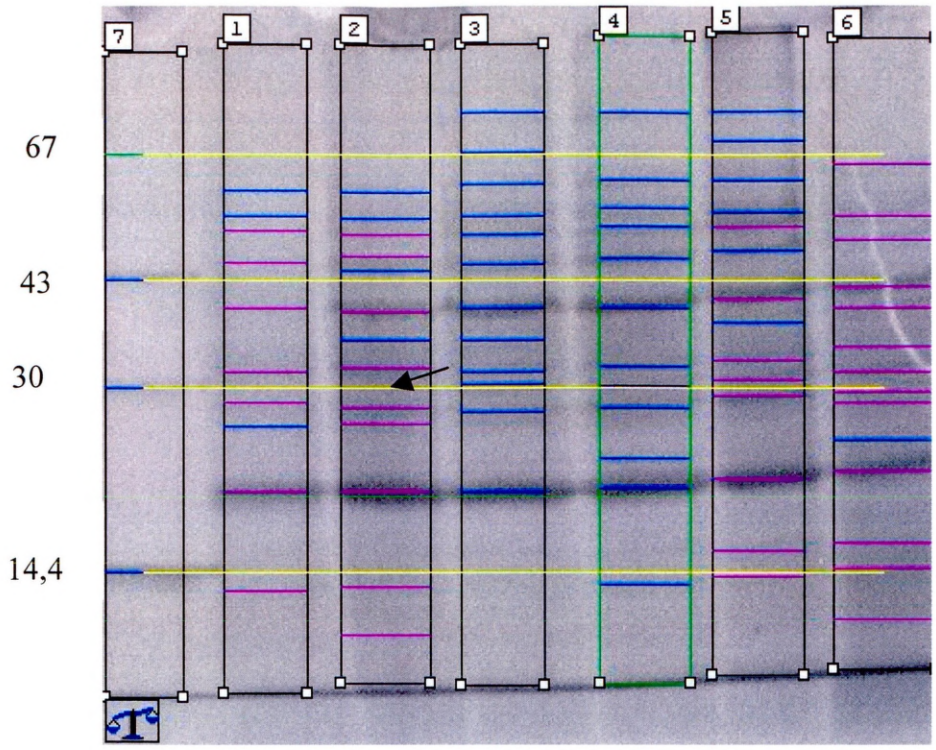


Figure 5.2: A Coomassi blue stained gel image of the outer membranes of ZnPT-sensitive and -resistant strains of *P. aeruginosa* PAO1 grown in R2A medium. L 7 is the molecular weight standards. Numbers on the left indicates the molecular weights in kD. Yellow lines show the matching according to the molecular weight. Blue and pink lines indicate the position of the individual bands in each lane as detected by the phoretix system. L1 represents the outer membrane of passage 2 cells. L2 represents the outer membrane of passage 11 cells. Lanes 3, 4 and 5 represent the outer membranes of passages 12, 13, and 14 respectively (biocide-free passages). Lane 6 is the outer membrane of wild type *P. aeruginosa* PAO1 strain (unexposed control). The protein concentration in each of the lanes was as follows: 210μg mL^{-1}, 240μg mL^{-1}, 330μg mL^{-1}, 540μg mL^{-1}, 240μg mL^{-1}, 560 μg mL^{-1} respectively. The arrow points at the missing protein band from OM of P11.

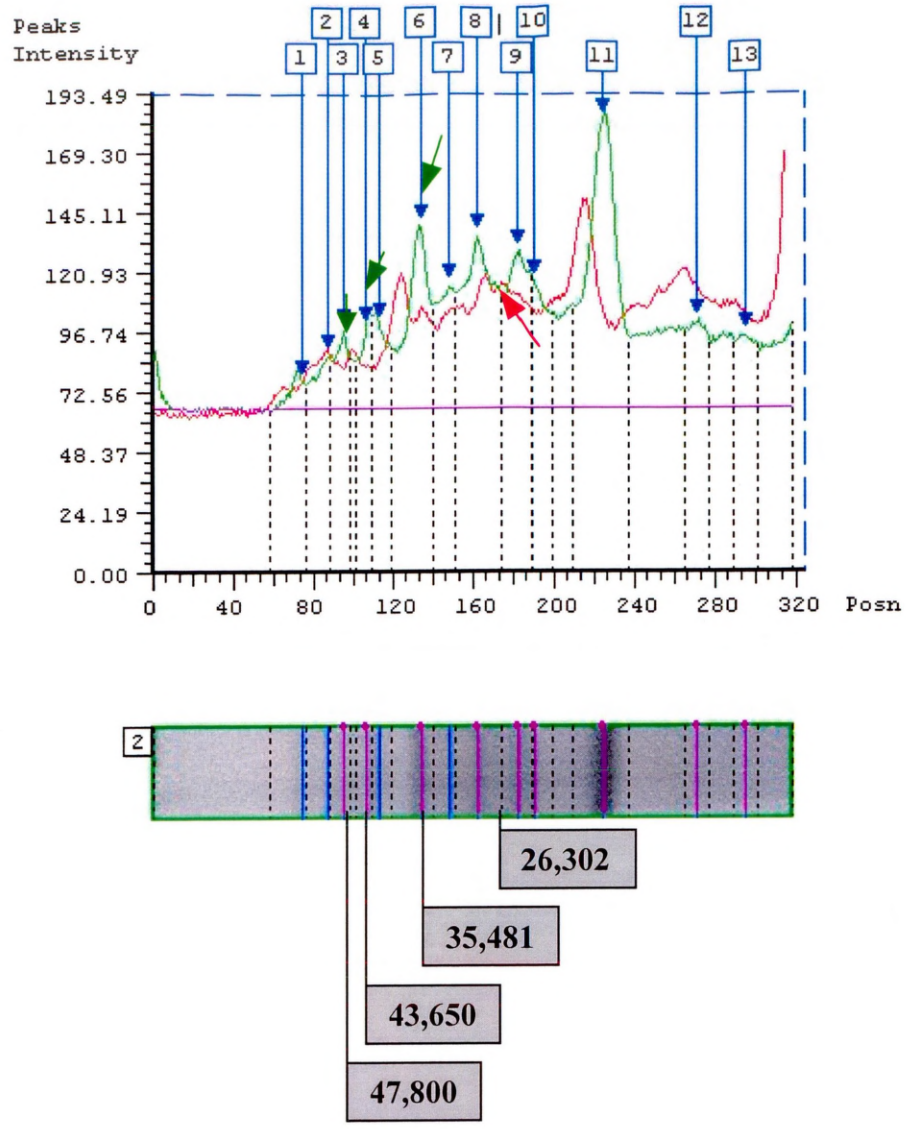


Figure 5.3: Comparison between the peak analysis of the outer membrane proteins of *P. aeruginosa* adapted to 6 μg ml $^{-1}$ of ZnPT (P11) (green line) and the peak analysis of the outer membrane proteins of ZnPT-sensitive *P. aeruginosa* (red line). The upper graph indicates the peak intensity in relation to the pixel position. Blue arrows point at peaks that represent the bands in the lower lane image. Numbers in boxes on the graph indicates band position. Numbers in boxes on the image indicates molecular weight of the specific bands. The green arrow indicates the protein band that appears to be over-expressed in outer membranes of Passage11 cells. The red arrow indicates missing protein in the outer membranes of passage 11.

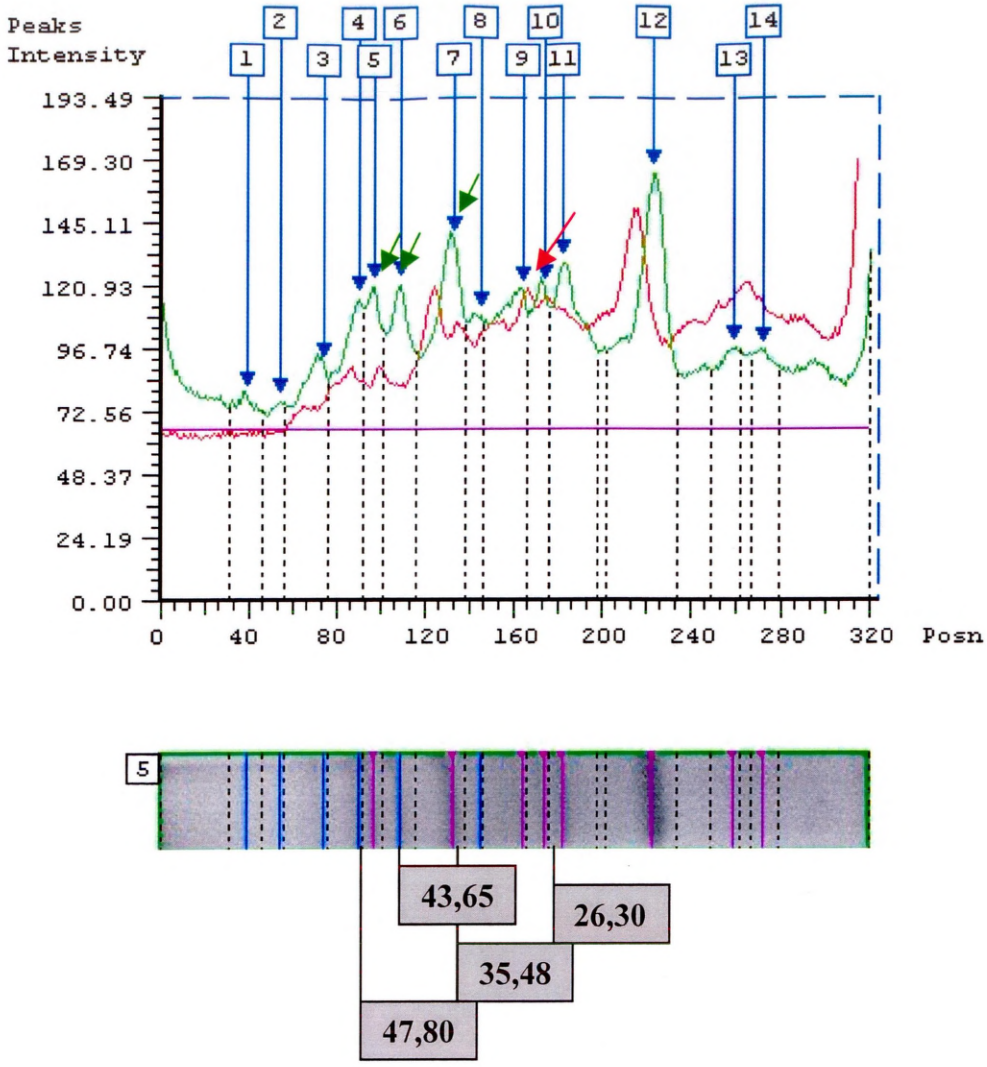


Figure 5.4: Comparison between the peak analysis of the outer membrane proteins of *P. aeruginosa* previously adapted to ZnPT (6 μg ml $^{-1}$) and grown in ZnPT-free media for three passages (P14) (green line), and the peak analysis of the outer membrane proteins of ZnPT-sensitive *P. aeruginosa* (red line). The upper graph indicates the peak intensity in relation to the pixel position. Blue arrows indicate peaks that represent the bands in the lower lane image. Numbers in boxes on the graph indicates band position. Numbers in boxes on the image indicates the molecular weight of specific bands. The green arrows indicate a protein band that appears to be over-expressed in outer membranes of Passage 14 cells. The red arrow indicates the missing protein in the outer membranes of passage 11 cells that reappeared in P14

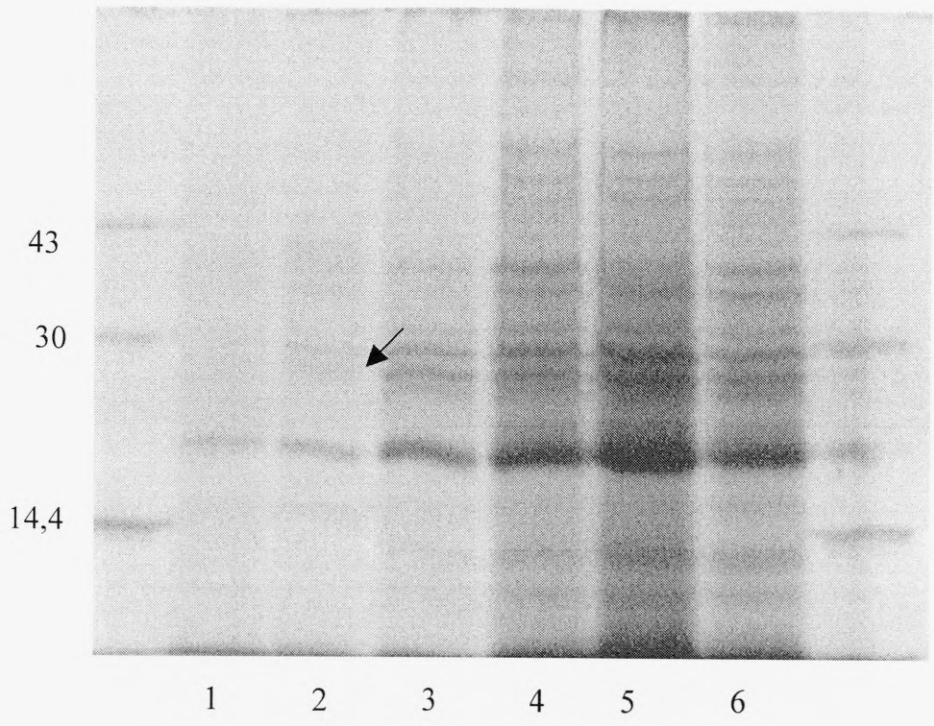


Figure 5.5: A Coomassie blue SDS-PAGE gel of the outer membranes Cetrimide-sensitive and -resistant *P. aeruginosa* grown in CDM. **(1)** P 2, **(2)** P 11, **(3)** P 12, **(4)** P13, **(5)** P 14, **(6)** unexposed *P. aeruginosa* PAO1 cells. Numbers on the left represents the molecular weight standards in kD. The arrow indicates the position of the "missing" band in the ZnPT-resistant cells which appears to be present in Cetrimide-resistant cells.

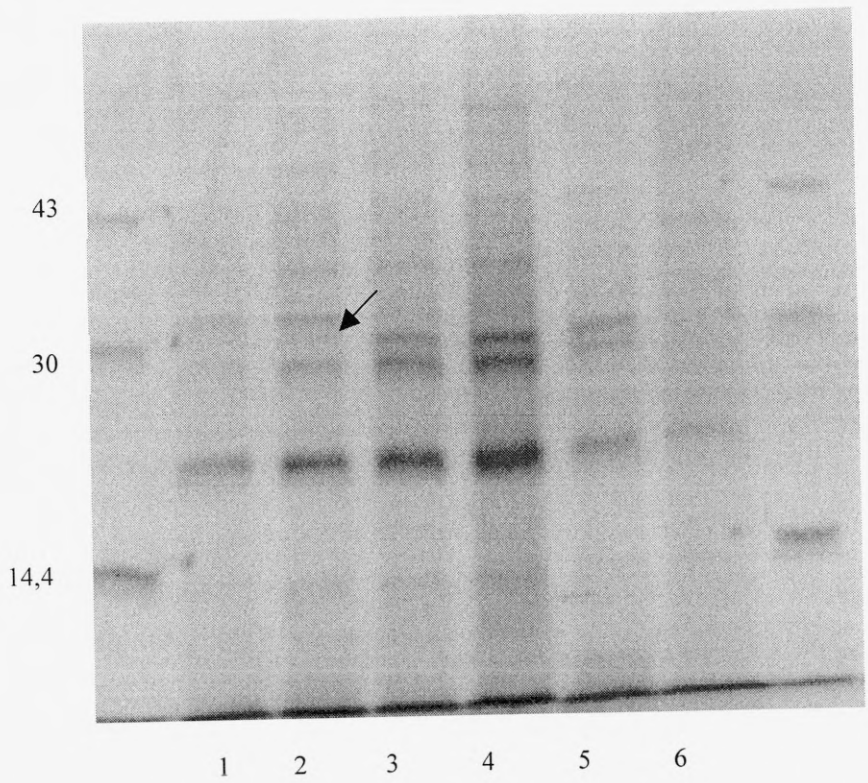


Figure 5.6: A Coomassie blue stained SDS-PAGE gel of the outer membranes *P. aeruginosa* grown in CDM and exposed to ZnPT. **(1)** P 2, **(2)** P11, **(3)** P12, **(4)** P 13, **(5)** P 14, **(6)** *P. aeruginosa* ZnPT-sensitive cells. Numbers on the left represents the molecular weight standards in kD. The arrowhead indicates the position of the missing protein.

These results are similar to the results reported by Masuda *et al* (1995), where the over-expression of a 50 kDa protein was observed in the outer membrane of *P. aeruginosa* cells grown in the presence of zinc ions. This protein was thought to be the outer membrane channel forming protein OprN of the efflux pump MexEF-OprN. However, they did not explain whether this protein persisted after removal of the zinc from the media, which is the case in the present study. The peak intensity of the two over-expressed proteins increased in passage 14. The sample of P14 had the same protein concentration as that of P11, which suggests that the cell increased the synthesis of these proteins even in the absence of ZnPT. These proteins could be efflux proteins that are over-expressed in response to the presence of the biocides, but the persistence of such proteins after removal of the biocide has not been discussed in the literature.

5.2 The effect of Pyrithione Biocides on the Outer Membrane of *P. aeruginosa* PAO1

It was suggested that the 26.30 kDa protein could be a direct chemical target for ZnPT, hence it is destroyed upon exposure with the biocide. In order to exclude this possibility the following experiment was done. Membrane preparations of sensitive *P. aeruginosa* were mixed with the pyrithione biocides. An aliquot (100μL) of the outer membrane of wild type *P. aeruginosa* PAO1 was mixed with 1μg mL^{-1} of ZnPT or 10μg mL^{-1} of NaPT. Vials were incubated for 1 hour at room temperature, at the end of the incubation period the mixture was centrifuged at 10000 *g* for 10 minutes and the supernatants were discarded. Pellets were washed twice in TRIS buffer pH7.4 (Chapter Two) for 10 minutes at 10000*g*. Pellets were then mixed with 150 μL of SDS-PAGE sample buffer (Chapter Two). The outer membranes were then heated in a boiling water bath for 5 minutes and left to cool at room temperature. The outer membrane protein profile was obtained using SDS-PAGE,Gels obtained were silver stained.

