Studies on T-OMP and the Development of Antimicrobial Tolerance in *Pseudomonas aeruginosa* PAO1

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

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Abstract

Pseudomonas aeruginosa displays high levels of tolerance and resistance to many antimicrobial agents. Much of this tolerance is related to the nature of the Gram-negative cell envelope and in particular, the outer membrane. The outer membrane plays an important role in excluding harmful molecules from the cell, whilst being selectively permeable to other solutes *via* its implanted proteins (outer membrane proteins or OMPs). In order to exert their antibacterial action, antimicrobial agents must enter the cell and attain sufficiently high concentrations at their target site(s). The OMPs are highly sensitive to environmental changes and have a physiological ability to respond to such changes. It is thought that the altered cell envelope structure contributes to the accessibility of antimicrobial agents into the cell interior and resistance to such agents is related to over expression or loss of certain OMPs.

Brözel and Cloete (1994) observed a gradual increase in tolerance to increasing concentrations of biocide upon exposure of *P. aeruginosa* to Kathon™, a commercial biocide containing 1.15% v/v 5-chloro-N-methylisothiazolone (CMIT) and 0.35% v/v N-methylisothiazolone (MIT). This adaptation was associated with the concurrent disappearance of a 35kDa OMP, designated T-OMP. Therefore, they concluded that the biocide entered the sensitive cells *via* the T-OMP and that the observed resistance was the result of the absence of this OMP. The aim of this investigation was to induce tolerance in cultures of *P. aeruginosa* PAO1 towards the pure active forms of the three isothiazolone biocides 1,2-benzisothiazolone (BIT), MIT, CMIT and the thiol-interactive agent thiomersal (used as a positive control).

An increase was observed in the minimum inhibitory concentrations (MIC) of all four biocides by at least 58% between the sensitive and resistant cultures. In some cases the percentage increase in MIC was in excess of 150%. However, when the tolerant cells were removed from the presence of the biocide, the MIC began to decrease, indicating a loss in tolerance. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the OMP profiles from the tolerant-induced cultures illustrated the loss of T-OMP in all cases. Analysis of the sensitive and resistant cultures using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) indicated that the T-OMP disappeared in the tolerant cultures. However, these observations also suggested that other outer membrane alterations occur concurrently in T-OMP depleted tolerant cells. Investigations into the cross-resistance of the resistant cultures towards the other test biocides, indicated that resistance was, to some extent, transferable, once it had been developed towards one member of the biocide group. Following routine passaging of the resistant cultures on gradient plates two distinct colonial morphologies were observed, mucoid and non-mucoid. An increase in the cell surface hydrophobicity was noted between the mucoid and non-mucoid cultures, which indicated a loss or reduction in the B-band O-Polysaccharide. However, there were no observable differences in the lipopolysaccharide banding patterns between the mucoid and non-mucoid cells. These observations suggested that other alterations were occurring in the tolerant cells upon exposure to biocide, over and above the simple disappearance of T-OMP. Therefore, it is suggested that the observed tolerant development in biocide exposed cells, was not solely due to the loss of T-OMP. Investigations into Gram-negative bacteria isolated from contaminated industrial samples preserved with isothiazolone compounds exhibited higher MICs towards the preservative biocides than would normally be expected in the species of bacteria isolated and identified. However, there were no observable alterations in their OMP profiles.

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Chapter One

Introduction: An Overview of Antimicrobial Resistance

Since the discovery of penicillin in 1929 a great variety of antibiotics have been developed, for example the aminoglycosides, tetracyclines and the 4-quinolones. These discoveries have made it possible to treat diseases such as endocarditis, meningitis, tuberculosis and pneumonia (Foley & Gilbert, 1996), and their use has revolutionised surgery by preventing post-surgical infections. Soon after the discovery of penicillin it was observed that not all bacteria were killed or inhibited by the presence of antibiotics. This may be due to either innate resistance (an inbred characteristic), the development of acquired resistance or decreased sensitivity towards antimicrobial agents. A decrease in the sensitivity of the microorganism towards an antimicrobial agent may result from a combination of resistance mechanisms. Resistance may be defined as the ability of the bacteria to evade the action of an antimicrobial agent. However, it is probably more accurate to define resistance in comparison to the minimum inhibitory concentration (MIC) values of the sensitive strains (Chapman, 1998). Sensitivity is defined as the ability of the antimicrobial agent to inhibit the growth of or kill bacteria. The method of determining the susceptibility of a bacterium to a particular agent is estimated by comparing the recently determined MIC of the isolate, to the original MIC of a non-biocide exposed strain of the isolate (Chapman, 1998). An isolate is deemed as resistant, when the MIC is significantly higher than that of the sensitive microorganisms, A bacterial strain which exhibits an MIC increase of 2- to 5-fold greater than the sensitive strains may be deemed resistant. However, this is thought, to be indicative of low level resistance (Chapman, 1998). When an antimicrobial agent becomes ineffective against a