5.2.1 Results

No changes were observed in the outer membranes of biocide treated cells as compared with the untreated cells (Figure 5.7). Which denies the assumption of possible destruction of the 26.30 kDa protein by direct chemical interaction with the biocide.

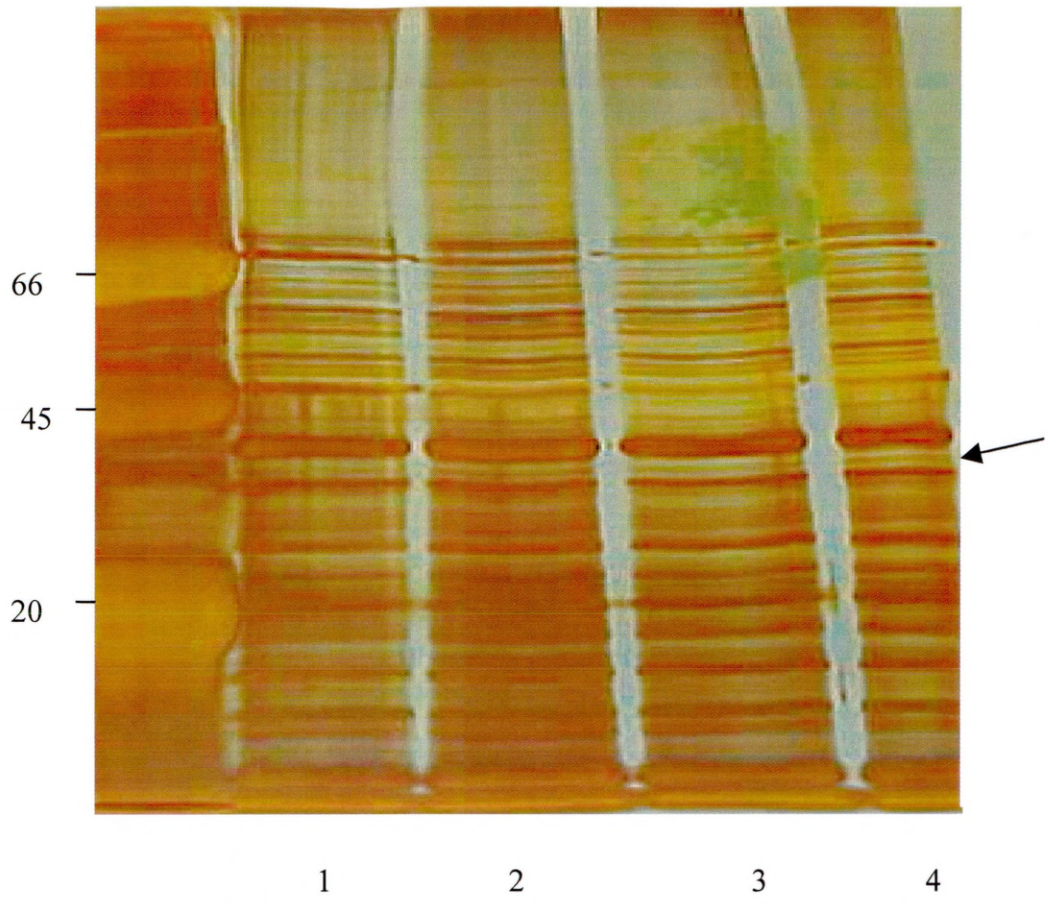


Figure 5.7: The effect of pyrithione biocides on the outer membrane of *P. aeruginosa* PAO1 **(1)** OMs mixed with 1μg ml^{-1} of ZnPT, **(2)** OMs mixed with 10μg ml^{-1} of NaPT, **(3& 4)** OMs control (without biocides). The arrowhead indicates the position of the 26.302 kD band in the control OMs.

5.3 Efflux systems as mechanisms of resistance

Penetration of hydrophilic low molecular weight drugs into the Gram-negative bacterial cell is so rapid, that reducing the number of porins or any alterations in the outer membrane protein structure will still produce a half equilibration time of just a few minutes. This period of time is long enough to allow for the accumulation of the drug at its site of action and hence, exerting its antimicrobial effect. *P. aeruginosa* is known to have the most effective permeability barrier, and, yet cannot prolong the half-equilibration time of most hydrophilic drugs beyond several minutes, nor can it shut out the influx of small molecules, but it can only reduce the influx of some compounds which makes efflux of these compounds an especially effective mechanism for resistance (Nikaido 1994). When there are clinically significant levels of resistance, low outer membrane permeability alone is unlikely to be responsible; a second contributor is needed for this purpose (Nikaido 1994). Efflux pumps in *P. aeruginosa* are coded for by chromosomal genes (Poole, 2001). An efflux pump of the RND-MFP-OMF type is made of three proteins (Figure 1.3); a cytoplasmic membrane associated drug-proton antiporter protein that belongs to the RND family (Resistance-Nodulation-Division) of transporters, a periplasmic membrane fusion protein (MFP) and an outer membrane channel-forming protein (OMF). If over-expressed such efflux systems will confer a much higher level of resistance to different types of antimicrobials (Nikaido, 1998). The only efflux system that is constitutive in *P. aeruginosa* is the MexAB-OprM (Poole 2001). Genetic regulation of the efflux operons controls their expression. Each of these operons has a regulator gene, the product of which either supresses or derepresses the operon. The regulator gene (*mexR)* is located upstream of the *mexAB-oprM* operon and is the target of the *nalB* mutation. MexR protein negatively regulates the expression of the *mexAB-OprM* operon (Poole 2001). A mutation (*nalC*) in an unidentified regulator

of the *mexAB-OprM* results in the hyperexpression of the operon. MexB and OprM topological analysis revealed that these two proteins are integral membrane proteins that span the membrane several times (Poole *et al* 1996). MexB possesses 12 hydrophobic membrane spanning helices with two very pronounced periplasmic loops between helices 1 & 2 and helices 7 & 8 that suggested the site of interaction with MexA (MFP) (Poole *et al* 1996). MexA is a periplasmic lipoprotein that is anchored to the cytoplasmic membrane *via* its hydrophobic tail (Poole 2001). This system contributes to the intrinsic resistance towards a number of antimicrobials including floroquinolones, β-lactams (other than Carbapenems), tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim, and sulphonamides (Kohler *et al* 1999). MexCD-OprJ is expressed as a result of a mutation in the *nfxB* regulator gene that occurs upstream of the *mexCD-OprJ* operon (Poole 2001). Expression of this efflux system confers resistance to quinolone antibiotics, macrolides, chloramphenicol, novobiocin, tetracycline, trimethoprim and β-lactams of the 4th generation (Poole 1996). This system is distinguished from the MexAB-OprM in the ability of the former to export additional β-lactams such as carbenicillin and aztreonam (Poole 2001). MexEF-OprN is inactive in wild type *P. aeruginosa* and is expressed by a mutation in the *mexT* gene, that is a positive regulator of that system and at the same time is responsible for the decrease in OprD expression in the *nfxC* mutants (Poole 2001). Expression of this system renders the bacteria more susceptible to β-lactams and aminoglycosides and resistant to imipenem antibiotics with the absence of OprD. The fourth efflux system in *P.aeruginosa* is MexXY-OprM. This system lacks a link outer membrane gene of its own; it utilizes OprM as its outer membrane component. When expressed, it exports aminoglycosides, tetracyclines and erythromycin (Poole 2001).

Ciprofloxacin resistant strains of *P. aeruginosa* were found to posses an altered A and or B subunits of DNA gyrase enzyme which are the target sites of that antibiotic (Cho *et al* 1995). Resistant strains expressed an outer membrane protein of a 51 kD molecular weight and were found to accumulate half the amount of ciprofloxacin intracellularly, in comparison to the amount accumulated in the sensitive strain (Cho, *et al* 1995). This type of resistance was attributed to an induced efflux mechanism in the cell (Cho, *et al* 1995). *P. aeruginosa* mutants that lacked the MexAB-OprM were not able to grow on the *Pseudomonas* isolation agar (PIA), which included in its constituents the broad spectrum antimicrobial Irgasan (Triclosan). Transformation of these mutants with the *mexA*$^{+}$-*mexB*$^{+}$-*oprM*$^{+}$ plasmid restored the ability of the mutant to grow on that media, suggesting the role of efflux pumps in Triclosan tolerance in *P.aeruginosa* (Schweizer, 1998). Li (2000b) found that the loss of MexAB-OprM system in *P. aeruginosa* correlated with an increase in MexCD-OprJ or MexEF-OprN, suggesting that the absence of MexAB-OprM might have promoted the increased accumulation of certain compounds which triggered the expression of other efflux systems.

Under metal ion excess in the growth medium of Gram-negative bacteria, Ion efflux system is induced (Nies and Silver 1989). The plasmid mediated CzcABC efflux system (Figure 1.4) for cadmium, zinc and cobalt raises the MIC for zinc by 100 fold when expressed. CzcA is an RND type transporter, CzcB is a cytoplasmic membrane bound protein that has binding sites for Zn^{2+}. The system is induced through a membrane bound sensor protein CzcD and a soluble regulatory protein CzcR (Nies and Silver 1989).

Efflux systems use energy for the transport of harmful substances (including antibiotics, numerous dyes, detergents, inhibitors, organic solvents and biocides) out of the cell (Poole 2001). Attacking the energy source will certainly apprehend the efflux

pump and allow the accumulation of harmful substances to a level that would be toxic for the bacteria and hence, reduce its tolerance to such substances. The effect of efflux pump inhibition could be manifested in the form of reduced MIC values for certain antibiotics and biocides or increased accumulation of the harmful substance intracellularly. Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) is an extremely effective uncoupler of oxidative phosphorylation (Heytler & Prichard 1962). Hence, CCCP has been used by several researchers as an active efflux blocker (Piddock *et al* 1999; Piddock *et al* 2001 and Cho *et al* 1995). CCCP exhibits the uncoupling effects at concentrations down to 10^{-8} molar (Heytler & Prichard 1962). Cho *et al* (1995) measured the intracellular concentration of Ciprofloxacin in both Ciprofloxacin sensitive and resistant cells of *P. aeruginosa*. They then subjected the sensitive and resistant cells to the effect of 1mM of CCCP. The authors found that resistant cells accumulated half the amount of the antibiotic when CCCP was added. This result suggested that decreased accumulation of the antibiotic might have contributed to the bacterial resistance to the antimicrobial action of Ciprofloxacin (Cho *et a*l 1995).

In the present study the presence of active drug efflux systems as a mechanism of resistance was investigated. CCCP was added together with ZnPT in the growth medium of both ZnPT-sensitive and -resistant *P. aeruginosa* PAO1 strain. Sodium Orthovanadate (OVN) is another efflux modulator that is a potent and selective inhibitor of protein-phosphotyrosine-phosphatase and alkaline phosphatase in micromolar concentrations (Swarup *et al* 1982). OVN was added together with ZnPT to both ZnPT sensitive and resistant cells, and a combination of the two inhibitors (CCCP and OVN) was also added together with ZnPT to both the ZnPT-sensitive and-resistant cultures. Growth curves and MICs for the various cultures were determined and analyzed.

5.3.1 Materials and Methods

5.3.1.a Chemicals and Strains

CCCP (Carbonyl cyanide m-chlorophenylhydrazone) (Sigma) was prepared by dissolving 10mg ml^{-1} in Methanol (MeOH).

OVN (Sodium Orthovanadate $NaVO_4$) (Sigma) was prepared as a 25mM stock solution by dissolving 0.299 g in sterile distilled water and adjusting the solution to pH 10 after boiling until translucent. The stock was stored in glass bottles at 4°C.

A fresh 24hrs culture of *P. aeruginosa* PAO1 strain in R2A broth was prepared. This culture was used as the sensitive strain throughout the experiment. ZnPT resistant strains were obtained from passage 11 ZnPT-resistant cultures maintained on R2A gradient plates (Chapter Three).

5.3.1.b Methods

The experiment was performed in triplicate. R2A medium (25 mL) was inoculated with ZnPT-sensitive or-resistant strains of *P. aeruginosa*. ZnPT and CCCP or OVN were added simultaneously with inoculation. The following cultures were prepared:

3 R2A media flasks inoculated with ZnPT-sensitive *P. aeruginosa* PAO1.

3 R2A flasks inoculated with ZnPT-resistant *P. aeruginosa* PAO1 (P11) cells and containing ZnPT.

3 R2A flasks inoculated with P11 cells and containing both ZnPT and OVN.

3 R2A flasks inoculated with P11 cells and containing ZnPT and CCCP.

3 R2A flasks inoculated with P11 cells and containing ZnPT +CCCP+OVN.

3 R2A flasks inoculated with P11 cells and containing MeOH (control).

Cultures were incubated in an orbital-shaking incubator (180 rpm, Gallenkamp INA-305) at 37°C. Growth curves for all cultures were determined at hourly intervals (Chapter Two). At the end of the 24 h incubation period, the same cultures were used as inoculums for MIC determination by the tube dilution method (Chapter Three).

***5.3.1.c** Determination of ZnPT antibacterial activity by disc diffusion method.*

Whatman filter paper (0.5mm thick) was cut by a puncher into discs and autoclaved. The dry sterile discs were then impregnated with 6µg mL^{-1} of ZnPT and were left to dry. The same cultures, prepared previously, were streaked across the surface of R2A agar plates using sterile swabs. ZnPT impregnated discs were placed in the center of each inoculated plate. Plates were then incubated for 24 hrs at 37°C.The diameter of the zone of inhibition was measured.

5.3.2 Results and Discussion

When grown in the presence of CCCP and/or OVN, ZnPT-sensitive strain of *P. aeruginosa* did not show any changes in the MIC of ZnPT, which suggested the absence of an active efflux mechanism in the wild type *P. aeruginosa* PAO1 strain. This result is further confirmed by the absence of the relatively over-expressed proteins (35.48 kDa, 43.65 kDa and 47.8 kDa) from the peak intensity profiles of the sensitive strain (Figure 5.3 the red line). On the contrary, the ZnPT-resistant strain exhibited a 30% reduction in the MIC when the two chemicals (OVN + CCCP) were added together and when CCCP was added alone, while a 16% reduction in MIC for ZnPT was observed when OVN was added alone (Table 5.1). When measuring changes of the relative activity of ZnPT in response to the efflux inhibitors using the disc diffusion method, there was an 11% reduction in the diameter of the zone of inhibition when the

ZnPT resistant cells were grown in the presence of CCCP alone. A reduction of 18.5% was observed when resistant cells were grown in the presence of CCCP + OVN (Table 5.2). The combination of the two inhibitors CCCP and OVN expressed the greatest inhibitory effect suggesting a synergistic effect between the two chemicals. These results indicate that the mechanism of ZnPT resistance is energy-dependant. Energy consumption is known to be the hallmark of efflux processes (Poole *et al.* 1996).

Growth curves in the presence of the efflux inhibitors (Figures 5.8, 5.9 and 5.10) showed a significant reduction ($p \leq 0.05$) in the growth rate upon the addition of efflux inhibitors. Efflux inhibitors are known to interfere with the energy building mechanisms of the cell (Heytler & Prichard 1962; Gordon 1991). Cyanide acts as an O_2 analogue that competes with the electron carriers being the ultimate electron acceptor itself, hence, inhibiting the oxidative phosphorylation and ATP synthesis. On the other hand, OVN at micromolar concentration, is known to be an inhibitor of the enzyme protein-phosphotyrosin-phosphatase (Swarup, *et al.* 1982) affecting the availability of inorganic phosphate for ATP synthesis .

An 80% increase in MIC of ZnPT occurred as *P. aeruginosa* adapted to that biocide. Such an increase in MIC could not be achieved by alterations in the outer membrane protein profile alone. Efflux systems could be a second contributor to the development of such resistance. Components of a zinc efflux pump have not yet been identified. However, the over-expressed proteins in the outer membrane of the ZnPT- resistant cells could be members of a novel efflux system that involves the export of the perthiolate ions from the cell, since ZnPT is suggested to enter the cell in the dissociated form (Dinning *et al*1998).

Efflux systems are composed of a tripartite complex, a cytoplasmic membrane associated drug-proton antiporter of the RND family (Nikaido & Hall 1998), membrane

fusion protein that belongs to the MFP family (that connects the transporter protein to the outer membrane) and an outer membrane channel forming protein (Figure 1.3). Results of efflux inhibitor studies suggest that in case of ZnPT the energy driven transporter could be an ABC transporter, since it was affected by the use of OVN. However, known efflux systems in *P. aeruginosa* use transporters that belong to the RND family and the zinc efflux system in Gram-negative bacteria is a proton-cation antiporter (Nies & Silver 1995).

These results suggest that an efflux system could contribute to the mechanism of ZnPT-resistance. Determination of the accumulation of the ZnPT in the cells prior to and after the incorporation of the efflux inhibitors in the growth medium would give a precise answer to the involvement of the efflux pumps in the resistance against ZnPT.

Table 5.1: MIC for ZnPT sensitive and resistant *P. aeruginosa* PAO1 strain subjected to CCCP, OVN and CCCP+OVN.

Cells	MIC(μg ml^{-1})*	Standard error (+/-)
ZnPT- sensitive *P. aeruginosa*	7	0.8
ZnPT-resistant *P. aeruginosa* (P 11)	17	0.81
P11 + OVN	14	1.6
P11 + CCCP	12	0
P11 + OVN+ CCCP	12	0
ZnPT-sensitive PAO1 +OVN	7	0
ZnPT-sensitive PAO1 +CCCP	7	0
ZnPT-sensitive PAO1+OVN+CCCP	7	0

* Mean of three experiments

Table 5.2: MIC determined by the disc diffusion method

Cells	Zone of inhibition (mm)*	Standard error (+/-)
ZnPT-sensitive *P.aeruginosa*	1.7	0
ZnPT-resistant *P.aeruginosa* (P11)	1.3	1.44
P11+ CCCP	1.16	.09
P11+ OVN	1.43	.048
P11+ CCCP+OVN	1.06	1.7

*Mean of three measurements of the zone of inhibition in mm.

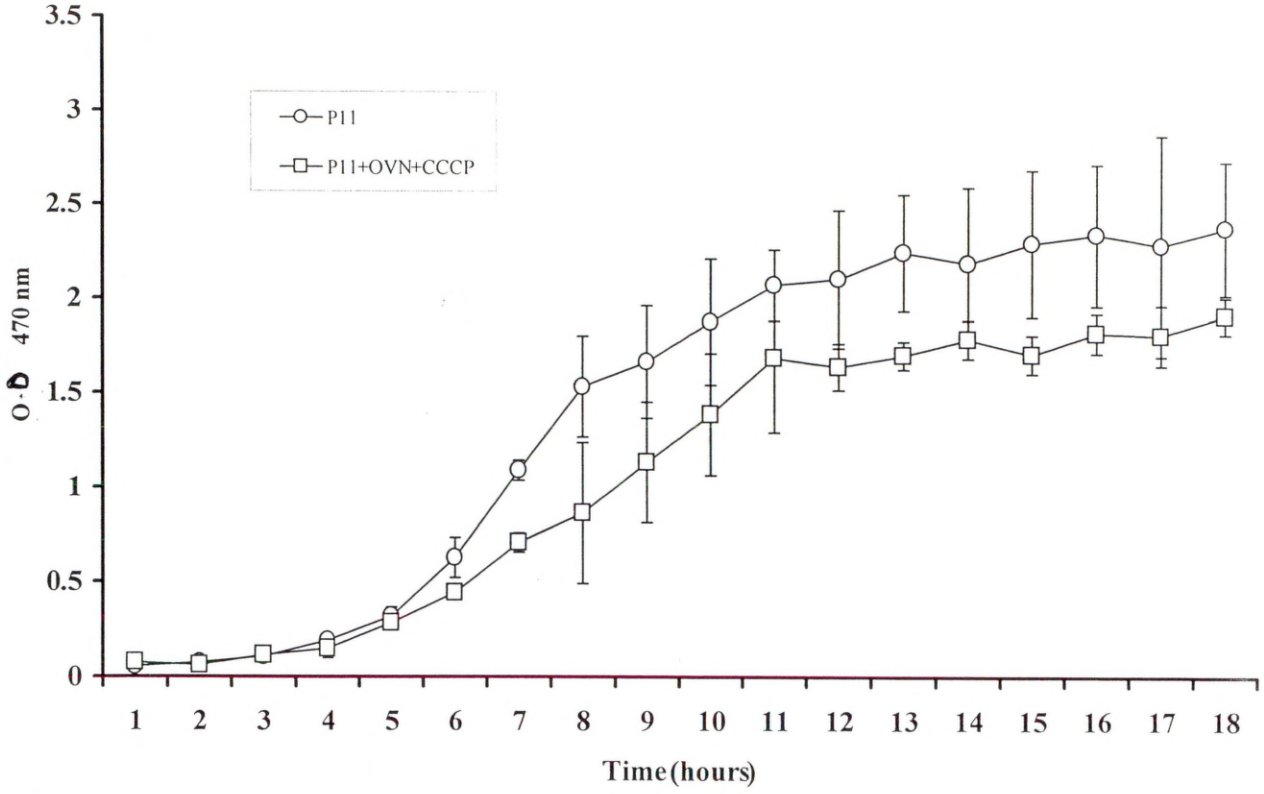


Figure 5.8: Comparison between the growth curves of ZnPT-resistant *P. aeruginosa* (P11) grown in R2A medium containing 6μg mL^{-1} of ZnPT and the growth curve of ZnPT-resistant (P11) *P. aeruginosa* grown in R2A medium containing 6μg mL^{-1} of ZnPT and CCCP (15 μg mL^{-1}) and OVN (250μM). Error bars are the standard deviation of each data point.Statistics for these data were determined using triplicate experiments ($p \leq 0.05$).

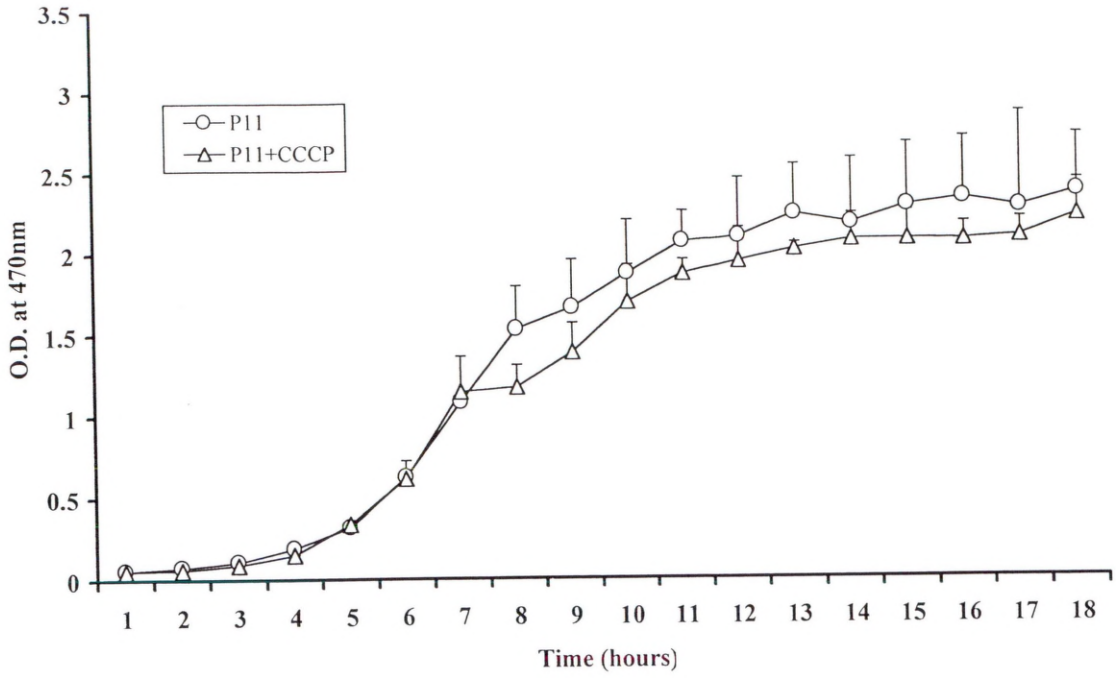


Figure 5.9: Comparison between the growth curves of ZnPT-resistant *P. aeruginosa* (P11) grown in R2A medium containing 6μg mL^{-1} of ZnPT and the growth curve of ZnPT-resistant (P11) *P. aeruginosa* grown in R2A medium containing 6μg mL^{-1} of ZnPT and CCCP (15μg mL^{-1}). Error bars are the standard deviation of each data point. Statistics for these data were determined using triplicate experiments ($p \leq 0.05$).

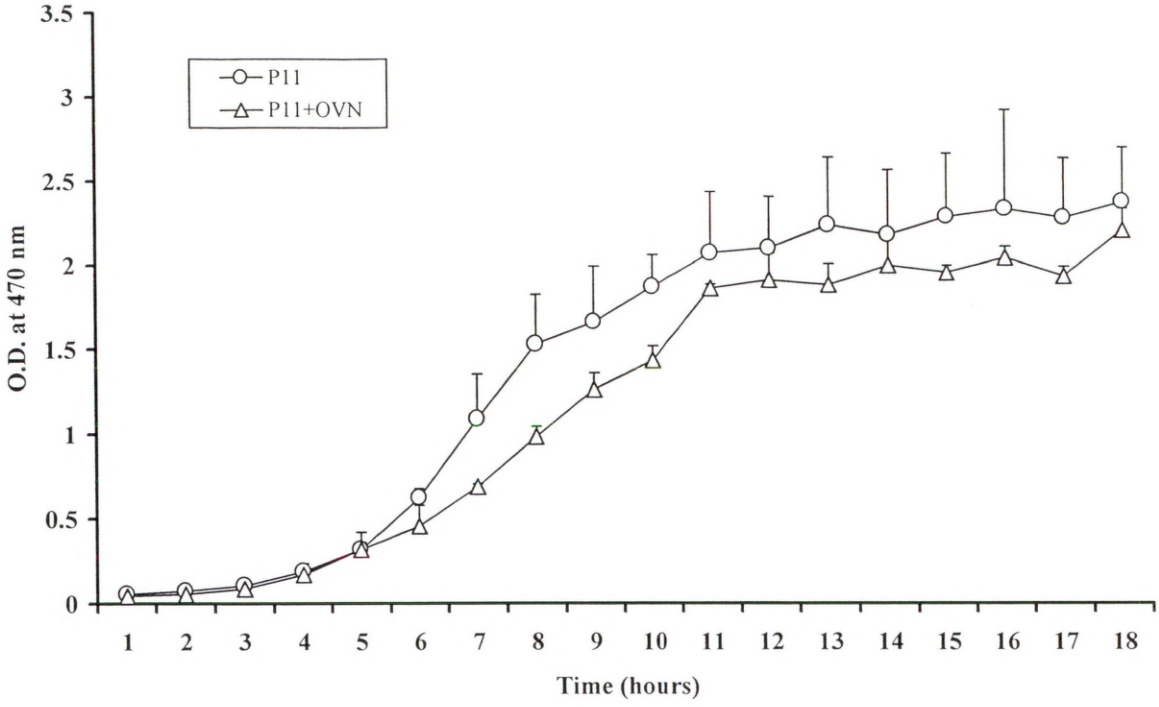


Figure 5.10: Comparison between the growth curves of ZnPT-resistant *P. aeruginosa* (P11) grown in R2A medium containing 6μg mL^{-1} of ZnPT and the growth curve of ZnPT-resistant (P11) *P. aeruginosa* grown in R2A medium containing 6μg mL^{-1} of ZnPT and OVN (250μM). Error bars are the standard deviation of each data point. Statistics for these data were determined using triplicate experiments ($p \leq 0.05$).

5.4 Investigation of Stress Response to ZnPT

When a living cell is exposed to a sudden environmental change, it suffers a shock or stress (Maccario 1995). Bacteria and other forms of life respond to a wide range of environmental stresses by inducing the synthesis of a small set of proteins called stress proteins (Watson 1990). Environmental stressors vary between physical (temperature, UV, osmotic stress and pH) and chemical stressors (Ethanol, dyes, antibiotics and biocides). The hallmark of the stress response is visible in the protein patterns of cell free extracts revealed by SDS-PAGE. Most normal protein bands either fade or disappear after a heat shock, while others become prominent or appear as new bands (Maccario 1995). Apparently stress causes a rapid inhibition of the initiation step of protein synthesis accompanied by the selective expression of stress proteins or heat shock proteins (Hu *et al* 1993). The synthesis or the over-expression of this subset of proteins allows the bacteria to face harsh conditions through maintaining essential cellular functions (Vasseur *et al* 1999) and to withstand the presence of higher concentrations or further but lethal stress challenges (Watson 1990).

Acquired thermotolerance refers to the transient, non-heritable acquisition of resistance to normally lethal temperature induced by a short exposure to a non-lethal heat shock (Watson 1990). Incubation of relatively short periods at non-lethal temperatures induces synthesis of heat shock proteins and produces transient tolerance to normally lethal temperatures. The presence of the heat shock proteins is not always required for the development of thermotolerance. Temperature stress in *E. coli* was measured by the increase in the rate of synthesis of heat shock proteins. The heat induced (a temperature shift-up from 30 to 43°C) increase in the rate of synthesis of the heat shock protein GroEL, peaked after 5-10 minutes of the temperature up-shift, and then rapidly declined to a steady state level that was five fold higher than the rate of

synthesis observed at the initial exposure (30°C). Stressed *E. coli* was subsequently tested for acquired thermotolerance (as measured by percentage of survivors). Stressed *E. coli* was exposed to a higher temperature (55°C) for 10 minutes. Acquired thermotolerance peaked (10% survivors) after 30 minutes incubation at 42°C, and then declined to a steady state level approaching the endogenous values (less than 1% survivors) after two hours at 42°C. These results suggest that stress proteins had no or little effect on induced thermotolerance, but on the other hand they may have a protective effect during heat inactivation or growth at high temperature (Watson 1990). Acquired thermotolerance in *E. coli* could be induced by stresses other than a temperature shock; including addition of ethanol, Cadmium chloride or hydrogen peroxide (Watson 1990). Bacterial response at the level of protein synthesis can be completely different and produce variations in the different subsets of induced proteins according to the nature of the stress. Some stresses induce the same sets of proteins, but with few qualitative or quantitative variations according to the nature and the magnitude of the stress. For example out of the 17 heat shock proteins induced in *E. coli,* only six are induced in response to exposure to cadmium chloride and only one is induced after exposure to H_2O_2 (Watson 1990). Allan *et al* (1988) detected 17 proteins (Table 5.3) that were over-expressed 10 minutes after transfer of log phase cells of *P. aeruginosa* PAO from incubation temperature of 30°C to 45°C. These over-expressed proteins had a molecular weight ranging between 15.7 kDa to 103 kDa. There were five major heat shock proteins and they had molecular weights of 86.40 kDa, 70.4 kDa, 67.4 kDa, 60.6 kDa and 15.7 kDa. In addition a marked suppression in the synthesis of other proteins occurred. The suppression and synthesis of these proteins remained for 60 minutes after the temperature shift. When *P. aeruginosa* PAO was exposed to ethanol,

the synthesis of 7 proteins was detected, 3 of which were heat shock proteins which were the 91 kDa, 76 kDa and 70 kDa.

Table 5.3: Relative mobilities of the heat shock proteins of *P. aeruginosa* (Allan *et al* 1988)

Protein no.	M_r in Daltons (No. of determinations)
1	103,400 (4)
2	94,900 (5)
3	90,400 (6)
4	86,400 (10)
5	81,400 (6)
6	76,000 (10)
7	70,400 (9)
8	60,900 (11)
9	50,100 (11)
10	42,600 (12)
11	39,800 (9)
12	35,100 (8)
13	27,500 (11)
14	25,800 (9)
15	21,000 (9)
16	17,800 (5)
17	15,700 (9)

Vasseur *et al* (1999) studied the stress response of *P. fragi*, the main spoilage species of refrigerated meat. *P. fragi* was subjected to various stresses that mimicked the cleaning and disinfection processes which included pH, osmotic and biocide shocks. A large number (91) of stress proteins were detected in the cell-free extracts using two dimensional gel-electrophoresis (2-DE). 25 of these proteins were considered as general stress proteins (Gsp), that were thought to provide a non-specific protective function and could contribute to cell survival by rapid adaptation of cellular machinery to transient variations in the environmental conditions. Two of the biocide (fatty amine) induced proteins, that were previously characterized as Hsps, were found to be immunologicaly related to the molecular chaperones DnaK and GroEL Hsps of *E. coli* (Vasseur *et al* 1999). *P. aeruginosa* induced GroEL- and DnaK-like proteins were also found to react immunologicaly with antibodies against the *E. coli* GroEL and DnaK proteins, with slight variations between the two species in the molecular weights of the DnaK protein. These results indicated that GroEL and DnaK were highly conserved between the two species (Allan *et al* 1988).

It is known that the heat shock response involves the synthesis of an alternative sigma factor that recognizes stress promoters. Purification of heat shock RNA polymerase of *P. aeruginosa* revealed the presence of a protein (86.4 kD) that failed to react with monoclonal antibodies prepared against σ^{70} of *E. coli* but reacted with monoclonal antibodies prepared against the σ^{87} of *P. aeruginosa* (Allan *et al* 1988). In *E. coli* an alternative sigma factor (σ^{32}), the product of the regulatory gene *rpoH*, was found to be involved in RNA polymerase recognition of the heat shock promoters (Allan *et al* 1988, Grossman *et al* 1987). Using immunoprecepitation Allan *et al* (1988) have detected a 40 kD protein which was suggested to be the *P. aeruginosa* heat shock sigma factor that corresponds to the σ^{32} of *E. coli* in that species.

In this section ZnPT-induced stress response was investigated. Cell-free extracts (CFE) of P2, P11, sensitive cells and heat shocked cells were used. The protein profile of these cells was analyzed after running on SDS-PAGE. Immunological relatedness of over-expressed proteins to other known stress proteins was performed using western blot.

5.4.1 Materials and Methods

5.4.1.a Bacterial cultures

Stationary phase cultures of ZnPT-sensitive and resistant (P11) *P. aeruginosa* PAO1 grown in R2A medium, and heat shocked ZnPT-sensitive *P. aeruginosa* were used.

5.4.1.b Preparation of heat shocked cultures

Stationary phase cultures of ZnPT-sensitive *P. aeruginosa* PAO1 were subjected to a temperature up-shift from 37°C to 44°C for 1 hour, to induce synthesis of Hsp (Hinode *et al* 1996). Cell free extracts were prepared immediately after removal from the 44°C incubator.

5.4.1.c Preparation of cell free extracts (CFE)

Cell-free extracts were prepared according to Duncan *et al* (2000), by pelleting 1.5 mL samples from the above mentioned cultures at 13000 *g* for 5 minutes. The pellets were resuspended in 100 µl of cell free extract buffer (Tris (pH7.5) 50mmol L^{-1}, EDTA (Sigma) 10mmol L^{-1}, sodium chloride 10mmol L^{-1}, PMSF (Sigma) 0.1 mmol L^{-1} and 5% v/v glycerol). The mixture was homogenized for 2.5 minutes (5 x 30s bursts with 10 s rests on ice) (Ultra Turrax T8, S8N-5G, IKA Labortechnik, Staufer, Germany). Insoluble debris was removed by centrifugation (13000 *g* for 15 min.) and

the protein content of each CFE sample was measured using the bicinchoninic acid method (Chapter Two).

5.4.1.d Preparation of CFE for SDS-PAGE

CFE samples were diluted 1:4 with the electrophoresis sample buffer (0.18 mol l^{-1} Tris-HCL (pH 6.8), 15% v/v glycerol, 5% v/v β-mercaptoethanol, 0.075% w/v bromophenol blue, 0.3% w/v SDS) and heated (100°C) for 5 min. After centrifugation (13000*g* for 5min) proteins in the supernatant fluids were separated on 10% polyachrylamide gel (mini-PROTEAN II, Biorad) at 35mA constant current for 1.5 hour or until the bromophenol blue front reached the bottom of the gell. All samples were loaded at equal protein concentration (300µg ml^{-1}).

5.4.1.e Immobilization of proteins

Protein bands on gels were immobilized on nitrocellulose membrane using Millipore Dry-blot system. Nitrocellulose membrane (NCM) were trimmed to the size of the gel and soaked in D.H_2O for 5 to10 min. Whatman chromatography filter paper (3mm) were trimmed to the size of the gel and soaked in transfer buffer (Tris 48mM, glycine 39mM, SDS .037% w/v, methanol 20% made up to 1 liter with D.H_2O). Three wet sheets of filter paper were laid on the anode plate followed by the wet NCM. The separating gel was removed from the casting plates and laid carefully (avoiding trapping air bubbles) on top of the NCM followed by three sheets of wet filter paper. The cathode lid was replaced and secured on top of the assembly. The blotter system was connected to the power pack at constant current equal to 0.8 mA cm^{-2} for 30 min.