microorganism it is presumed that the microorganism is resistant to the agent. However, the microorganism may be demonstrating tolerance rather than resistant towards the antimicrobial. It is difficult to accurately determine whether the microorganism is displaying decreased sensitivity, tolerance or resistance towards an antimicrobial agent. When considering antibiotics the National Committee for Clinical Laboratory Standards sets breakpoint MIC values for each antibiotic. Above this value the isolate may be classified as resistant. In other words, the bacteria may be deemed resistant in comparison to the original isolate or a pool of conspecific sensitive strains (Chapman, 1998). There is no regulatory or industrial organisation to determine breakpoint MIC values for biocide susceptibility, and there is limited opportunity to characterise pre-treatment isolates, when considering a contamination problem (Chapman, 1998). Hence, it is difficult to establish whether a microorganism displays tolerance or resistance towards a particular biocidal agent (Chapman, 1998). A moderate reduction in the susceptibility will be sufficient to cause a failure in the preservation system. However, this may be deemed tolerance rather than resistance (Chapman, 1998). A particular isolate demonstrating a high MIC to an antimicrobial may have an intrinsically low susceptibility to the agent or may have a truly resistant genotype (Chapman, 1998). In order to determine the susceptibility and hence, establish if resistance is present in an industrial contaminant, susceptible microorganisms without prior biocide exposure may be drawn from culture collections such as ATCC. BIT-resistance isolates identified by Chapman *et al,* (1997) demonstrated levels of resistance six- to nine-fold higher than the sensitive pool and were classified as high level resistant (Chapman, 1998). A high level of resistance was described as five-fold higher than the sensitive isolates in the Chapman (1998) study.

The development of resistance to a wide range of antimicrobial agents is now a considerable problem rendering many antimicrobial drugs ineffective. Therefore, in the

past few years there has been a resurgence of diseases which were previously controlled by antibiotics, for example, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin insensitive *Staphylococcus aureus* (VISA) and tuberculosis. Resistance to antibiotics has been studied for many years. However, resistance to non-antibiotic agents, otherwise known as biocides (a collective term for antiseptics, disinfectants and preservatives) is less well understood (Russell, 1995). The development of resistance to disinfectants and preservatives is not a new phenomenon, strains resistant to quaternary ammonium compounds were reported in 1952 (Chapman, 1998). It is unlikely that any antimicrobial agent is immune to the development of resistant (Chapman, 1998). Resistance may result from a genetic alteration or the acquisition of new genetic material. Hancock (1998) described three types of resistance: intrinsic, acquired and genetic. Some bacteria have an innate defence mechanism, which prevents the antimicrobial agent entering the cell. Thus the antimicrobial agent cannot gain entry to its target site and therefore cannot exert antibacterial action. Acquired resistance occurs when bacteria develop resistance following exposure to the antimicrobial or through some genetic event. Intrinsic resistance comprises the inherent features of the cell responsible for antimicrobial resistance, irrespective of exposure towards the agents. Acquired resistance is characterised by the induction of unstable resistance without any observable genotypic change. This resistance is reversible when the inducing conditions are removed (Hancock, 1998). Genetic resistance involves the stable acquisition of new genetic information *via* mutation or the acquisition of a drug resistance plasmid (Hancock, 1998). The high use of antibiotics particularly in the hospital environment exerts strong selective pressure on the microbial population to develop antimicrobial resistance (Brown, 1999).

One recent example of how the use of biocidal agents may lead to the development of resistance towards antibiotics is exampled with triclosan. Triclosan is an antimicrobial

which has been used for more than thirty years, it is routinely added to hand soaps, tooth pastes, cosmetics and plastic kitchenware (Levy *et al,* 1999). Triclosan has been shown to inhibit lipid synthesis in *E. coli* by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase, which is involved in fatty acid elongation (Levy *et al,* 1999; M^{c}Murray *et al,* 1998). Resistance in *E. coli* towards triclosan is conferred by a single amino-acid substitution in the enoyl-reductase enzyme. It has been discovered that the three substitutions affect residues that come into direct contact with triclosan. This will lead to a dramatic increase in the binding of triclosan (Levy *et al,* 1999). The InhA protein in *Mycobacterium smegmatis* is an enoyl reductase and is 35 % identical to the *E. coli* enzyme (M^{c}Murray *et al,* 1999). The mutated residues of *M. smegmatis* are situated close to the NADH cofactor and putative acyl substrate binding site (like those of the FabI of triclosan resistant *E. coli*; M^{c}Murray *et al,* 1999). This supports the theory that InhA is the triclosan target site. It is possible that triclosan may covalently bind to the NADH, which is the mode of action of the antibiotic isoniazid, an antituberculosis agent (M^{c}Murray *et al,* 1999). Resistance to isonazid occurs because of reduced binding of NADH to the enzyme enoyl reductase (Rozwarski *et al,* 1999). An alternative suggestion is that the triclosan non-covalently binds the protein and interferes with optimal binding of NADH or fatty acyl substrate in the active site (M^{c}Murray *et al,* 1999). The resistant mutants would therefore, be unable to bind triclosan.

Intrinsic Resistance to Antimicrobial agents

Intrinsic resistance is defined as a natural or innate property of the bacterial cell that enables it to evade the action of the antimicrobial agent. This phenomenon is more greatly pronounced in the Gram negative bacteria, mainly due to the nature of their cell envelope structure, than in Gram positive bacteria. However, exceptions to this do occur,

for example, chlorine is more active against *P. aeruginosa* and *Proteus mirabilis* than against *Staphylococcus aureus* (Russell, 1992a; Trueman, 1971). The Gram negative envelope provides a selectively permeable barrier. The hydrophilic outer membrane excludes hydrophobic antimicrobial agents from the cell. Whilst the outer membrane proteins create an exclusion limit (only allowing hydrophobic molecules up to a molecular weight of 600Da into the interior) (Denyer, 1995). This limits the entry of biocides into the cell interior and consequently its target site (Russell, 1995). Antimicrobial agents which exert their effect by inducing metabolic or structural lesions in the cytoplasmic membrane are noted as being less active against Gram negatives (Russell, 1992a; Hugo, 1967).