5.4.1.f Immunological detection of proteins

Nitrocellulose membranes were blocked overnight at 4°C in Marvel solution (Marvel^{TM} 5% w/v, Tween 20 0.2% v/v in phosphate buffered saline (PBS) (pH7.4)). Membranes were then washed in PBS for 3 times and incubated with the primary antibody (Monoclonal Rabbit anti-GroEL 1:5000 (Sigma) or polyclonal rabbit anti-GroEL 2μg mL^{-1} (StressGen Biotechnologies Corp.) or mouse monoclonal anti-σ^{70} 1:1000 (NeoClone Biotechnology) in antibody diluent (bovine serum albumin 3% in PBS) for 1 hour at 37°C. At the end of the incubation period membranes were washed in PBS and incubated with the second antibody (Rabbit anti-mouse horseradish peroxidase (1:1000) or mouse anti-rabbit horseradish peroxidase (1:1000) for 1 hour at 37°C. Color was developed by the addition of the Diaminobenzidine (DAB) solution (DAB 100 mg in citrate buffer (citric acid 0.035M, $Na_2HPO_4.2H_2O$ 0.067M in $D.H_2O$ (pH 5±0.2) and 10μL of 30% H_2O_2 added immediately before use). The reaction was stopped by the addition of $D.H_2O$.

5.4.2 Results and Discussion

In this section the possibility of a stress response mounted by the bacteria as a result of exposure to ZnPT was investigated. Protein profiles of P2 and P11 cell-free extracts were compared with CFE of both ZnPT-sensitive and heat-shocked ZnPT-sensitive cells. On SDS-PAGE, stress response is recognized from the absence or fading of most normally synthesized protein bands in a cell lysate and the appearance of some pre-existing or newly synthesized protein bands (Maccario 1995). Such a response was manifested in the protein profile of CFE from P11 cells. Figure 5.11 demonstrates the absence of several proteins from the CFE of P11 (67 kDa, 81 kDa, 46 kDa, 36.30 kDa, 26.91 kDa) and the over-expression of others (53.7 kDa, 47 kDa and 15.8 kDa). The 47 kDa protein appeared in P2 and was observed to be over-expressed in P11. This protein band did not exist in the ZnPT-sensitive cells nor did it exist in the heat shocked cells. This result suggests that this protein is expressed in response to the ZnPT stress. The other two proteins expressed in P11 cells (15.8 kDa, 53.70 kDa) were not present in CFE of P2 and both ZnPT-sensitive cells and heat shocked cells, which suggests that ZnPT stress response is concentration dependent, the higher the concentration of the biocide, the larger the stress response. These three protein bands did not have any corresponding bands in the *P. aeruginosa* heat shocked cells suggesting that none of the induced proteins were heat shock proteins, nor are they general stress proteins (Gsps), but instead they are biocide specific induced proteins. Allan *et al* (1988) subjected log-phase *P. aeruginosa* PAO to a heat shock, two of the induced proteins (15.7 kDa, 50.1 kDa (Table 5.3) closely resemble the ZnPT-induced stress proteins in the present study. However, the former two proteins were not detected in the heat shocked *P. aeruginosa* PAO1 in the present study. Such contradiction in results could be explained by the

growth phase of cells that were exposed to the heat shock. It is known that stationary phase cells are more resistant to heat shock than log phase cells (Watson 1990). Allan *et al* (1988) used log phase cells for the heat shock experiment. Protein synthesis in Log phase cells peaked after ten minutes from the temperature shift and then declined to levels that were slightly greater than the original. The suppression of protein synthesis on the other hand, continued for at least 60 minutes after the temperature shift (Allan *et al* 1988). In the present study stationary phase cells were used, these cells were subjected to a heat shock that lasted 60 minutes. The 60 minutes period of time was chosen because a 15 minutes heat shock did not cause heat shock response that could be recognized on SDS-PAGE gels (results not shown). The method of Hinode *et al* (1995) was then adopted, which involved a heat shock of 60 minutes. Thus, the absence of heat shocked proteins could be a result of the long period of temperature shock or the use of stationary phase cells, and hence the Hsps were missed or had faded away.

Biocides are known to exert stress on bacteria. When *P. fragi* was subjected to a biocide (fatty amine), the synthesis of 5 polypeptides was induced. Two of the biocide-induced proteins were characterized as Hsps and were found to be immunologicaly related to *E. coli* Dnak and GroEL Hsps (Vasseur *et al* 1999).

CFE of P2, P11 and the heat shocked cells failed to react immunologically with polyclonal antibody against *E. coli* GroEL and the monoclonal antibody against *E. coli* σ^{70} . However, the 33 kD band in the heat shocked cells showed a faint reaction with the monoclonal antibody against *E. coli* GroEL (Figure 5.12). The faint reaction could possibly be explained by the low concentration of proteins due to the long period of exposure to the temperature shift. On the other hand, the positive reaction with the monoclonal anti-GroEL is in accordance with the results in the literature, which have

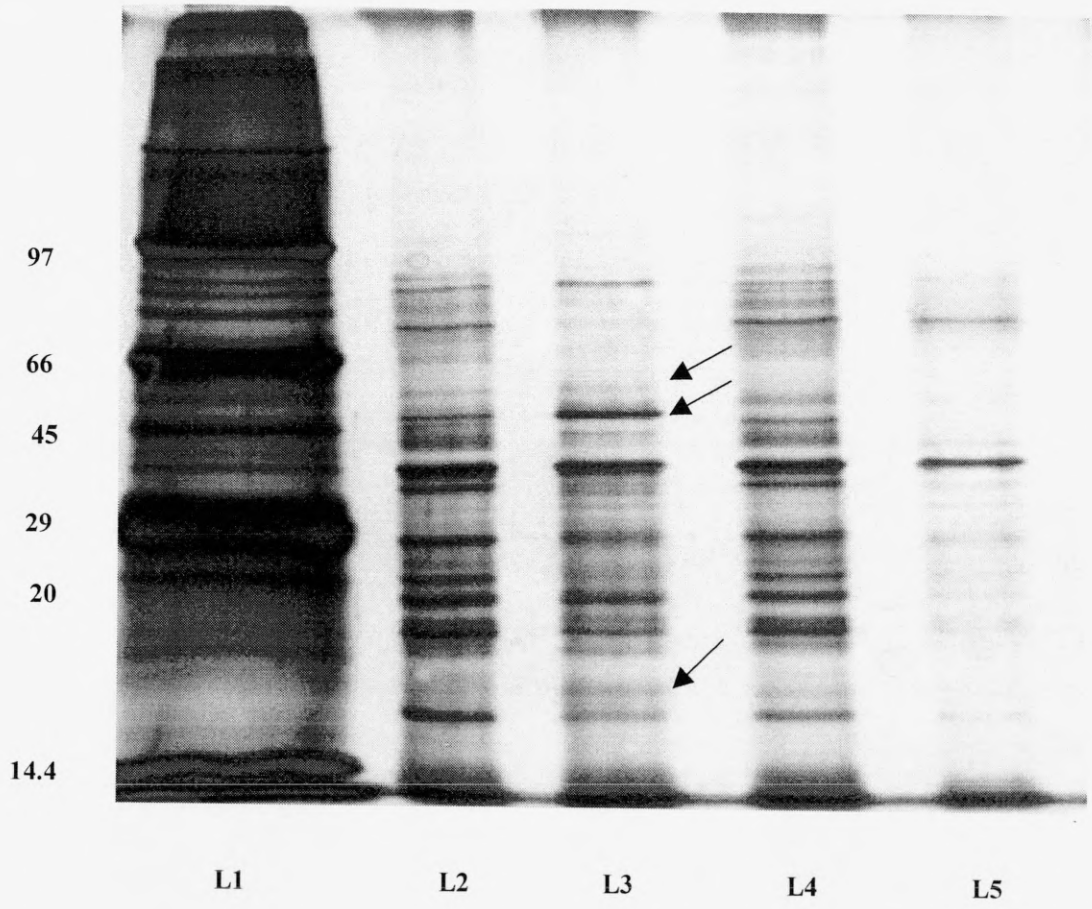


Figure 5.11: Silver stained SDS-PAGE gel of cell free extracts of ZnPT-sensitive, -resistant and heat shocked *P. aeruginosa* PAO1, showing comparison between **(L2)** P2 ZnPT-exposed cells, **(L3)** P11 ZnPT-resistant cells, **(L4)** ZnPT-sensitive *P. aeruginosa* PAO1 and **(L5)** heat-shocked ZnPT-sensitive *P. aeruginosa* PAO1. Protein concentration in each lane is equal to 300 μg mL^{-1}. Low molecular weight markers (Amersham Pharmacia Biotech) (L 1) are given in kD. Arrows indicate the over-expressed proteins in ZnPT-resistant cells.

The previous results indicate that the proteins detected in the resistant cells could be stress induced molecular chaperones synthesized in response to the introduction of ZnPT into the bacterial cell. The absence of several proteins from the CFE protein profile of P11 cells and the general fading of protein bands in comparison to ZnPT-sensitive cells suggests a stress response. However, the detection of the alternative σ^{87} or the heat shock σ^{40} will clarify the case. On the other hand these proteins could be part of the resistance mechanism adopted by the cell towards ZnPT. However, the identity and role of these proteins in resistance are yet to be explored.

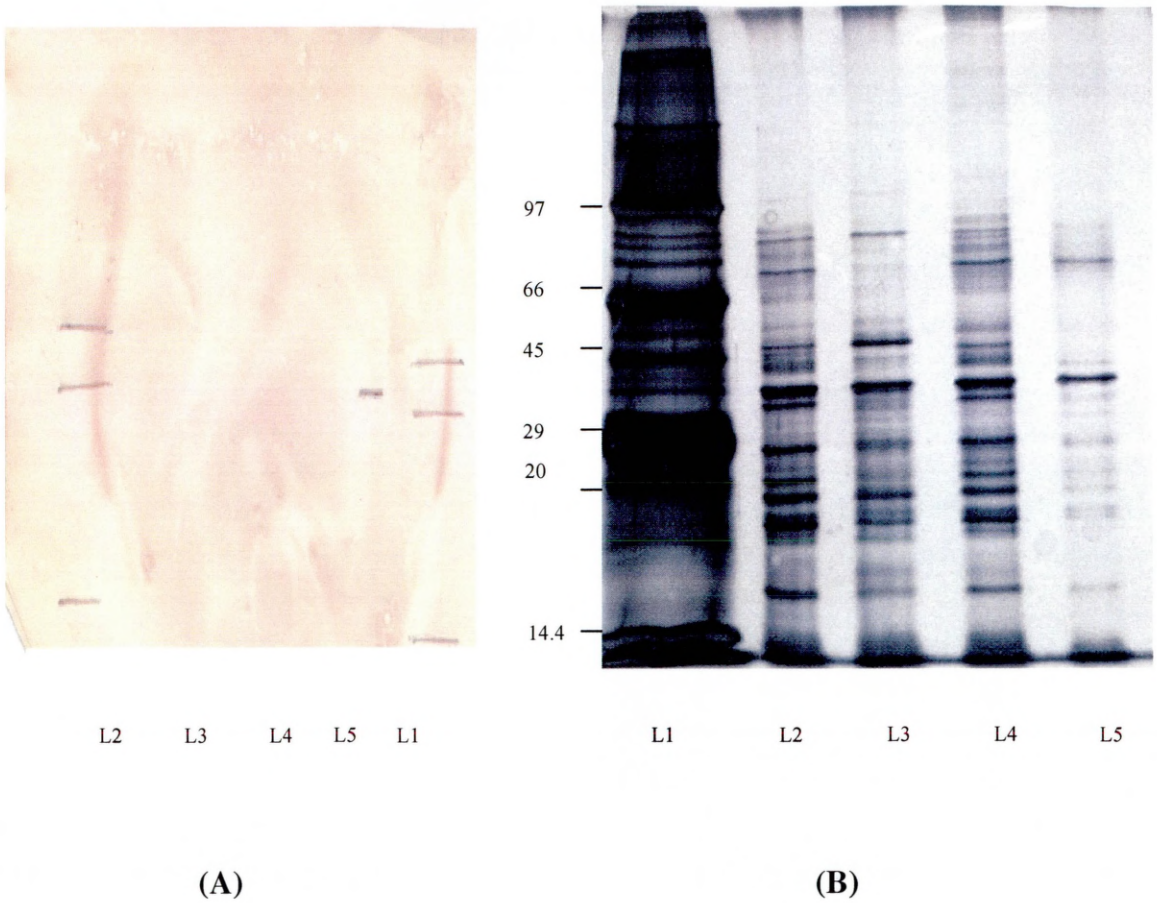


Figure 5.12: Identification of stress proteins by western blot analysis. **(A)** Blot was probed with rabbit monoclonal anti-GroEL of *E. coli* (1:5000). **(B)** A replica SDS-PAGE gel of the transferred proteins. **L1**, M_r markers; **L2**, (P2) ZnPT exposed cells; **L3**, (P11) ZnPT-resistant cells; **L4**, control ZnPT-sensitive *P. aeruginosa* PAO1 grown at 37°C; **L5**, heat shocked (44°C) ZnPT-sensitive *P. aeruginosa* PAO1.

5.5 General Discussion

Outer membrane protein alterations that accompanied the development of resistance towards Pyrithione biocides involved the absence of a protein and the over-expression of others. A low molecular weight (26.30 kDa) protein was observed to be missing from the outer membranes of ZnPT-resistant cells. The disappearance of that protein coincided with the increase in MIC towards that drug. Upon the transfer of resistant cells to a biocide free media the missing protein reappeared (Figure 5.2). However, results have shown that this protein was not destroyed by the presence of the biocide (Figure5.8). Thus, the previous observations suggest the involvement of this protein in the development of resistance towards ZnPT. The sharp increase in MIC values for ZnPT suggests the involvement of other mechanisms in the development of resistance towards this biocide. *P. aeroginosa* posses effective efflux mechanisms that are used to pump harmful substances out of the cell. These efflux systems have been the effective mechanism of resistance towards various groups of antimicrobials. In the present study it was observed that it is possible that an efflux mechanism be involved in the resistance towards ZnPT. The over-expression of outer membrane proteins that were observed in P11 ZnPT-resistant cells and the drop in MIC values (Tables 5.1 and 5.2) as a result of the incorporation of efflux inhibitors such as CCCP and OVN suggests that there is efflux involvement. The results show that such system is not expressed in the wild type strain, thus, it must be a ZnPT induced system similar to the CzcABC ion efflux system (Chapter One).

When SDS-PAGE gels for ZnPT-sensitive and -resistant cell lysates were examined, specific protein bands were observed to be expressed in the ZnPT-resistant CFE and were not detected in the ZnPT-sensitive CFE. This led to the assumption that these proteins were the product of a stress response. However, when the electrophoretic

profile of ZnPT-resistant cell lysates were compared with that of the heat shocked *P. aeruginosa* CFE, non of these proteins were immunologicaly similar to the heat stress proteins of heat shocked *P. aeruginosa* or *E. coli.* Which led to the assumption that these proteins could be molecular chaperones that assist the cell to recover from the effect of ZnPT in the cell or these proteins are ZnPT specific stress proteins.

Chapter Six

Identification of the mobile Protein

Proteomics aim at the identification and characterization of all proteins expressed in an organism, through linking amino acid sequence of proteins to DNA sequences of genes (Wilkins and Gooley 1997). Protein identification is the first step towards studies on protein co and post-translational modification and ultimately protein function. The process of identification starts with the isolation and purification of a protein through various methods, most important of which is gel electrophoresis. 1D and 2D-gel electrophoresis, if not followed by protein identification, remain a tool of isolation and description or classification of proteins. These tools become more useful if such isolated proteins are further identified by proteomics. Thousands of proteins have been identified todate. Protein and DNA databases that contain millions of sequences have been established. These databases assist in the identification of unknown proteins, providing that the correct parameters are supplied to the search tools. Protein identification involves assigning a name or database accession code to a protein band or spot on a gel, which is followed by linking the amino acid sequence of this protein to DNA sequences of genes in databases.

6.1 Protein identification strategies

The identification of a protein through the use of proteomics requires the definition of one or more attributes of a protein. Protein attributes are characteristics of a protein that help in its identification. They fall into two main categories depending upon the nature of the protein on which the analysis is performed. If the analysis is

performed on the intact protein, attributes that are supplied to database are considered primary and these include the protein's species of origin, it's isoelectric point (pI), mass, N- and C-terminal sequence tags and extensive N-terminal protein sequence. Secondary protein attributes on the other hand, are generated from fragments of the whole protein such as peptide mass fingerprinting, peptide fragmentation data and *de novo* mass spectrometry (MS) sequencing and amino acid composition of the protein. Most attributes relate directly or indirectly to a protein's sequence. However, they vary in the way they are generated and hence, there usefulness as protein identifiers. After defining protein attributes for a certain protein, they are fed into Internet bioinformatics search tools and are matched against protein databases. Protein databases store information concerning identified proteins such as protein's species of origin, it's accession number, amino acid sequence, secondary and tertiary structure, function, and post translational modifications. Examples of some known databases are the SWISS-PROT (URL 1), EMBL (URL 2) PROSITE (URL 3) and NCBInr (BLAST) (URL 4) (Attwood and Parry-Smith 1999). In conclusion, protein identification involves the matching of analytically-gained protein attributes against sequences in databases predicted from the translation of genomic or complementary DNA (Wilkins and Gooley 1997).

In the present study the outer membranes of *P. aeruginosa* PAO1 ZnPT-resistant cells were found to be deficient in a 26.30 kDa protein. This protein (26.30 kDa) was expressed in the outer membranes of ZnPT-sensitive cells. The protein band in the ZnPT sensitive strain that corresponded to the missing protein in the ZnPT-resistant strain was excised in an attempt to identify it. *P. aeruginosa* PAO1 genome has recently been published (Stover *et al* 2000). Sequencing has proved it to posses the largest bacterial genome ever sequenced. It contains 6.3 million base pairs. DNA

sequencing has provided insights into the basis of intrinsic drug resistance in this species. Thus, it appeared tempting to identify the 26.30 kDa protein using the proteomics databases to gain some information about its role in resistance towards ZnPT.

6.2 Materials and Methods

The outer membranes of ZnPT-resistant and -sensitive cells were electrophoresed using large casting gel plates (15x15cm). An aliquot (1mL) of the outer membrane of ZnPT-sensitive cells and ZnPT-resistant cells (300 µl) in electrophoresis sample buffer (Chapter Two) were loaded on the stacking gel wells. The resistant strain was used as an indicator for the missing protein. Gels were run at 35mA constant current until the blue front reached the bottom of the gel. Gels were Coomassie blue stained followed by destaining (Chapter Two) until the bands were clear. The band in the ZnPT-sensitive outer membrane that corresponded to the missing protein in the ZnPT-resistant strain was excised carefully and placed in a clean Eppendorf tube with a few drops of water. The Eppendorf tube was stored frozen at -20°C until sent to the protein identification lab at the University of Aberdeen.

6.2.1 Peptide Mass Fingerprinting and Internet Search Tools

The protein sample was digested with trypsin enzyme that cleaves at the C-terminal side of arginine and /or lysine. The exact masses of peptides that result from this cleavage are then measured in a mass spectrometer. The resulting peptide masses were fed into Internet search tools and matched against theoretical peptide masses of proteins in databases. In this study peptide masses obtained from University of Aberdeen were fed into the MASCOT search tool that can be accessed through the

Matrix science lab web site (http://www.matrixscience.com). The search tool was asked to perform the search in different protein databases (OWL (URL 5), NCBInr (URL 4)). The output is a list of proteins ranked according to the percentage of peptides matched from the unknown protein against that matched with a known protein in the databases (Table 6.1, 6.2 and 6.3). A score is given for each protein hit. The MOWSE tool, in the Matrix science web site, was used to calculate the probability of an observed match being a random protein. Once a *P. aeruginosa* protein had been identified in the initial results all *P. aeruginosa* proteins in the NCBInr (BLAST) were searched using the mass spectrometry data. A MOWSE score was also calculated.

6.3 Results and Discussion

Two gel samples were sent for identification to the University of Aberdeen. The two samples were prepared separately using freshly prepared outer membranes. The isolation of the protein on gels was performed for each sample. The results of mass spectrometry obtained from the sample number one included 60 peptides with no missed cleavages. Peptide masses were fed into the MASCOT search form and the OWL database was searched. According to the MOWSE tool, calculations for significance of the protein hits, protein scores greater than 56 were significant ($p< 0.05$). The top 5 proteins are listed in Table 6.1. The fact that the first protein (accession number AF128399) had a score of 59 and it is a *P. aeruginosa* protein makes it a significant hit. When the accession number of that protein was activated, a protein view screen was obtained that showed the amino acid sequence of the 7 peptides that matched with the AF128399 protein and the sequence of the AF128399 protein with the matched peptides shown in bold red (Figure 6.1). The protein that has the accession number AF128399

was identified by the NCBInr database as *P. aeruginosa* succinyl-CoA synthetase beta subunit.

In the second sample the peptide masses were 59 with 1 missed cleavage. Peptide masses were fed into a different search tool, the MS-Fit. NCBInr and OWL database was searched for protein matches. Results obtained are summarized in Tabels 6.2 and 6.3 respectively.

Table 6.1: Protein list obtained from the OWL database for sample (1) using the Mascot search.

Rank	Accession number	MW (Da)	Score	Peptide matched	Species of Origin
1	AF128399	41899	59	7/60	*P. aeruginosa*
2	PVU605222	44793	46	6/60	*Paracoccus versutus*
3	H64593	114739	39	5/60	*Helicobacter pylori*
4	C64543	11186	37	4/60	*Helicobacter pylori*
5	AF0274052	47872	35	5/60	*Campylobacter fetus*

Table 6.2: Protein list obtained from NCBInr database for the sample (2) using the MS-Fit search.

Rank	Accession number	MW (Da)	Score	Masses matched	Species of Origin	Protein name
1	AF128399	41925.4	1.14e+003	13	*P. aeruginosa*	Succinyl-CoA synthetase β-subunit
2	U57969	51873.9	17.1	6	*P. aeruginosa*	OprJ
3	AF126491	94964.5	11.3	6	*P. aeruginosa*	Mismatch repair system component
4	M88627	43237.7	6.62	6	*P. aeruginosa*	Phenylalanine hydroxylase
5	U47920	99570	5.12	6	*P. aeruginosa*	Pyruvate dehydrogenase

Table 6.3: Protein list obtained from the OWL database for sample (2) using the MS-Fit search.

Rank	Score	MW(Da)	Masses matched	Accession number	Species of Origin
1	789	41925.4	9/59	AF128399	*P. aeruginosa*
2	389	74959.3	10/59	PAALGYGEN	*P. aeruginosa*
3	67.2	34992.6	5/59	S77670	*P. aeruginosa*
4	67.1	35069.7	5/59	AF0022224	*P. aeruginosa*
5	35.7	35462.8	4/59	AF0119228	*P. aeruginosa*

Figure 6.1: Protein view as printed from the MASCOT search results

Nominal mass (M_r): **41899**; Calculated pI value: **6.57**
NCBI BLAST search of AF128399 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Pseudomonas aeruginosa

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **60**
Number of mass values matched: **7**
Sequence Coverage: **17%**