Intrinsic resistance is also observed in Gram positive bacteria, the most notable examples are the spore forming genera *Bacillus* and *Clostridium*. The bacterial spore is resistant to concentrations of antimicrobial agents, which would normally kill the bacterial cell. However, at higher concentrations the antimicrobial agent may demonstrate sporicidal activity (Russell, 1995). For example, freshly prepared hypochlorite solutions demonstrate a rapid sporicidal action (Cook & Pierson, 1983). Intrinsic resistance in mycobacteria is thought to be intermediate between sporing and non-sporing bacteria (Russell, 1995). This is thought to be associated with the cell wall structure providing a physical and functional barrier, similar to that found in the Gram negative cell envelope structure (Russell, 1995). The cell walls possess a high lipid content, which is thought to be connected to the intrinsic resistance. The lipids in the wall have an organised structure with hydrocarbons set perpendicular to the cell wall plane, hence acting as a barrier to hydrophilic biocides and antibiotics (Russell, 1995).

Intrinsic Resistance of Pseudomonas aeruginosa

The intrinsic resistance demonstrated by *P. aeruginosa* to various antimicrobial agents is thought to be largely due to the low permeability rate of the outer membrane of the Gram negative cell envelope (Nikaido, 1992). *P. aeruginosa* exhibits a number of features distinctive from the enterobacteria. These features significantly increase resistance to a variety of antimicrobial agents. For example, wild type *P. aeruginosa* cells have a 12-fold decreased permeability to ß-lactams antibiotics in comparison to *E. coli* and a subsequently higher resistance to hydrophilic antibiotics (Hancock, 1984). The primary reason for the intrinsic resistance in *P. aeruginosa,* is the low permeability of the outer membrane to a variety of hydrophilic substances (Russell, 1992a; Angus, *et al,* 1982; Yoshimura & Nikaido, 1982; Nicas & Hancock, 1983). In addition to this, the Gram negative cell demonstrates general resistance to hydrophobic antimicrobials, due to the presence of the lipopolysaccharide (LPS) in the outer membrane (Helander & Mattila-Sandholm, 2000). The exclusion limits for the outer membrane proteins are significantly greater than in enterobacteria (Decad & Nikaido, 1976), excluding molecules with a molecular weight greater than 600Da (Nikaido, 1992). Many bacteria have the ability to produce polysaccharide capsules, which are important during the establishment of invasive infections and evading the patient's immune defences. *P. aeruginosa* produces a capsule-like exopolysaccharide, sometimes called alginate, which is thought to be of particular importance in patients suffering from cystic fibrosis (Hacker, 1999). Bacteria have a number of individual cell structure characteristics, which helps to evade the action of antimicrobial agents. The bacteria can also exist as a biofilm formation. A bacterial biofilm is a structured community of cells encased in a polymeric glycocalyx, which allows the community to survive in hostile environments and demonstrate greater

resistance towards antimicrobial agents than sessile bacteria (Costerton *et al,* 1999; Ayres *et al*, 1998).

Acquired Resistance to Antimicrobial Agents

Acquired resistance occurs when resistant strains emerge from previously sensitive strains. This results from a genetic change occurring either by mutation or by the acquisition of genetic material in the form of a plasmid or transposons from another cell (Russell, 1995). Alternatively, this can result from the bacterial cell adapting to changes in the external environment, and often exploiting some innate defence mechanism.

Genetic Resistance and Mutation: adaptation, exchange and recombination

The natural existence of efficient horizontal gene transfer between bacteria, and the plasticity of the bacterial genome allows exchanged genetic material to become incorporated into its own genome. This in turn constitutes a prominent role in bacterial evolution (Mitsuhashi, 1993). Genetic variation in bacteria proceeds at a rapid rate, especially in the development of resistance to antibiotics. The selection process for the resistant strains has been amplified as a result of intensive and multiple use of antibiotics (Son *et al*, 1997).

The Genetic Basis of Drug Resistance

Intrinsic resistance is the bacteria's innate defence mechanism and is expressed by the chromosomal genes. Whereas, acquired resistance results from a mutation or the acquisition of new genes from plasmids and transposons. Acquired resistance is of particular importance in the clinical situation. For example, strains of *Staphylococcus aureus* demonstrate resistance to a variety of antibiotics, eg: methicillin. The spread of methicillin-resistant *Staphylococcus aureus* (MRSA) is a problem in the hospital

environment particularly when considering invasive procedures such as, indwelling vascular lines or surgery (Brown, 1999). Resistance can be conferred by the genes resident in the host cell and transferred by plasmids or transposons (Mitsuhashi, 1993). Plasmids are now considered to have greater importance in resistance to non-antibiotic antimicrobial agents. For example, numerous reports have linked resistance to chlorohexidine and the quaternary ammonium compounds to the presence of plasmids (Russell, 1997). It is not thought that plasmids confer a great reduction in the susceptibility of Gram negative bacteria to biocides in comparison to intrinsic resistance mechanisms (Russell, 1997).

Biochemical mechanism of Antibiotic Resistance

There are a variety of mechanisms of biochemical resistance that are exhibited by bacteria. For a bacteria to acquire resistance to a particular antibiotic several of these mechanisms must be operative, especially in the Gram negative bacteria whereby the slow permeability of the outer membrane and the subsequent inactivation may participate (Nikaido, 1988).