Matched peptides shown in **Bold Red**

```
    1 MNLHEYQGKQ LFAEYGLPVS KGFAVDTPEE AAEACDKIGG SEWVVKAQVH
   51 AGGRGKAGGV KLVKSKEDAK AFAQQWLGKN LVTYQTDANG QPVSKILVES
  101 CTDIDKELYL GAVVDRSSRR IVFMASTEGG VDIEKVAHDT PEKILKATID
  151 PLVGAQPYQG RELAFQLGLK GDQIKQFTHI FVGLAKLFQD YDLALLEVNP
  201 LVIKKDGNLH CLDAKINIDS NALYRQPKLR AMHDPSQDDA REAHAQKWEL
  251 NYVALEGNIG CMVNGAGLAM GTMDIVNLHG GKPANFLDVG GGATKERVTE
  301 AFKIILSDSN VKAVLVNIFG GIVRCDMIAE GIIGAVKEVG VKVPVVVRLE
  351 GNNAELGAKV LAEKRPEHHR GNQPDRRCPA SLKAAEGK
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
147 - 161	1585.77	1584.76	1584.83	-0.06	0	ATIDPLVGAQPYQGR
162 - 170	1018.56	1017.55	1017.59	-0.03	0	ELAFQLGLK
176 - 186	1260.71	1259.70	1259.70	-0.00	0	QFTHIFVGLAK
216 - 225	1178.60	1177.59	1177.61	-0.02	0	INIDSNALYR
313 - 324	1257.74	1256.73	1256.76	-0.03	0	AVLVNIFGGIVR
338 - 348	1180.69	1179.68	1179.73	-0.05	1	EVGVKVPVVVR
343 - 348	668.44	667.43	667.44	-0.00	0	VPVVVR

No match to: 606.29, 615.32, 616.33, 617.29, 618.27, 619.35, 620.27, 627.29, 628.32, 630.32, 634.30, 635.29, 718.38, 747.34, 842.51, 856.52, 870.52, 879.49, 909.60, 947.54, 1045.56, 1049.54, 1089.65, 1129.61, 1141.67, 1194.55, 1217.64, 1263.55, 1277.67, 1308.62, 1324.61, 1360.67, 1375.75, 1390.67, 1414.64, 1598.80, 1623.76, 1704.77, 1707.72, 2186.84, 2210.86, 2283.10, 2619.00, 2707.20, 2721.14, 2750.16, 2779.50, 2808.21, 3310.52, 3323.42, 3337.61, 3339.38, 3341.53

The results obtained for the two samples from different databases seemed to agree with regard to the first protein that has the accession number AF128399. This protein was the succinyl CoA synthetase β-subunit. This agreement ensures that the protein sent in the two samples was a pure protein. Variations in the protein lists that follow the succinyl CoA synthetase β-subunit between different databases depend upon the relative richness of the different databases with protein information. However, the only significant protein was always the protein ranked number one, which was the Succinyl CoA synthetase β-subunit. Succinyl CoA synthetase (Scs) is an enzyme that is involved in metabolic processes such as carbohydrate metabolism. The enzyme is made of 2 α and 2 β-subunits. The Scs α subunit is a 30 kD protein and the β-subunit is a 40 kD protein (Kapatral *et al* 2000). The enzyme is involved in the only step in the tricarboxylic acid cycle (TCA) where a molecule of GTP or ATP is generated by substrate level phosphorylation. In *P. aeruginosa* Scs catalyzes the synthesis of both ATP and GTP from succinyl-CoA, P_i and an equimolar mixture of ADP and GDP, it was also able of catalyzing the synthesis of UTP and CTP when exposed to succinyl-CoA, P_i and an equimolar mixture UDP and CDP (Kapatral *et al* 2000). Alginate is a polymer of D- mannuronic acid and L-guluronic acid. GDP-mannuronic acid, derived from GDP-mannose is the building block of alginate, which requires mannose-1-phosphate and GTP as precursors. Thus alginate synthesis demands a steady supply of GTP for every molecule of mannuronic acid incorporated into a molecule of alginate. Succinyl CoA synthetase catalyzes the synthesis of high-energy molecules required for alginate production in *P. aeruginosa (*Kapatral *et al* 2000).

These results suggest that this enzyme might also be supplying energy required for the active transport of the ZnPT molecule and that its absence arrests this transport

process. Obtaining the *algR2* mutants, that are deficient in Scs enzyme, and observing the protein profile for its outer membrane could test this theory. The same mutants could be challenged with ZnPT in growth media and the ZnPT MIC measured and compared with MIC of P11 ZnPT resistant *P. aeruginosa*. Due to funds and time limitations this work was not done.

However, until this work is done the identification of this protein remains uncertain due to several points. The first point is the molecular weight, as the observed difference in molecular weights is significant. The missing protein has a molecular weight of 26.302 kDa whilst Scs-β-subunit has a molecular weight of 40 kDa. The second point is the location of the protein, Scs-β-subunit was isolated from the cytosol (Kapatral *et al* 2000), whilst the missing protein was located in the outer membrane. The third point t is that ZnPT is a membrane active compound that enters the cell after dissociation into Zn^{2+} and perthiolate anions through diffusion. Thus, energy for transport is unlikely to be involved here. However, the function of Succinyl-CoA-synthetase (Scs) is still under research and is not fully explored.

The results obtained depending upon the mass fingerprinting data were not highly significant, since only 8 peptides out of 59 or 60 peptides matched with known proteins in the databases. This fact suggests that it is inappropriate to depend upon mass fingerprinting as a sole protein attribute for protein identification. Protein sequence, even if only a few amino acids in length is more specific and is more desirable. Short lengths of sequences from the N- and C- termini of intact proteins (terminal sequence tags) makes excellent attributes for protein identification. It was shown that 60% of *E. coli* proteins have unique N-terminal sequence tags of length 4 amino acids and 90% of all *E. coli* proteins have unique C-terminal tags of 4 amino acids in length (Wilkins and Gooley 1997). Therefore, given more time and funds it would be desirable to undertake

N-terminal sequence analysis on the purified 26.30 kDa outer membrane protein, in an attempt to identify its probable full amino acid sequence, mRNA, cDNA and genome mapping site.

Chapter Seven

General Discussion

Pyrithiones are a group of antimicrobials that have been shown to inhibit the growth and viability of both bacteria and yeasts at a species dependant concentration, that are also medium influenced (Khattar and Salt 1993; Ermolayeva and Sanders 1995; Al-Adham *et al* 1998; Dinning *et al* 1998a,b,c). The Zinc and Sodium salts of Pyrithiones are also known as Zinc and Sodium Omadine. These antimicrobials have been used commercially in cosmetic and pharmaceutical preparations intended for topical use (Khattar and Salt 1993). Pyrithiones are membrane active agents that act by the chelation of the divalent cations on the outer membrane of Gram-negative bacteria and react with the phospholipid head groups resulting in the disruption of the outer membrane and the diffusion of the biocide into the cell. It has been suggested that ZnPT reduces protein synthesis, through chelation of intracellular cations that are important for enzyme function (Chandler and Segel 1978; Khattar and Salt 1993). Resistance towards this group of biocides has not been previously reported. Previous studies by Br.zel and Cloete (1994) and Winder *et al* (2000) have reported the possible induction of resistance to Isothiazolone biocides in the opportunistic pathogen *P. aeruginosa* PAO1. In the present study the aim was to investigate whether *P. aeruginosa* PAO1 can develop resistance towards the Pyrithione salts, Zinc Pyrithione (ZnPT) and Sodium Pyrithione (NaPT). If resistance could be detected, then the subsequent goal was to determine the mechanism(s) of resistance adopted by that pathogen. The QAC Cetrimide is known to be a membrane active compound hence, it was used as a control biocide in the studies of the mechanism of resistance.

In order to assess the effect of media components and growth conditions on adaptation to the biocides, *P. aeruginosa* PAO1 was cultured on two types of media that

were nutritionally dissimilar. R2A medium is an enriched, iron deficient, low nutrient medium (Reasoner & Geldreich, 1985) and CDM is nutritionally limited (Dinning 1995). Growth of *P. aeruginosa* (Figures 3.1 and 3.2) differed on both types of media. R2A is a relatively rich medium that supported a normal growth (Figure 3.2), with a short lag phase (1hour) followed by a long (15 hour) exponential phase. In an iron deficient medium low growth rate is expected (Brown *et al* 1990). However, this curve showed a rapid growth that resembled that in normal enriched media, with a doubling time of approximately 1 hour. R2A is supposed to be iron depleted, however, no specific measures have been taken to assure iron deprivation since iron is a ubiquitous contaminant of most materials. On the other hand the observation of flourescent green pigmentation in R2A cultures is indicative of the presence of Pyoveridines. Pyoveridine is a fluorescent green pigment produced by *P. aeruginosa* that also function as a siderophore (iron chelator). Pyoveridine has a high affinity for Fe^{3+} (Sokol & Woods 1983), which explains the strong fluorescent green colour of the *P. aeruginosa* R2A cultures. Growth in CDM exhibited a long lag phase (9h) and a short (6h) exponential phase. Nutritionally limited media often support this type of growth (Brown & Williams 1985; Gilbert *et al* 1987; Brown *et al* 1990). Bacteria in nutrient limited media are actually going through stationary phase, where induction of specific transport systems takes place and susceptibility to antimicrobials may be altered. However, when outer membranes of *P. aeruginosa* PAO1 cultured on both types of media (R2A and CDM) were compared (Figure 3.3) no variations in the outer membrane protein profile were observed. This indicates that, changes in growth conditions do not necessarily affect OMP profiles and that other components in the cell envelope might be affected.

Initial assessment of MIC for the three biocides (ZnPT, NaPT and Cetrimide) was performed on both types of media. MIC for ZnPT was 4 $\mu g\ mL^{-1}$ and 3.4 $\mu g\ mL^{-1}$

on R2A and CDM respectively. MIC for NaPT was 50 μg mL^{-1} and 30 μg mL^{-1} on R2A and CDM respectively. For Cetrimide the MIC on CDM was 10 μg mL^{-1}, while on R2A the MIC was ≥ 256 μg mL^{-1} and hence, it was considered resistant on that medium and as a result no further studies were performed for Cetrimide on R2A medium. Results in table 3.3 & 3.4 showed that ZnPT is an antimicrobial that is effective in low concentrations, while higher concentrations of NaPT are required for its antimicrobial activity. ZnPT is a stronger antimicrobial agent, which is in accordance with other results from previous studies (Al-Adham *et al* 1998; Dinning *et al* 1998c).

Bacterial cultures in nutrient limited media have been reported to exhibit altered susceptibility to antimicrobials (Gilbert *et al* 1987). Cultures grown in CDM exhibited increased susceptibility to the biocides manifested in the low MIC values when compared to MIC values obtained from R2A medium (Tables 3.3 and 3.4). However, it is interesting that on R2A medium *P. aeruginosa* was resistant to Cetrimide while on CDM it was sensitive. This result by itself proves that cells grown under starvation conditions induce alterations in their cell envelope. Although outer membrane protein alterations were not observed (Figure 3.3), but such alterations could be present in other components of the outer membrane.

It has been reported that low concentrations of biocides could induce resistance in bacteria towards these biocides (Brozel and Cloete 1994; Winder *et al* 2000). Accordingly, *P. aeruginosa* PAO1 cultures prepared in both R2A medium and CDM were subjected to low concentrations (MIC/4) of the three biocides (ZnPT, NaPT and Cetrimide). ZnPT-, NaPT- and Cetrimide-resistant *P. aeruginosa* PAO1 phenotypes were obtained. The acquisition of resistance has been the result of the gradual exposure of *P. aeruginosa* to sub-MIC (MIC/4) concentrations of the two Pyrithione salts and Cetrimide. The MIC increased from 3.4 μg mL^{-1} to 12.5 μg mL^{-1} for ZnPT when *P.*

aeruginosa was grown on CDM medium, and from 4 μg mL^{-1} to 19.3 μg mL^{-1} when grown in R2A medium. As for NaPT the MIC increased from 30μg mL^{-1} to105 μg mL^{-1} when grown on CDM, while it increased from 40 μg mL^{-1} to 170 μg mL^{-1} when grown on R2A medium (Table 4.1). When CDM bacterial cultures were exposed to sub-MIC concentration of Cetrimide the MIC increased from 10 μg mL^{-1} to 130μg mL^{-1}. It was noted that the acquisition of resistance (Increase in MIC) in R2A medium was faster than the acquisition of resistance in CDM. Similarly the loss of the resistance as indicated by the decrease in MIC was slow in CDM, in comparison to R2A (Figures 4.1 to 4.5). Results in Table 4.1 have shown that the percentage increase in MIC from P1 to P11 in R2A media (382% and 325% for ZnPT and NaPT respectively) is higher than the percentage increase in MIC in CDM (267.6% and 250% for ZnPT and NaPT respectively). Interestingly, the percentage reduction in MIC after the removal of the biocide from the media was higher in R2A medium (63.7% and 55% for ZnPT and NaPT respectively) in comparison to the percentage reduction in CDM medium (20% and 28.5% for ZnPT and NaPT respectively). These results indicate that R2A medium allows the rapid selection of the resistant phenotype. It seems that the bacteria growing in R2A medium use all their genetic and metabolic machinery to overcome the lethal effect of the Pyrithione biocides. As the biocide is removed from the media the bacteria revert easily and rapidly to their normal phenotype. However, bacteria growing on CDM are challenged with two factors nutrient limitation and the antimicrobial effect of the biocides. These bacteria first have to slow their growth rate (Figure 3.1) and adjust their associated metabolic pathways to suit the conditions in CDM and then they have to overcome the presence of the biocide. Obtaining the resistant phenotype from cultures growing on CDM was a relatively slow procedure in comparison with cultures growing on R2A medium. However, if the number of passages in CDM medium was increased

to exceed 10 passages the percentage increase in MIC may have reached the same figure obtained in the 10^{th} passage in R2A medium for both biocides.

A reduction in MIC after the removal of the biocides (P12, P13 and P14) was variable between the biocide treatments. ZnPT-and Cetrimide-resistant cells exhibited a gradual decrease in MIC over this period of three biocide-free passages. This result suggests that if the number of biocide free passages was increased, a complete reversion to the sensitive phenotype may have been observed. However, the case is different with NaPT, where there was a reduction in the MIC when the cells were passaged to a NaPT-free media. The MIC remained stable in passages 13 and 14, suggesting that if the number of biocide-free passages had increased a further reduction in the MIC might not be observed and the cells may retain a low level of NaPT resistance. The previous results suggest that the state of resistance towards ZnPT and Cetrimide is unstable and is likely to end with the removal of the biocides. Similar results were obtained with Isothiazolone-resistant *P. aeruginosa* PAO1 (Winder *et al* 2000). The authors reported that when Benzisothiazolone-(BIT), 5-Cloro-methylisothiazolone-(CMIT) and methylisothiazolone-(MIT) resistant cells were transferred into three biocide-free passages a gradual decrease in MIC was observed but not a complete reversion to the sensitive phenotype. These results and the results obtained from the present study suggest that this type of acquired resistance is unstable.

The MIC for NaPT and Cetrimide of the previously induced ZnPT-resistant cultures on CDM was obtained. ZnPT-resistant *P. aeruginosa* PAO1 was found to exhibit cross-resistance towards NaPT and Cetrimide. However, when similar experiments were performed on Cetrimide-resistant cells, only small increases in MIC towards the Pyrithione biocides were observed (Figure 4.6). The previous result suggests that mechanism of resistance adopted by *P. aeruginosa* towards Pyrithiones

conferred resistance towards Cetrimide. *P. aeruginosa* resistance towards the QAC Cetrimide is known to be due to the increased concentration of divalent cations on the outer leaflet of the outer membrane, which results in the tight interactions between LPS molecules. These tight interactions prevent the subsequent action of the biocide. Jones *et al* (1989) adapted *P. aeruginosa* (CMC2730) to the QAC Bardac 22. Subsequent resistance was lost in the presence of EDTA, which suggested the involvement of the outer membrane of the cell in the resistance mechanism.

"Super-ZnPT-resistant" cells (Chapter Four) that exhibited a mucoid phenotype and had a higher value of MIC for ZnPT when compared with the MIC value of the rest of the population in the same passage were investigated for alterations in their LPS structure. LPS SDS-PAGE analysis (Figure 4.8) for the mucoid colonies showed that these colonies had an increased B-band structure in the form of extra high molecular weight bands. Rottem and Leive (1977) considered an increase in the structure of LPS to be a mechanism used by bacteria to reduce the mobility of the acyl chains in the Lipid A region, thus increasing the stability of the outer membrane and increasing its impermeability properties. Thus, results obtained with the LPS gel analysis could explain the observed cross-resistance with Cetrimide. Cross-resistance studies also indicated that NaPT resistant cells exhibited resistance towards ZnPT and Cetrimide. However, Cetrimide-resistant cells exhibited low level of resistance, in the form of a slight increase in MIC, towards ZnPT and NaPT. The MIC changed for ZnPT from 4.8μg mL^{-1} to 6 μg mL^{-1} and for NaPT from 50 μg mL^{-1} to 57.5 μg mL^{-1}, which suggests that the mechanism of resistance exhibited by *P. aeruginosa* towards Cetrimide conferred only partial resistance towards Pyrithiones. Cross-resistance results indicate that mechanism of resistance towards Pyrithiones is multifactorial and is dissimilar to the mechanism of resistance towards Cetrimide in some aspects.

It was observed that during the process of ZnPT adaptation on both R2A medium and CDM, the MIC values paused (no increase) for 3 passages. A similar result was observed with NaPT on CDM (Figures 4.1, 4.3 and 4.4). The end of this period coincided with the appearance of the previously mentioned mucoid colonies that exhibited higher MIC values than the rest of the cells in the passage. This result could be explained by the possibility that at a certain concentration (1.5 to 2 $\mu g\ mL^{-1}$ in the case of ZnPT) the biocide presence induces the cell to go through some structural or metabolic changes to overcome the biocide challenge. These changes might have been manifested by the emergence of these mucoid "super-resistant" cells. Water-Xylene phase partitioning of ZnPT-resistant cells showed that resistant cells exhibited an increased hydrophobicity in comparison with ZnPT-sensitive cells (Figure 4.9). This is a non-specific mechanism adopted by the bacteria to interfere with the penetration of hydrophilic antimicrobials into the cells. Although ZnPT is a hydrophobic molecule, this increased hydrophobicity will not increase its chances of penetration, since it is accompanied by the increased stability in the LPS.

When outer membrane profiles of ZnPT-resistant cells were investigated for their protein profile using SDS-PAGE, it was observed that upon exposure of the cells to the biocide (P2), an outer membrane protein (26.30 kDa) appeared to be reduced in concentration. This protein was later observed to be missing when the cells reached passage 11. Interestingly, this outer membrane protein reappeared when the cells were transferred to the biocide-free medium (Figure 5.2). These results indicate that this missing protein could contribute to the resistance towards ZnPT. *P. aeruginosa* PAO1 genome was found to contain a large number of regulating genes, the products of which are proteins that regulate protein synthesis in the cell (Stover *et al* 2000). It is possible that this protein is a receptor porin protein (Figure 7.1), which allows the penetration of

ZnPT into the cell. Low concentrations of ZnPT could be suppressing the synthesis of that protein. ZnPT might be a structural analogue of a substrate of the 26.30 kDa OMP. At low concentrations, ZnPT crosses the outer membrane to the periplasmic area where it binds a metal binding regulator protein through its Zinc moiety. This process would alter the conformation of the regulator protein in such a way that it would prevent it from binding to the OMP promotor site on the chromosomal DNA. The regulator protein up-regulates the synthesis of the outer membrane protein (26.30 kDa) when it binds to the promoter sequence. The absence of the biocide from the media would free the regulator protein allowing for the up-regulation of the outer membrane gene. If this theory was true this mechanism would be a specific resistance mechanism against ZnPT. This theory is further strengthened by the fact that the presence of the 26.30 kDa protein in Cetrimide resistant cells was unaffected during the passage period (Figure 5.5). The previous theory suggests that the synthesis of the outer membrane protein is inhibited at the transcriptional level. However, it would be of interest to know the mechanism by which the 26.30 kDa protein is inhibited. This could be easier if the identity of the missing protein was revealed.