The inactivation of antibiotics is significant in intrinsic resistance to ß-lactam antibiotics, whereby the conversion of an active agent to an inactive agent is catalysed by enzymes produced by the bacteria. Many biochemically distinct ß-lactamases have been reported in Gram-negative bacteria (Paul *et al,* 1997). The ß-lactamases are responsible for resistance to ß-lactams. Attempts have been made to develop ß-lactam antibiotics which are not inactivated by these enzymes. However, there has been little success with the penicillin family, although cephalosporins and cephamycins compounds have been developed that resist hydrolysis by ß-lactamases (Paul *et al,* 1997).

Modification of the target site is accompanied by decreased sensitivity to the antimicrobial. Resistance to the antimicrobial agent is observed whilst the target site retains its physiological function, for example, acquired resistance to the quinolones. Alternatively the biochemical mechanism of the bacterial cell may be modified by the loss of cell permeability to antimicrobial agents. For example, acquired resistance to the tetracycline, where Gram-positive and Gram-negative bacteria demonstrate decreased cellular accumulation of the antibiotic. The acquired resistance to trimethoprim is a result of the overproduction of the target site, therefore, a greater concentration of the antimicrobial agent would be required to exert bactericidal effects. The final mechanism of biochemical resistance is the absence of an enzyme or metabolic pathway; this is associated with the intrinsic resistance to a variety of antibiotics (Rattan, 1999).

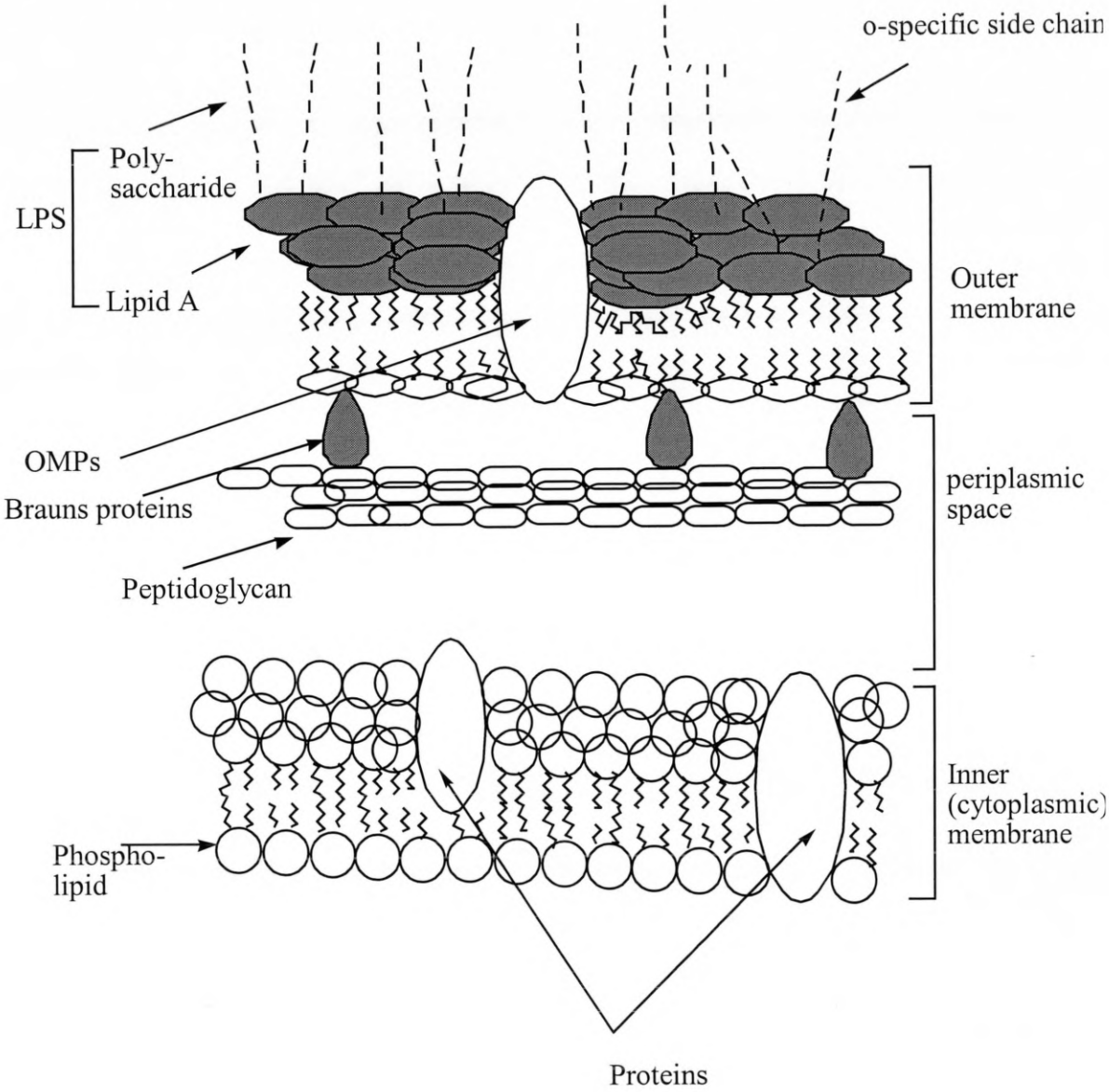
Cell Envelope Structure of Pseudomonas aeruginosa

P. aeruginosa PAO1 Holloway 1C Stanier 131 (NCIMB 10548, ATCC 15692) was isolated from an infected wound by P.J. Stewart. It is characterised as a phage host and is a FP- recipient and its optimum growth temperature is 37°C. *P. aeruginosa* is considered to be highly resistant to a variety of antimicrobial agents. For example *P. aeruginosa* demonstrates tolerance levels to quaternary ammonium compounds and to chlorhexidine acetate (CHA) than other Gram negative bacteria (Russell, 1995). This resistance is thought to be related to the nature of the *Pseudomonas* Gram negative envelope. The Gram negative cell envelope is a complex structure comprised of two membranes, the outer membrane (OM) and inner or cytoplasmic membrane (CM), separated by a layer of peptidoglycan (the cell wall) and the periplasmic space (Figure 1). The outer membrane is a highly specialised structure 6 to 10 nm thick, providing a physical and functional barrier to the external environment (Mayer, 1999; Hancock, 1984). It excludes harmful molecules

from the cell, for example, hydrophobic antibiotics. This is due to the highly charged bacterial surface which is stabilised by divalent cations, whilst being selectively permeable to other solutes *via* it's implanted proteins (outer membrane proteins; OMPs) (Rocchetta *et al,* 1999; Osborn & Wu, 1980; Nikaido & Vaara, 1985; Hancock *et al,* 1990). The outer membrane is a bilayered structure and is chemically distinct from other biological membranes, due to the presence of the lipopolysaccharide (LPS), a unique molecule not found elsewhere in nature (Russell, 1992a). Upon initial electron micrograph studies the outer membrane appears similar to the cytoplasmic membrane. However, its biochemical composition is considerably different (Russell, 1992a). It contains less phospholipids and fewer types of proteins than the CM and the LPS. The outer leaflet is comprised of bacterial lipopolysaccharide and proteins, whilst the inner leaflet resembles the cytoplasmic membrane containing phospholipids and proteins

(Hancock, 1984). The layers of the membrane are extremely asymmetrical adding to these unusual properties, (Sleytr & Messner, 1983).

LPS is an ampihilic molecule consisting of three portions, a lipid, (lipid A, an endotoxin), a short series of core sugars and a long carbohydrate chain, (the O antigen) (Neidhardt *et al,* 1990). Lipid A comprises the hydrophobic interior region, it secures the LPS in the outer membrane and is linked to the O-antigen by the core oligosaccharide (Rochetta *et al,* 1999). It is an unusual glycoprotein because the fatty acids attached to the disaccharides are 14 carbons long instead of 16 or 18. It is the most highly conserved part of the LPS reflecting its role in anchoring the LPS to the outer membrane leaflet (Hammond *et al,* 1984). Lipid A has typically 5 or 6 fatty acids linked to the diglucosamine phosphate with a covalently attached rough oligosaccharide core containing an unusual sugar, 3-deoxy-D-manno-octulosonic acid (KDO) (Rocchetta *et al,* 1999; Hancock, 1984). The KDO contributes a structural unit and strongly binds divalent cations



LPS = Lipopolysaccharide

Figure 1: Schematic Diagram of the Gram Negative Bacterial Cell Envelope.

(Rocchetta *et al,* 1999;). In addition to this, a variety of heptose and hexose sugars are present (Rocchetta *et al,* 1999; Hancock, 1984). The rough oligosaccharide may be substituted with a number of repeated tri- to penta-saccharide units called the O antigen (Hancock, 1984). This creates a hydrophilic chain that extrudes from the bacterial surface and is highly effective in excluding hydrophobic compounds from the cell (Rocchetta *et al,* 1999). The LPS carries a net negative charge and anchors the outer membrane by binding to the implanted proteins. This binding occurs either *via* hydrophobic interactions with Lipid A or by noncovalent cross-bridging of adjacent LPS molecules with divalent cations (Rocchetta *et al,* 1999; Hancock, 1984). When Gram negative cells are treated with ethylenediaminetetraacetate (EDTA), chelation of the divalent cations occurs resulting in the subsequent disruption of the outer membrane (Hancock, 1984). The lipid nature of the outer membrane excludes hydrophilic compounds, this property is considered to account for the intrinsic resistance of *P. aeruginosa* to a wide range of antimicrobial agents (Rocchetta *et al,* 1999; Hammond *et al,* 1984; Hancock, 1984). However, the low permeability of the outer membrane could pose a problem in transporting nutrients into the cell, therefore the outer membrane has special channels consisting of proteins (the outer membrane proteins; OMPs; porins) which allow the passive diffusion of hydrophilic compounds into the cell (Nikaido, 1992). For example, *Pho D* transports glucose across the outer membrane in Pseudomonads (Kramer, 1999). It is thought that some antimicrobial agents enter the bacterial cell via a hydrophilic pathway utilising the OMPs (Rocchetta *et al,* 1999; Nikaido & Nakae, 1979).