Proteomics aims at the identification of proteins in an organism, through linking amino acid sequences of these proteins with the corresponding DNA sequence of genes. These approaches use several characteristics of the missing protein in order to conclude the identity of that protein. One of these characteristics is mass fingerprinting data. Mass fingerprinting data depends upon the enzymatic cleavage of the unknown protein.

The masses of the obtained fragments are then matched against known masses of peptides that are held in the databases. According to the percentage of peptides matched from the missing protein with known proteins in the databases, an identity for the unknown protein can be suggested. In the present study the identity of the missing

protein was investigated using the mass fingerprinting data and a protein identity was given to the 26.30 kDa protein. According to different database results (Tables 6.2 and 6.3 and Figure 6.1) the protein was identified to be Succinyl-CoA- synthetase β-subunit. Succinyl-CoA-synthetase β-subunit is a subunit of the enzyme Succinyl CoA synthetase, which is located in the cytosol and has a molecular weight of 41.92 kDa (Table 6.2). These findings do not make that protein a good candidate for the identity of the 26,30 kDa outer membrane protein. Mass fingerprinting data by itself does not make a good attribute for protein identification. Performing an N-terminal analysis for the isolated 26.30 kDa protein would be a better approach for the identification of this protein. Unfortunately, due to time and funding limitations this part of the work could not be completed.

The absence of the 26.30 kDa OMP could prevent the access of ZnPT into the cell. However, it has been shown by others (Al-Adham *et al* 1998; Dinning *et al* 1998, Chandler and Segel 1978; Khattar and Salt 1993) that ZnPT is a membrane active agent that causes disruption of the outer membrane followed by its diffusion into the cell. Thus, the sharp increase in MIC towards these biocides (resistance) could not be acquired only as a result of the absence of the 26.30 kDa outer membrane protein. Another mechanism must be involved to achieve such high level of resistance.

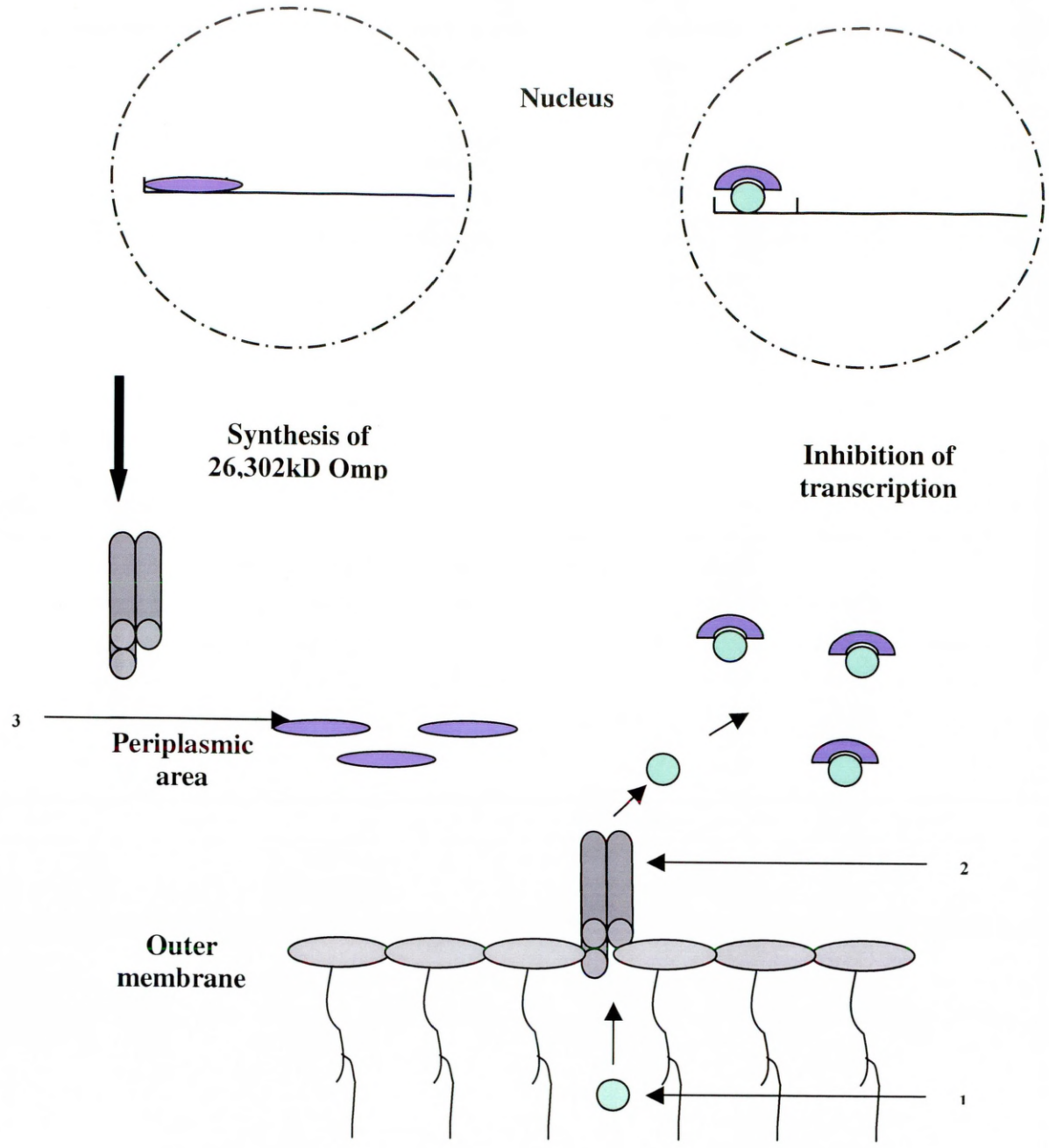


Figure 7.1: Illustration of the theory of the disappearance of the 26.302 kD OMP from the outer membranes of ZnPT resistant *P. aeruginosa* PAO1. The 26.302 kD OMP (**2**) is a receptor protein that allows the penetration of the ZnPT (**1**) molecule into the cell. ZnPt then is bound to a metal binding protein (**3**) in the periplasm resulting in conformational changes, which will prevent its binding to the promotor sequence of the OMP on the chromosomal DNA and as a result its synthesis is inhibited.

Multi-drug efflux systems (Figure 1.3) have recently been recognized as efficient mechanisms of resistance in *P.aeruginosa* (Nikaido 1998; Aires *et al* 1999). Efflux pumps assist in the acquisition of resistance by preventing the accumulation of biocide molecules that succeed in penetrating the outer membrane. Efflux systems use energy for the export of substances out of the cell. Thus, attacking the energy source would apprehend the transport process. In the present study the level of resistance towards ZnPT was determined by the increase in MIC. Thus, a decrease in MIC would be suggestive of increased susceptibility towards the biocide. ZnPT-resistant *P. aeruginosa* when grown in R2A medium containing the efflux inhibitors CCCP and OVN, exhibited a significant reduction in MIC (Tables 5.1, 5.2), which suggests the possible involvement of an efflux system in the observed resistance. SDS-PAGE Outer membrane protein profiles of ZnPT-resistant cells have demonstrated the over-expression of 43.650 and 47.80 kDa proteins. The presence of these proteins, together with the reduction in MIC levels when efflux inhibitors were incorporated in the growth medium, suggests the possible presence of an efflux pump component in the outer membrane. The 47.800 kDa protein might be similar to the 50 kDa (OprN) protein detected by Masuda *et al* (1995) in the *nfxC* type mutant. The *nfxC* type mutant of *P. aeruginosa* is a form of the MDR type of resistance in which OprN outer membrane protein (50 kDa) was over-produced and OprD protein was suppressed. OprD protein is a Carbapenem specific porin. Hence, the *nfxC* mutants exhibited cross-resistance to Carbapenems and Quinolones (Masuda *et al* 1995). However, when ZnPT-resistant and -sensitive cells were challenged with various antibiotics, ZnPT-resistant cells were as sensitive as the wild type cells to all tested antibiotics (Table 4.3). This result excludes the possibility that ZnPT is an inducer of the *mar* regulon in *P. aeruginosa* PAO1. This finding disagree with the results obtained with Triclosan and Pine oil biocides that were

found to select for *mar* mutants when used in low concentrations (Moken *et al* 1997; McMurry *et al* 1998). This result also suggests that the efflux pump detected in this study does not belong to any of the four efflux pumps characterized in *P. aeruginosa* so far (Poole 2001).

Increased expression of efflux transporters in the outer membrane is often accompanied by the repression of OmpF synthesis in Gram-negative bacteria (Nikaido 1994). Hence, the disappearance of the 26.30 kDa protein could be the result of the over-expression of the 47.800 kDa and the 43.65 kDa proteins. But, the 26.30 kDa protein reappeared after the removal of the biocide (biocide-free passages) while the over-expressed proteins (47.80 kDa and 43.65 kDa) remained expressed. This result, together with the exclusion of the possibility of a biocide-induced damage for that protein, further emphasize the involvement of the 26.30 kDa protein in the development of resistance towards ZnPT.

SDS-PAGE of ZnPT-sensitive and -resistant cell lysates showed that as *P. aeruginosa* PAO1 cells were exposed to ZnPT, a 47 kDa protein was synthesized which increased in concentration with the increase of ZnPT concentration. It was also observed that when the concentration of the biocide increased in the growth media more proteins were synthesized and others were depressed (Figure 5.11). The previous response is similar to the stress response mounted by the bacteria as a result of exposure to environmental stressors. When living cells are subjected to environmental stressors they mount a stress response, which is manifested in the form of increased synthesis of specific proteins and depression of synthesis of other proteins (Maccario 1995). In the present study the primary exposure of *P. aeruginosa* to ZnPT (P2) was confronted with increased protein synthesis. A 47 kDa protein band appeared to be present in low concentration in the SDS-PAGE gels of (P2) cell lysates. The former band appeared

thicker (higher concentration) in P11 cell lysates and was accompanied with the synthesis of other proteins (53.7 kDa and 15.8 kDa). The previously mentioned protein bands were not detected in the cell lysates of the ZnPT-sensitive *P. aeruginosa* PAO1, nor did they correspond to any of the heat shock induced proteins in the same organism. It is known that stress responses involve the synthesis of an alternative sigma factor that recognizes specific stress proteins sites on the bacterial chromosome and initiates their transcription. In *P. aeruginosa* a heat shock sigma factor (σ^{40}) have been isolated (Allan *et al* 1988). Thus, the detection of that sigma factor in the cell lysates of the ZnPT-resistant cells would prove the presence of a stress response. However, *Pseudomonas* anti-σ^{40} was not available during the performance of the experiments and *E. coli* anti-σ^{32} antibodies were used instead. Cross reactivity between the two alternative sigma factors from the two species was expected. However, Western blot analysis (Figure5-12) yielded negative results. These negative results indicate one of two things: either that σ^{40} protein is present but the antibodies were not specific to it, or that the increase in protein synthesis, which was observed in the form of new bands in the SDS-PAGE gels was not a result of a stress response. However, it is known that some of the stress proteins are constitutively present in the cell, but increase in concentration upon exposure to a stressor (Forreiter and Nover 1998). These proteins are called heat shock cognate (Hsc). It is also known that some of the stress proteins act as molecular chaperones. Molecular chaperones protect the cell from the damage imposed on it as a result of biocide presence. This protection is through the repair of damaged proteins. It is clear that the bacteria have synthesized new proteins upon exposure to ZnPT, but the identity and function of these proteins remains to be elucidated.

7.1 Conclusion

P. aeruginosa PAO1 acquired an adaptive resistance to ZnPT. Resistance was acquired as a result of exposure of *P. aeruginosa* to sub-MIC concentrations of the biocide. Resistance was unstable and showed tendency towards reverting to the susceptible state when the biocide was removed. Adaptive resistance is known to be uninheritable and shows high levels of resistance as long as the biocide is present, but is lost upon removal of the biocide (Conrad and Galanos 1989). Adaptation to Zinc pyrithione was a complicated and a multifactorial phenomenon that resulted in profound structural perturbations in the cell. Resistance involved specific and non-specific mechanisms. Non-specific mechanisms involved increases in LPS structure and cellular hydrophobicity. While, specific mechanisms involved the loss of a 26.30 kDa outer membrane protein. It is suggested that the outer membrane protein is a receptor protein that allows the penetration of ZnPT into the cell. The subsequent presence of ZnPT down-regulates the outer membrane protein synthesis. ZnPT binds to a regulator protein in the periplasm and, as a result, prevents this regulator from binding to the OMP promotor site on the chromosomal DNA. The former process will inhibit the synthesis of the 26.30 kDa protein. This theory explains the reappearance of the outer membrane protein after the removal of ZnPT from the growth media. The large increase in MIC values for ZnPT suggests the involvement of other mechanisms. When efflux inhibitors were included in the growth medium, MIC values fall significantly, which indicates the possible involvement of an efflux pump. This proposal was further strengthened by the over-expression of outer membrane proteins. Results in the present study propose a mechanism of resistance similar to Carbapenem resistance (*nfxC* mutants), which involves the absence of an outer membrane protein specific for the antimicrobial and the

over-expression of another protein that is a component of an efflux pump. Outer membrane alterations have been backed up by cytoplasmic changes in the form of synthesis of new proteins and suppression of others. Newly synthesized proteins could be molecular chaperones.

Suggestions for Further work

This project could have been extended further to complete the whole picture of ZnPT resistance in *P. aeruginosa* but due to limited funding and time the work had to stop at this stage. However, a few more studies could be performed, including:

- N-terminal analysis for the 26.30 kDa outer membrane protein and the use of the proteomics to identify the protein and determine its function.
- Purification and identification of the cytoplasmic proteins expressed in the ZnPT- resistant cells.
- Identification of the over-expressed outer membrane proteins of ZnPT-resistant cells and the investigation of their possible role in efflux pumps.

Appendix 1

URL 1: http://expasy.hcuge.ch/sprot/sprot-top.html

URL 2: http://www.ebi.ac.uk/ebi_docs/embl_db/ebi/topembl.html

URL 3: http://expacy.hcuge.ch/sprot/prosite.html

URL4: http://www.ncbi.nlm.nih.gov/

URL 5: http://www.biochem.ucl.ac.uk /bsm/dbbrowser/OWL/

Appendix 2

Antimicrobial susceptibility changes and T-OMP shifts in pyrithione-passaged planktonic cultures of *Pseudomonas aeruginosa* PAO1

(Published paper)

Pages 181-189 have been removed due to copyright restrictions. The citation to the published paper is given below.

Malek, S M A A, Al - Adham, I.S.I., Winder, C.L., Buultjens, T.E.J., Gartland, K.M.A. and Collier, P.J. (2002) 'Antimicrobial susceptibility changes and T - OMP shifts in pyrithione - passaged planktonic cultures of *Pseudomonas aeruginosa* PAO1', Journal of applied microbiology, 92(4), pp. 729-736. doi: 10.1046/j.1365-2672.2002.01575.x.

Refrences

Aires, J. R., Kohler, T., Nikaido,H. , and Plesiat, P. (1999). Involvement of an Active Efflux System in the Natural Resistance of *Pseudomonas aeruginosa* to Aminoglycosides. *Antimicrobial Agents and Chemotherapy* **43**(11): 2624-2628.

Al-Adham, I. S. I., Dinning, A. J. Eastwood, I. M. Austin, P., and Collier, P. J. (1998). Cell Membrane Effects of Some Common Biocides *Journal for Industrial Microbiology and Biotechnology* **21**: 6-10.

Alekshun, M. N., and Levy, S.B. (1999). The mar Regulon: Multiple Resistance to Antibiotics and Other Toxic Chemicals. *Trends in Microbiology* **7**(10): 410-413.

Allan, B., Linseman, M. ,Macdonald, L. A. ,Lam, J. S. and Kropinski A. M. (1988). Heat Shock Response of *Pseudomonas aeruginosa. Journal of Bacteriology* **170**(8): 3668-3674.

Anderes, E. A., Sandine W. E. and Elliker P. R. (1971). Lipids of Antibiotic-Sensitive and -Resistant Strains of *Pseudomonas aeruginosa. CAN. J. Microbiology* **17**: 1357-1365.

Attwood, T. K. and Parry-Smith, D.J. (1999)In : Introduction to Bioinformatics (Cell and Molecular Microbiology in action series) Prentice Hall, London, England.

Ayres, H. M., Furr, J. R., and Russell, A. D. (1998). Effect of Divalent Cations on Permeabilizer-Induced Lysozyme Lysis of *Pseudomonas aeruginosa*. *Letters in Applied Microbiology* **27**: 372-374.

Ayres, H. M., Furr, J. R., and Russell, A. D. (1999). Effect of Permeabilizers on Antibiotic Sensitivity of *Pseudomonas aeruginosa*. *Letters in Applied Microbiology* **28**: 13-16.

Beveridge, T. J. and Kadurugamuwa, J. L. (1996). Periplasm, periplasmic spaces, and their relation to bacterial wall structure : Novel secretion of selected periplasmic proteins from *Pseudomonas aeruginosa* . *Microbial drug resistance* **2**(1): 1-8.

Black, J. G., and Howes, D. (1978). Toxicity of Pyrithiones. *Clinical Toxicology* **13**(1): 1-26.

Bloomfield, S. F. (1991). Methods for Assessing Antimicrobial Activity. Mechanisms of Action of Chemical Biocides. S. P. a. H. Denyer, W. B., Blackwell Scientific Publications: 1-22

Brannan, D. K. J. (1995). Cosmetic preservation. *J. Sco. Cosmet. Chem.* **46**: 199-220.

Braun, V. (1995). Energy coupled transport and signal transduction through outer membrane *via* TonB-ExbB-ExbD-dependant receptor proteins. *FEMS Microbiological reviews* **16**: 295-307.

Brooun ,A., Liu, S. and Lewis, K. (2000) A Dose Response Study of Antibiotic Resistance in *Pseudomonas.aeruginosa* Biofilms. *Antimicrobial Agents and Chemotherapy* **44** (3): 640-646

Brown, M. R. W. and Melling, J (1969). Loss of Sensitivity to EDTA by *Pseudomonas aeruginosa* Grown under Conditions of Mg-Limitations. *Journal of General Microbiology* **54**: 439-444.

Brown, M. R. W. (1979) Nutrient Depletion and Antibiotic Susceptibility. *Journal of Antimicrobial Chemotherapy* **3**:198-200

Brown, M. R. W., Collier, P. J. and Gilbert , P. (1990). Influence of growth rate on susceptibility to antimicrobial agents: Modification of the cell envelope and batch and continuous culture studies. *Antimicrobial Agents and Chemotherapy* **34**(9): 1623-1628.

Brown, M. R. W. and Williams, P. (1985). The Influence of Environment on Envelope Properties Affecting Survival of Bacteria in Infections. *Annual Review Microbiology* **39**: 527-556.

Brozel, V. S. and Cloete, T. E. (1994). Resistance of *Pseudomonas aeruginosa* to Isothiazolone. *Journal of Applied Bacteriology* **76**: 576-582.

Bryan, L. E. (1991). Microbial Persistance or Phenotypic Adaptation to Antimicrobial agents : Cystic Fibrosis as an Illustrative Case. in Microbial Resistance to Drugs, New York, Springer Verlag: 411-420

Bryan, L. E., O'hara, K. and Wong, S. (1984). Lipopolysacharide Changes in Impermeability-Type Aminoglycosides Resistance in *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy* **26**(2): 250-255.

Buchanan, S. K., Smith, B.S. ,Venkatramani, L. , Xia, D.Esser,L., Palnitkar,M. ,Chakraborty, R., Van der helm , D. ,Deisenhofer J. (1999). Crystal structure of the outer membrane active transporter FepA from *E.coli. Nature structural biology* **6**(1): 56-63.

Buscher, K. H., Cullmann, W., Dick, W.,Wendt, S. , Opferkuch, W. (1987). Imipenem Resistance in *Pseudomonas aeruginosa* is due to Diminished Expression of Outer Membrane Proteins. *The Journal of Infectious Diseases* **156**(4): 681-684.

Chandler, C. J., and Segel, H. I. (1978). Mechanism of the Antibacterial Action of Pyrithione: Effects on Membrane Transport, ATP Levels, and Protein Synthesis. *Antimicrobial Agents and Chemotherapy* **14**(1): 60-68.

Chaplin, C. E. (1951). Bacterial resistance to quaternary ammonium disinfectants. *Journal of bacteriology* **63**: 453-458.

Chapman, J. S., Diehl, M.A., Fearonside K.P. (1998). Preservative tolerance and resistance. *International Journal of Cosmetic-Science* **20**: 31-39.

Cho, M., Kim, D., Kong, J. Y., and Yang, S. (1995). Ciprofloxacin Resistance by Altered Gyrase and Drug Efflux System in *Pseudomonas aeruginosa*. *Arch. Pharm. Res.* **18**(3): 173-178.

Clarke, T. (2001). Groceries trip triclosan switch/ American society for microbiology General meeting, Florida Lifelines. *Nature science update*, May 2001

Conrad, R. S., Galanos, C. (1989). Fatty Acid Alterations and Polymyxin B Binding by Lipopolysacharides from *Ps.aeruginosa* Adapted to Polymyxin B Resistance. *Antimicrobial Agents and Chemotherapy*: 1724-1728.

Cooney, J. J. (1969). Effects of Polyurethane Foams on Microbial Growth in Fuel-Water Systems. *Applied Microbiology* **17**: 227-231

.

Costerton, J. W. (1995). Overview of Microbial Biofilms. *Journal of Industrial Microbiology* **15**: 137-140.

Delcour, A. H. (1997). Functions and modulations of bacterial porins ;insights from electrophysiology. *FEMS Microbiological letters* **151**: 115-123.

Deretic, V., Dikshit, R., Konyecsni, W.M., Chakrabarty, A.M.,,and Misra, T.K. (1989). The *algR* Gene , which Regulates Mucoidy in *Pseudomonas aeruginosa*, Belongs to a Class of Environmentally Responsive Genes. *Journal of Bacteriology* **171**(3): 1278-1283.

Deretic, V. S., M. J. Boucher, J.C. and Martin, D. W. (1994). Conversion of *Pseudomonas aeruginosa* to Mucoidy in Cystic Fibrosis; Environmental Stress and Regulation of Bacterial Virulence by Alternative SigmaFactors. *Journal of Bacteriology* **176**(10): 2773-2780.

Dinning, A. (1995) Studies on the Mode of Action of Pyrithione Biocides A PhD Thesis. University of Abertay Dundee,Dundee

Dinning, A. J., Al-Adham, I.S.I., Eastwood, I.M., Austin, P.and Collier, P.J. (1998 b). Pyrithione Biocides as Inhibitors of Bacterial ATP Synthesis. *Journal of Applied Microbiology* **85**: 141-146.

Dinning, A. J., Al-Adham, I. S. I., Austin, P. and Collier, P. J. (1998 c). A Novel Assay for the Distribution of Pyrithione Biocides in Bacterial Cells. *Letters in Applied Microbiology* **27**: 1-4

.

Dinning, A. J., Al-Adham, I.S. I., Austin, P., Charlton, M. and Collier, P. J. (1998a). Pyrithione Biocides Interaction with Bacterial Phospholipid Head Groups. *Journal of Applied Microbiology* **85**: 132-140.

Duncan, A. J., Bott, C. B., Terlesky, K. C. and Love, N. G. (2000). Detection of GroEL in Activated Sludge: a Model for Detection of System Stress. *Letters in Applied Microbiology* **30**: 28-32.

Dunnick, J. K., O,Learry, W. M. (1970). Correlation of Bacterial Lipid Composition with Antibiotic Resistance. *Journal of Bacteriology*. **101**: 892-90

Edrissi, M., Nanaie, H., and Barrett, J. (1973). Photosensetivity and some Spectral Properties of 1-hydroxy 2- Pyridinthion and 1-hydroxy-4- methyl-2- Pyridinthione Reagents . *Microchemical Journal* **18**: 59-65.

El-Falaha, B. M. A., Rogers, D.T., Furr, J. R. and Russel, A.D. (1985). Surface Changes in *Pseudomonas aeruginosa* Exposed to Chlorahexidine Diacetate and Benzalkonium Chloride. *Int. J. of Pharmaceutics* **23**: 239-243.