Outer membrane proteins (OMPs) are water-filled pores set transversely across the lipid bilayer, which allow the entry of low molecular weight hydrophilic compounds into the cell (Rocchetta *et al,* 1999). The molecular weight exclusion limits of the OMPs vary between organisms. *P. aeruginosa* excludes compounds with a molecular weight greater

than 600 Daltons (Mayer, 1999; Hancock, 1984). The OMPs are synthesised as preproteins on polysomes situated on the inner side of the cytoplasmic membrane. They are translocated across the membrane with a chaperone molecule at which point, they are converted into mature proteins by Peptidase I (Benz, 1988). An internal stop-transfer signal indicates the exact orientation of the OMP and also results in multiple looping of the protein across the outer membrane with several separate ß-sheets (Benz, 1988). The channel-type proteins in the outer membrane can be divided into three classes, the general OMPs, the more or less specific OMPs and the active transport mechanisms related to the OMPs (Nikaido, 1992). The expression of the general proteins is regulated by a two-component system (classified as *osm*) which is sensitive to the osmolarity of the external environment. The expression of the specific proteins is different and can be reduced by cerulenin, which inhibits the production of fatty acids and hence, reduces LPS synthesis. Therefore, the expression of OMPs C and F can be correlated with the synthesis of LPS (Nikaido, 1992). The solute specificity in relation to specific OMPs may be due to the presence of ligand-binding sites in the channel, for example, the *LamB* outer membrane protein in *E. coli* which increases the influx of maltose into the periplasm (Kramer, 1999). Maltose is accepted by the specific binding protein-dependent uptake system (Kramer, 1999). Examples of active transport systems related to porins include uptake systems for iron chelators and vitamin B12. The systems are energised by conformational coupling to the plasma membrane through proteins spanning the periplasm (Kramer, 1999).

The major porin of *P. aeruginosa* (OMP F) forms a substantially larger channel than those found in enteric organisms. However, its activity is substantially lower and it is estimated that only 0.2 to 1% of the porin forms an open channel (Angus *et al,* 1982; Nicas & Hancock, 1983). *P. aeruginosa* is observed to demonstrate only between 1 to 5% the permeability of *E. coli* to the ß-lactams (Rocchetta *et al,* 1999) and a general permeability

of 100 to 500 times lower than that observed in *E. coli* (Wylie & Worobec, 1995). This lower permeability has been attributed to the unusual channel-forming properties of the OMP F (Wylie & Worobec, 1995), which may be due to either heterogeneity in channel formation or inefficient channel architecture (Hancock, 1998). This may account for an enhanced impermeability of *P. aeruginosa* to antimicrobial agents (Rocchetta *et al,* 1999). The diffusion rate of hydrophilic compounds is proportional to the area of water available in the water filled OMP and therefore, the uptake rate of hydrophilic compounds is reduced (Hancock, 1984). The OMPs are small enough such that hydrophobic compounds would come into contact with the polar wall and be excluded from the cell. A small number of 'major' proteins anchor the outer membrane to the underlying peptidoglycan using two types of interactions. The most important interaction involves the outer membrane lipoprotein known as Braun's lipoprotein. About one third of the lipoprotein molecules are covalently linked to the peptidoglycan layer (Neidhardt *et al,* 1990). The other type of interaction (non-covalent) is a tight association between the OMPs and peptidoglycan layer (Neidhardt *et al,* 1990). The cell wall peptidoglycan is less substantial than in the Gram positive cell wall and less closely associated with the cytoplasmic membrane (Figure 1; Hammond *et al,* 1984). However, the peptidoglycan layer is not a thin layer. It forms a loosely organised three-dimensional network filling the entire periplasmic space (Beveridge, 1995). Bayers proteins connect the outer membrane to the cytoplasmic membrane. The periplasmic space or periplasm constitutes approximately 20 to 40 % of the cell volume (Beveridge, 1995). The periplasm is thought to be a highly hydrated gel-like matrix containing a variety of proteins (Beveridge, 1995). The peptidoglycan layer is no longer thought to be a thin-layer, but a loosely organised three-dimensional network that appears to almost fill the periplasmic space (Beveridge, 1995).

The inner or cytoplasmic membrane is essentially a phospholipid bilayer containing a variety of polypeptides (Hancock, 1984). The cytoplasmic membrane acts as a barrier towards hydrophilic or charged molecules. However, even moderately hydrophobic molecules can cross the lipid bilayer (Hancock, 1984).

The Gram Positive Cell Wall

The most striking difference between the Gram positive and Gram negative cell wall is that the Gram positive wall lacks an outer membrane and has a substantially thicker peptidoglycan layer. The thick peptidoglycan sacculus is located directly outside the cytoplasmic membrane, it is covalently linked to the polymer teichoic acid. Teichoic acids are polyhydric alcohol phosphates, often with side-chains of oligosaccharide units and ester-linked D-alanine residues. In addition, teichuronic acid and lipoteichoic acids may be present in which a glycerol teichonic acid is linked to a glycolipid. This enables the molecule to become linked to the cytoplasmic membrane, the hydrophilic part reaches into the cell wall (Mayer, 1999).

Physiological (Phenotypic) Adaptation of the Gram Negative Cell Envelope Structure

Following exposure to antibiotics, bacteria are physiologically damaged. The cell size distribution of a bacterial population may be significantly reduced even when the viable count is increasing at a normal rate (Hostacka & Karelova, 1997a). Intrinsic resistance can be exploited by the ability of the bacteria to adapt to its external environment. The cell envelope structure demonstrates high levels of plasticity, constantly interacting with its external environment and hence, providing a survival mechanism (Gilbert *et al,* 1987). Alterations in cell envelope components such as phospholipids, fatty acids, metal cations, envelope-associated proteins and enzymes, are notably accompanied

by growth rate changes and / or nutrient limitation (Gilbert *et al,* 1987). Such phenomena can alter bacterial susceptibility to antimicrobial agents (Brown & Williams, 1985). The susceptibility of bacteria to antibiotics and biocides is significantly affected by nutrient limitation (Gilbert, 1988), reduced growth rate (Brown & Williams, 1985), temperature, pH and exposure to sub-effective concentrations of antimicrobial agents (Foley & Gilbert, 1996).A wider stress response may be observed during these conditions resulting in increased susceptibility towards antimicrobial agents. This is thought to be the expression of intrinsic resistance brought on by external environmental conditions (Russell, 1995) and is of greater significance when considering sessile microorganisms.

Outer Membrane Proteins and Associated Resistance

The OMPs are thought to participate in the observed intrinsic resistance that *P. aeruginosa* demonstrates to a wide variety of antimicrobial agents. It is suggested that *P. aeruginosa* contains fewer trimeric OMPs (non-specific OMPs) than are found in enteric bacteria, but instead contains many specific OMPs (Wylie & Worobec, 1995). Therefore, the diffusion rate of solutes into and out of *P. aeruginosa* is greatly reduced, because the entry of compounds into the cell is more restricted (Nikaido, 1992). *P. aeruginosa* possesses only one non-specific OMP, OMP F, which is thought to be a homologue of the OMP A found in *E. coli.* (Sugawara *et al,* 1996). OMP F, like OMP A of *E. coli* has a very slow diffusion rate. It is thought that many molecules gain access across the outer membrane of *P. aeruginosa via* specific proteins. Therefore, resistance to certain antimicrobial agents is often associated with a specific channel or OMP (Nikaido, 1992).

The fluoroquinolones were developed as a group of antibiotics clinically effective against *Pseudomonas aeruginosa* (Hostacka *et al,* 1995). However, resistance in clinical isolates to the fluoroquinolones was reported by Diver *et al,* in 1991. Investigations have

indicated that alterations in DNA gyrase or cell permeability, are associated with decreased susceptibility to the fluoroquinolones (Hostacka *et al.* 1995). The *nfxB, nfxC* and *nalB* mutations are connected with decreased outer membrane permeability and have the unusual presence of 54, 50 and 49 kDa outer membrane proteins respectively (Hostacka *et al,* 1995). However, Chamberland *et al,* (1990) reported that the appearance of the 54 kDa protein was not associated with fluoroquinolone resistance in clinical isolates. The overproduction of Opr M (49 kDa) was observed to be associated with multidrug *nalB* mutants (Gotoh *et al,* 1994). It was suggested that Opr M plays an important role in both intrinsic and acquired resistance in *P. aeruginosa* and that the *nalB* mutant is thought to endow quinolone and ß-lactam resistance, as a result of a decreased outer membrane permeability. Therefore, this system prevents the accumulation of the antimicrobial agents in *P. aeruginosa* (Gotoh *et al,* 1994).

In a study carried out by Hostacka and Karelova (1997a) OMP profiles of *P. aeruginosa,* when compared to controls, were found to be unaffected by sub-inhibitory concentrations of the antibiotic amikacin. This contradicts a previous study where an overproduction in the 41 kDa protein and a reduction in the 23 and 45 kDa proteins was observed following exposure to norfloxacin (Hostacka & Karelova, 1997b).

Few antimicrobial agents are now thought to be effective against *P. aeruginosa* these include carbapenems and quinolones. However, a number of clinical isolates have demonstrated resistance to the quinolones (Masuda *et al,* 1995). This resistance has been associated with *gyrA* and *nalB* mutations. The *nalB* mutation infers cross-resistance to a variety of agents including the quinolones, cephens (for example, cefsulodin, ceftazidime and cefoperazone), carbenicillin and chloramphenicol and is associated with the overproduction of an OMP with a molecular mass of 49 kDa (Opr M) (Masuda *et al,* 1995). The overproduction of Opr J has also been associated with cross-resistance in *P.*

aeruginosa to the new cephens (for example, cefpirome and cefozopran) and the quinolones (Masuda *et al,* 1995). The new cephens have a positively charged substitution at position C-3 and a negatively charged carboxyl group at position C-2. Whereas the old cephens posses an additional negative charge in the substitution at position C-7 (Masuda *et al,* 1995). The function was not previously identified, however, it is probably associated with an alteration in efflux transport of the new cephens and quinolones due to this association with it's overproduction and cross resistance to these agents (Masuda *et al,* 1995).

P. aeruginosa cells resistant to polymyxin B illustrate an increased level of the OMP H1 and a subsequent decrease in cell envelope Mg^{2+} concentration. This indicates that the H1 protein is replaced by Mg^{2+} at the LPS Mg^{2+} cross bridging site. This is the proposed site of interaction with the polymyxin B, aminoglycosides and EDTA. Therefore, this, may explain the observed increase in cross-resistance between the aminoglycoside antibiotics and EDTA (Hancock, 1984). Evidence to support this theory was provided when wild-type cells grown in Mg^{2+} deficient medium developed a polymyxin resistant phenotype. Upon addition of Mg^{2+} to the medium the effect was reversed and the wild-type phenotype was found to be present (Hancock, 1984).