Ermolayeva, E. and Sanders, D. (1995). Mechanism of Pyrithione Inducesd Membrane Depolarization in *Neurospora crassa*. *Applied and Enviromental Microbiology*. **61**: 3380-3390.

Favero, M. S., Carson, L. A., Bond, W. W., Petersen, N. J. (1971). *Pseudomonas aeruginosa*: Growth in distilled water from hospitals. *Science* **173**: 836-838.

Ferguson, A. D., Hofmann, E., Coulton, K. D., Welte, W. (1998). Siderophore-Mediated Iron Transport: Crystal structure of FhuA with Bound Lipopolysaccharide. *Science* **282**: 2215-2220.

Forreiter, C. and Nover, L. (1998). Heat Induced Stress Proteins and the Concept of Molecular Chaperones. *Journal of Bioscience* **23**(4): 287-302.

George, A. M. (1996). Multidrug Resistance in Enteric and Other Gram-negative Bacteria . *FEMS Microbiology Letters* **139**: 1-10.

Gilbert, P., Brown, M. R. W. and Costerton, J. W. (1987). Inocula for Antimicrobial Sensitivity Testing:a Critical Review. *Journal of Antimicrobial Chemotherapy* **20**: 147-154.

Giordano, A., Magni, A., Trancassini, M. and Cipriani, P. (1993). Outer Membrane Proteins and Lipopolysaccharide Changes After Exposure of *Pseudomonas aeruginosa* to Antibacterial Drugs. *Microbiologica* **16**: 281-286.

Govan, J. R. W. and Fyfe, A. M. (1978). Mucoid *Pseudomonas aeruginosa* and Cystic Fibrosis: Resistance of the Mucoid Form to Carbenicillin, Flucloxacillin and Tobramycin and the Isolation of Mucoid Variants *in vitro*. *Journal of Antimicrobial Chemotherapy* **4**: 233-240.

Gordon, J. A. (1991). Use of Vanadate as Protein-Phosphotyrosine Phosphatase Inhibitor. *Methods in Enzymology* **201**: 477-482.

Gotoh, M., Wakebe, H. and Yoshimara, E., (1989). Role of Protein F in Maintaining Structural Integrity of the *Pseudomonas aeruginosa* Outer membrane. *Journal of Bacteriology* **171**(2): 983-990.

Grossman, A. D., Straus, D.B., Walter, W. A., and Gross, C. A. (1987). σ^{32} Synthesis can Regulate the Synthesis of Heat Shock Proteins in *Escherichia coli. Genes Development* **1**: 179-184

.

Hammond, S. M. L.,Lambert P.A. and Rycroft, A. N. (1984). The Bacterial Cell Surface. Washington, Kapitan Szabo Puplishers.57-118

Hancock, R. E. W., Siehnel, R., and Martin, M. (1990). Outer Membrane Proteins of *Pseudomonas aeruginosa. Molecular Microbiology* **4**(7): 1069-1075.

Hancock, R. E. W. (1997). The Bacterial Outer Membrane as a Drug Barrier. *Trends in Microbiology* **5**(1): 37-42

.

Hancock, R. E. W. (1998). Resistance Mechanisms in *Pseudomonas aeruginosa* and Other Nonfermentative Gram-negative bacteria. *Clinical Infectious Diseases* **27**(suppl.1): S93-S99.

Hancock, R. E. W. and Nikaido, H. (1978). Outer membrane of Gram negative bacteria .XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. *Journal of Bacteriology* **136**(1): 381-390.

Hasegawa, M., Kobayashi, I., Saika, T. and Nishida, M. (1997) Drug-Resistance Patterns of Clinical Isolates of *Pseudomonas aeruginosa* with Refrence to their Lipopolysaccharide Composition. *Chemotherapy* **43**: 323-331

Heytler, P. G.and Prichard, W. W. (1962). A New Class of Uncoupling Agents: Carbonyl Cyanide Phenylhydrazones. *Biochemical and Biophysical Research Communications* 7(4): 272-275.

Hinode, D., Grenier, D., and Mayrand, D. (1995). Purification and Characterization of a DnaK-Like and a GroEL-Like Protein from *Porphyromonas gingivalis*. *Anaerobe* **1**: 283-290.

Hoffmann, H., Geftic, S. G. , Gelzer, J. Heymann, H. and Adair, F.W. (1973). Ultrastructural alterations associated with the growth of resistant *Pseudomonas aeruginosa* in the presence of Benzalkonium chloride. *Journal of Bacteriology* **113**(1): 409-416.

Hu, B. R., Yang, Y. B. O. and Wieloch, T. (1993). Heat-Shock Inhibits Protein Synthesis and eIF-2 Activity in Cultured Cortical Neurons. *Neurochemical Research* **18**(9): 1003-1007.

Hyde, G. A. and Nelson, J. D. (1984). Sodium and Zinc Omadine. in Cosmotics and drug preservation principles and practices, Kabara, J.J., New york, Marcel Dekker.115-128

Jarrell, K. and Kropinski, A. M. (1977). The Chemical Composition of the Lipopolysaccharide from *Pseudomonas aeruginosa* strain PAO and a Spontaneously Derived Rough Mutant. *Microbios* **19**: 103-116.

Jones, M. V., Her, T. M. and Christie, H. J. (1989). Resistance of *Pseudomonas aeruginosa* to Amphoteric and Quaternary Ammonium Biocides. *Microbios* **58**: 49-61.

Kapatral, V., Bina, X. and Chakrabarty, A. M. (2000). Succinyl Coenzyme A Synthetase of Pseudomonas aeruginosa with a Broad Specificity for Nucleoside Triphosphate (NTP) Synthesis Modulates Specificity for NTP Synthesis by the 12-Kilodalton form of Nucleoside Diphosphate Kinase. *Journal of Bacteriology* **182**(5): 1333-1339..

Kenward, M. A., Brown, M. R W., Hesslewood, S. R., and Dillon, C. (1978). Influence of R-Plasmid RP1 of *Pseudomonas aeruginosa* on Cell Wall Composition, Drug Resistance, and Sensitivity to Cold Shock. *Antimicrobial Agents and Chemotherapy* **13**(13): 446-453.

Khattar, M. M. and Salt, W. G. (1993). Aspects of the Mode of Action of Pyrithione Against *Klebsiella Pneumoniae*. *Journal of Chromatography* **5**(S1): 175-177.

Koebnik, R. Locher, K. P. and Patrick, V. (2000). Structure and Function of Bacterial Outer Membrane Proteins: Barrels in a Nutshell. *Molecular Microbiology* **37**(2): 239-253.

Kohler,T., Michea-Hamzehpour,M., Epp, S. F. and Pechere, J. (1999). Carbapenem Activities against *Pseudomonas aeruginosa*: Respective Contributions of OprD and Efflux Systems. *Antimicrobial Agents and Chemotherapy* **43**(2): 424-427

.

Kolawole, D. O. (1984). Resistance mechanisms of mucoid-grown staphylococcus aureus to the antibacterial action of some disinfectants and antiseptics. *FEMS microbiology letters* **25**: 205-209.

Kropinski, A. M. B., Lewis, V. and Berry, D. (1987). Effect of Growth Temperature on the Lipids, Outer Membrane Proteins, and Lipopolysaccharides of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **169**(5): 1960-1966.

Kropinski, A.M., Jewell, B., Kuzio, J., Milazzo, F. and Berry, D. (1985) tructure and Functions of *Pseudomonas aeruginosa* Lipopolysaccharide. *Antibiotic. Chemother.* 36: 58-73

Lawrence, J. V. and. Maier, S. (1977). Correction for the Inherent Error in Optical Density Readings. *Applied and Environmental Microbiology* **33**(2): 482-484

Legnani, P., Leoni, E., Rapuano, S., Turin, D., Valenti, C. (1999). Survival and Growth of *Pseudomonas aeruginosa* in Natural Mineral water: a 5-year study. *International Journal of Food Microbiology* **53**: 153-158.

Levy, C. W., Roujeinikova, A., Sedelnikova,SA, s., Baker, P. J., Stuitje, A. R., Slabast, A. R., Rice, D. W., Rafferty, J. B. (1999). Molecular Basis of Triclosan Activity. *Nature* **398**: 383-384.

Levy, E. (1987). Insights into Microbial Adaptation to Cosmetic and Pharmaceutical Products. *Cosmetic Preservation* **102**: 69-74

Li, X.-Z., Barre, N., and Poole, K. (2000 b). Influence of the MexA-MexD-OprM Multidrug Efflux System on Expression of the MexC-MexD-OprJ and MexE-MexF-OprN Multidrug Efflux Systems in *Pseudomonas aeruginosa. Journal of Antimicrobial Chemotherapy* **46**: 885-893.

Macario, A. J. L. (1995). Heat-Shock Proteins and Molecular Chaperones: Implications for Pathogenesis, Diagnostics, and Therapeutics. *International Journal of Clinical Laboratory Research* **25**: 59-70.

Mason, C. A., Colangelo, T., and Indra, P. (1994). Chemicaly Induced Stress Proteins in Bacteria as Markers of Environmental Contamination. *Abstracts of Papers of the American Chemical Society* **207**(75).

Masuda, N., Sakagawa, E., and Ohya, S. (1995). Outer Membrane Proteins Responsible for Multiple Drug Resistance in *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy* **39**(3): 645-649.

Matchett, A. A., Nickson, P. B., Wainwright, M. and Percival, S. L. (2001). Control of Biofilms by Photoactivation of Exogenous Phenothiaziniums. The Fifth Meeting of the Biofilm Club, Gregynog, Powys, Wales

McMurry, L. M., Oethinger, M. and Levy, S. B. (1998) Over-expression of *marA*, *soxS* or *acrAB* Produces Resistance to Triclosan in Laboratory and Clinical Strains of E scherichia coli. *FEMS Microbiology Letters* 166: 305-309

Mcdonnell, G. and Russell, D. A. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology reviews* **12**(1): 147-179.

Michel, V., Labadie, J., Hebraud, M. (1996). Effect of Different Temperatures Up-shifts on Protein Synthesis by the Psychrotrophic Bacterium *Pseudomonas fragi*. *Current Microbiology* **33**: 16-25.

Michel-Briand, Y., Laporte, J. M., Bassignot, A., and Plesiat, P. (1986). Antibiotic Resistance Plasmids and Bactericidal Effect of Chlorhexidine on Enterobacteriaceae. *Letters in Applied Microbiology* **3**: 65-68.

Moken, M. C., McMurry, L. M.,and Levy, S. B. (1997). Selection of Multiple-Antibiotic-Resistance (Mar) Mutants of *Escherichia coli* by using the Disinfectant Pine oil: Roles of the *mar* and *acrAB* loci. *Antimicrobial Agents and Chemotherapy* **41**(12): 2770-2772.

Moore, G. F. H., M. S.; Holah, J.; Woodward,M. J.; Humphrey, T. J. and Lappin-scott, H. M. (2001). Identification of Genes Involved in Biofim Formation. Fifth meeting of Biofilm club, Gregynog, UK

Nelson, J. D. and Hyde, G. A. (1981). Sodium and Zinc Omadine Antimicrobials as Cosmetic Preservatives. *Cosmetics & toiletries* **96**: 87-90.

Nichols, W. W., Evans, M. J., Slack, M. P. E. and Walmsley, H. L. (1989). The Penetration of Antibiotics into Aggregates of Mucoid and non-Mucoid *Pseudomonas aeruginosa*. *Journal of General Microbiology* **135**: 1291-1303

Nies, D. H., and Silver, S. (1989). Metal Ion Uptake by a Plasmid-Free Metal-Sensitive *Alcligenes eutrophus* Strain. *Journal of Bacteriology* **171**(7): 4073-4075.

Nies, D. H. and Silver, S. (1995). Ion Efflux Systems Involved in Bacterial Metal Resistance. *Journal of Industrial Microbiology* **14**: 186-199.

Nikaido, H. (1988). Bacterial Resistance to Antibiotics as a Function of Outer Membrane Permeability. *Journal of Antimicrobial Chemotherapy* **22**(A): 17-22.

Nikaido, H. (1989). Role of Outer membrane of Gram-negative Bacteria in Antimicrobial Resistance. In Microbial Resistance to Drugs, New York, Springer Verlag. 1-34

Nikaido, H. (1990). Permeability of the Lipid Domains of Bacterial Membranes. *Membrane Transport and Information Storage*: 165-190.

Nikaido, H., Nikaido, K., and Harayama, S. (1991). Identification and Characterization of Porins in *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry* **266**(2): 770-779.

Nikaido, H. (1994). Prevention of Drug Access to Bacterial Targets: Permeability Barriers and Active Efflux. *Science* **264**: 382-388.

Nikaido, H. and Rosenberg, E. Y. (1990) CIR and FIU Proteins in the Outer-Membrane of Escherichia coli Catalyze Transport of Monomeric Catechols- Study with Beta- Lactam antibiotics Containing Catechol and Analogous Groups. *Journal of Bacteriology* **172** (3): 1361-1367

Nikaido, H. (1998). The Role of Outer Membrane and Efflux Pumps in the Resistance of Gram-negative Bacteria. Can we Improve Drug Access? *Drug Resistance Update* **1**: 93-98.

Nikaido, H., and Hall, J. A. (1998). Overview of Bacterial ABC Transporters. *Methods in Enzymology* **292**: 3-20

Nikaido, H. and Vaara, M. (1985). Molecular Basis of Bacterial Outer Membrane Permeability. *Microbiological Reviews* **49**(1): 1-32.

Pansy, F. E., Stander, H., Koerber, W. L., and Donovick, R. (1953). *In vitro* Studies with 1-Hydroxy-2(1H) Pyridinethione. (20041). *Proc. Soc. Exp. Bio. Med.* **82**: 122-124.

Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1996). Proton-Dependent Multidrug Efflux Systems. *Microbiological Reviews* **60**(4): 575-608

Paulus, W. (1993). Microbiocides for the Protection of Materials. A handbook, Chapman & Hall. 1st ed. 445-469

Pearce, H., Messager, S., and Maillard, J.-Y. (1999). Effect of Biocides Commonly used in the Hospital Environment on the Transfer of Antibiotic-Resistance Genes in *Staphylococcus aureus. Journal of Hospital Infection* **43**: 101-107.

Pechey, D. T. and James, A. M. (1974). Surface Properties of Cells of Gentamycin-Sensitive and Gentamycin Resistant Strains of *Pseudomonas aeruginosa. Microbios* **10**(A): 111-126.

Piddock, L. J. V., Jin, Y.F., Ricci, V., and Asuquo, A.E. (1999). Quinolone Accumilation by *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli. Journal of Antimicrobial Chemotherapy* **43**: 61-70.

Piddock, L. J. V., Jin, Y. F., and Griggs, D. J. (2001). Effect of Hydrophobicity and Molecular Mass on the Accumilation of Fluoroquinolones by *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy* **47**: 261-270.

Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Ymasaki, T., Neshat, S., Yamagishi, J., Li, X., and Nishino T. (1996). Overexpression of the *mexC-mexD-oprJ* Efflux Operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa.*" *Molecular Microbiology* **21**(4): 713-724.

Poole, K. (2001). Multidrug Efflux Pumps and Antimicrobial Resistance in *Pseudomonas aeruginosa* and Related Organisms. *Journal of Molecular Microbiology and Biotechnology* **3**(2): 255-264.

Pugsley, A. P., Moreno, F., and De Lorenzo, V. (1986). Microcin-E492-Insensitive Mutants of *Escherichia coli* K12. *Journal of General Microbiology* **132**: 3253-3259.

Preston, M. A. and Penner, J. L. (1987). Structural and Antigenic Properties of Lipopolysaccharides from Serotype Refrence Strains of *Campylobacter jejuni. Infection and Immunity* **55**(8): 1806-1812.

Reasoner, D. J. Geldreich, E. E. (1985). Anew medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology **49**(1): 1-7.

Rehm, B. H. A., Hancock, R. E. W. (1996). Membrane Topology of the Outer Membrane Protein OprH from *Pseudomonas aeruginosa*: PCR-Mediated Site-Directed Insertion and Deletion Mutagenesis. *Journal of Bacteriology* **178**(11): 3346-3349

Rocchetta, H. L. and Lam, J. S. (1997) Identification and Functional Characterization of an ABC Transport System Involved Polysaccharide export of A-Band Lipopolysaccharide in *Pseudomonas aeruginosa. Journal of Bacteriology* **179** (15): 4713-4724.

Rosenberg, M., Gutnuik, D., and Rosenberg, E. (1980). Adherence of Bacteria to Hydrocarbons: A simple Method for Measuring Cell Surface Hydrophobicity. *FEMS Microbiological Letters* **9**: 29-33.

Rossouw, F. T., and Rowbury, R. J. (1984). Effects of the resistance Plasmid R124 on the Level of the OmpF Outer Membrane Protein and on the Response of *Escherichia coli* to Environmental Agents. *Journal of Applied Bacteriology* **56**: 63-79.

Rottem, S., and Leive, L. (1977). Effect of Variations in Lipopolysaccharide on the Fluidity of the Outer Membrane of *Escherichia coli*. *The Journal of Biological Chemistry* **252**(6): 2077-2081.

Russell, A. D. (1997). Plasmids and Bacterial Resistance to Biocides. *Journal of Applied Microbiology* **83**: 155-165

Russell, A. D., Hugo, W. B., Ayliffe, G. A. J. (1999). Preservation of Medicines and Cosmetics in Principles and Practice of Disinfection, Preservation and Sterilization. 3 rd ed. Blackwell Scientific publications London, UK457-477

Russell, A. D. (2000). Do Biocides Select for Antibiotic Resistance.*J. Pharm. Pharmacol.* **52**: 227-233.

Russell, A. D. (2001). Mechanisms of Bacterial insusceptibility to Biocides. *American Journal for Infection Control* **29**(4): 259-261.

Russell, A. D. and Chopra, I. (1996). Understanding antibacterial action and resistance, 2nd (ed.)Ellis Horwood Ltd.

Russell, A. D. and Day, M. J. (1996). Antibiotic and biocide resistance in bacteria. *Microbios* **85**: 45-65.

Russell, A. D. and Furr, J. R. (1977). The Antibacterial Activity of a New Chloroxylenol Preparation Containing Ethylenediamine Tetraacetic Acid. *Journal of Applied Bacteriology* **43**: 253-260.

Schweizer, H. (1998). Intrinsic Resistance to Inhibitors of Fatty Acid Biosynthesis in *Pseudomonas aeruginosa* is due to efflux: Application of a Novel Technique for Generation of Unmarked Chromosomal Mutations for the Study of Efflux Systems. *Antimicrobial Agents and Chemotherapy* **42**(2): 394-398.

Shaw, E. (1949). Analogs of Aspergillic Acid. 1. The Tautomerism of the Hydroxypyridine-N-Oxides. *J. Am. Chem. Soc.* **71**: 67-70

Shand , G. H., Anwar, H. and Brown, M. R. (1988) Outer Membrane Proteins of Polymyxin Resistant *Pseudomonas aeruginosa* : Effect of Magnesium Depletion. *Journal of Antimicrobial Chemotherapy* **(22)** : 811-821

Siehnel, R. J., Martin, N. L., and Hancock, R. E. W. (1990). Function and Structure of the Porin Proteins OprF and OprP of *Pseudomonas aeruginosa*, in *Pseudomonas:*

Biotransformations, Pathogenesis and Evolving Biotechnology. American Society for Microbiology.328-342

Singer, M. (1976) Laboratory Procedures for Assessing the Potential of Antimicrobial Agents as Industrial Biocides. Process Biochemistry **July/August**: 30-36

Sokol, P. A. and Woods, D. E. (1983). Demonstration of an Iron-Siderophore-binding protein in the outer membrane of *Pseudomonas aeruginosa*. *Infection and Immunity* **40**(2): 665-669.

Sokol, P. A., and Woods, D. E. (1986). Monoclonal antibodies to Pseudomonas aeruginosa Ferripyochelin-Binding Protein. *Infection and Immunity* **53**(3): 621-627.

Soprey, P. R. and Maxcy, R.B. (1968) Tolerance of Bacteria for Quaternary Ammonium Compounds. *Journal of Food Science* **33**: 536-540

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H. , Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry* **150**: 76-85.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M.J., Brinkman, F. S. L., Ohufnagle, W., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y.,

Brody, L. L., Coulter, S. N., Folger (2000). Complete Genome Sequence of *Pseudomonas aeruginosa* PAO1, an Opportunistic Pathogen. *Nature* **406**: 959-964.

Swarup, G., Vincent, S. Jr., Cohen, S., and Garbers, D.L. (1982). Phosphotyrosyl-Protein Phosphatase of TCRC-2 Cells. *The Journal of Biological Chemistry* 257 (13): 7298-7301

Szita, G., Tabajdi, V., Fabian, A., Biro, G., Reichar, O., Kormoczy, P.S. (1998). A Novel Selective Synthetic Acetamide Containing Culture Medium for Isolating *Pseudomonas aeruginosa* from Milk. *International Journal of Food Microbiology* **43**: 123-127.

Trias, J., Rosenberg, E.Y. and Nikaido, H. (1988). Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. *Biochemica et Biophysica Acta* **938**: 493-496.

Trias, J. and Nikaido, H. (1990). Outer Membrane Protein D2 Catalyzes Facilitated Diffusion of Carbapenems and Penems through the Outer Membrane of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **34**(1): 52-57.

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological reviews* **56**(3): 395-411.

Vasseur, C., Labadie, J. and Hebraud, M. (1999). Diffrential Protein Expression by *Pseudomonas fragi* Submitted to Various Stresses. *Electrophoresis* **20**: 2204-2213.

Vilganen, P. and Boratynski, J. (1991). The Susceptibility of Conjugative Resistance Transfer in Gram-negative Bacteria to Physicochemical and Biochemical Agents. *FEMS Microbiological Reviews* **88**: 43-54.

Visca, P. Colotti, G., Serino, L., Verzili, D., Orsi, N., and Chiancone, E. (1992) Metal Regulation of Siderophore Synthesis in Pseudomonas aeruginosa and Functional Effects of Siderophore-Metal Complexes. *Applied and Environmental Microbiology* **58** (9): 2886-2893

Walker, J. T., Bradshaw, D. J., Fulford, M.R., Martin, M.V. and Marsh, P.D. (2001) Controlling Mixed Species Biofilm Contamination in Dental Unit Water Systems (DUWS) Using a Laboratory Model-A Choice of Products, The Fifth Meeting of the Biofilm Club, Gregynog, Powys, Wales, UK

Watson, K. (1990). Microbial Stress Proteins. *Advances in Microbial Physiology* **31**: 183-223

.

Williams, S. G., Greenwood J. A., and Jones, C. W. (1996). Physiological and Biochemical Changes Accompanying the Loss of Mucoidy by *Pseudomonas aeruginosa*. *Microbiology* **142**: 881-888

.

Wilson, L. A. and Ahearn, D. G. (1977). *Pseudomonas*-Induced Corneal Ulcers Associated With Contaminated Eye Mascaras. *AM. JNL OF Opthalmology* **84**: 112-119.

Wilkins, M. R. a. Gooley, A. A. (1997) Protein Identification in Proteome Projects in Proteome Research: New Frontiers in Functional Genomics, Wilkins, M. R., Williams, K. L., Appel, R. D. and Hochstrasser, D. F.(eds), Springer,35-63

Winder, C. L., Al-Adham, I. S. I., Abdel Malek, S. M. A., Buultjens T. E. J., Horrocks A.J., and Collier P. J. (2000). Outer Membrane Protein Shifts in Biocide-Resistant *Pseudomonas aeruginosa* PAO1. *Journal of Applied Microbiology* **89**: 1-8.

Yoshihara, E. and Nakae, T. (1989). Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. *The Journal of Biological Chemistry* **264**(11): 6297-6301.

Zgurskaya, H. I. and. Nikaido, H. (2000). Multidrug Resistance Mechanisms: Drug Efflux Across Two Membranes. *Molecular Microbiology* **37**(2): 219-225.