During conditions of iron deprivation the majority of Gram-negative bacteria produce between 3 and 9 extra OMPs, these are termed iron-repressible outer membrane proteins (IROMP; Hancock *et al,* 1990). The IROMPs are thought to act as receptors for the binding of complexes of iron with specific complexes called siderophores, which are commonly associated with bacteria during iron starvation (Hancock *et al,* 1990). Other OMPs are associated during periods of nutrient limitation. For example, Opr P is only observed during periods of growth in phosphate-limited conditions of 0.15M or less (Hancock *et al,* 1990).

The Hydrophilic Pathway

Hydrophilic compounds gain entry across the outer membrane *via* the water filled OMPs (Figure 2: Kramer, 1999). The OMPs in *E. coli* consist of trimeric complexes of identical subunits with molecular masses of approximately 35 kDa and a channel diameter of 1nm (Nikaido & Saier, 1992). *P. aeruginosa* appears to contain only one trimeric (specific) OMP, which is thought to be a homologue of OMP A of *E. coli* (Nikaido, 1992). The rate of uptake is determined by the channel size and ionic selectivity relative to the size and charge of the compound, in addition to the total number of available channels per cell (Hancock, 1984). The OMPs are water filled channels with charged amino-acid residues, hence the passage of solutes is a function of the intrinsic viscosity or the hydrophobicity of the permeating molecule (Hancock,1984).

The major OMP (OMP F) of *P. aeruginosa* is suggested to have substantially greater maximal exclusion limits than those found in *E. coli.* (Bellido, 1992). However, it has been estimated that only 0.2 to 1% of the channel has a functional role (Hancock, 1984). The diffusion rate of the hydrophilic compound is proportional to the available area of the water-filled channel, with a large proportion of the channel not playing a functional role, the uptake of the hydrophilic compound is reduced (Hancock, 1984).

The Hydrophobic Pathway

The hydrophobic pathway is ineffective in Gram-negative bacteria in comparison to the Gram-positive bacterial cell (Figure 2; Hancock, 1984). This is due to the nature of the outer membrane. Various lipid bilayers allow the passive uptake of hydrophobic compounds into the cell interior. However, the outer membranes of many wild type Gram

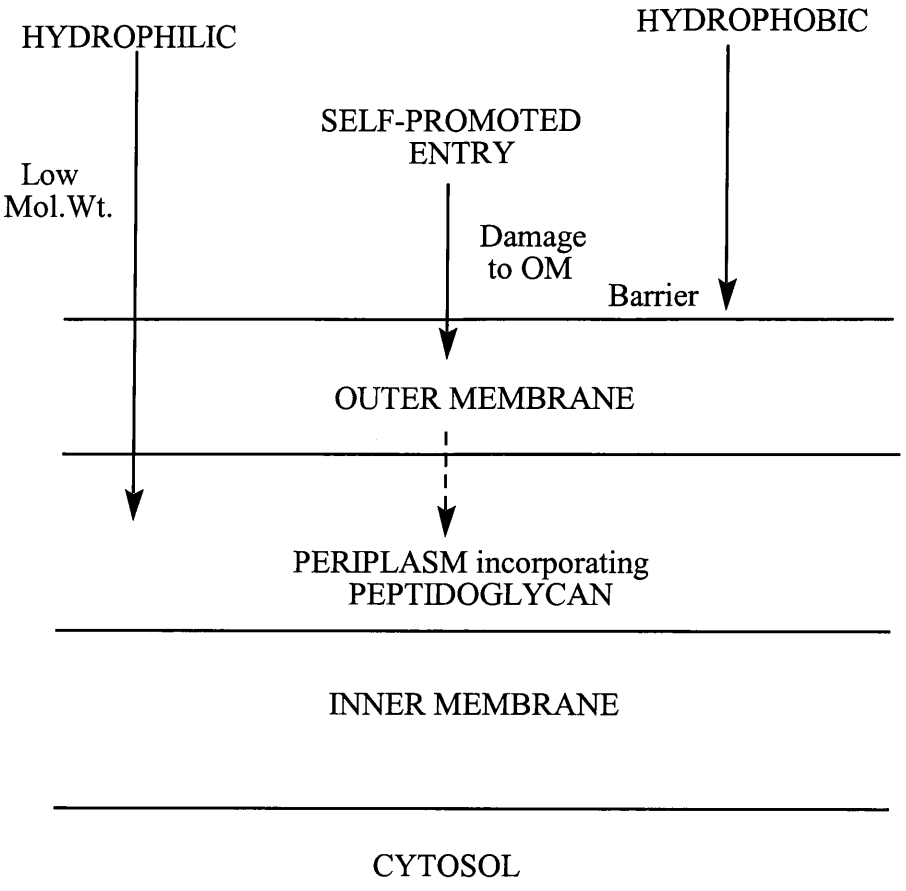


Figure 2: Schematic Representation of the possible Interactions of biocides with the Gram Negative Bacterial Cell Envelope. Diagrammatic representation of uptake pathways and exclusion barriers. Demonstrated area hydrophilic biocide of low molecular weight, a hydrophobic biocide and a biocide which promotes its own entry. OM: outer membrane.

negative bacteria, including, *Salmonella typhimurium* and *Escherichia. coli* are not thought to allow the passage of hydrophobic molecules (Hancock, 1984). This absence of a hydrophobic pathway promotes resistance towards hydrophobic antimicrobials, for example, actinomycin D (Hancock, 1984). It is suggested that the combined effects of divalent cation bridging of lipopolysaccharide (LPS) molecules and the high-surface negative charges, result in this apparent absence of the hydrophobic pathway (Hancock, 1984).

Biofilms and Resistance to Antimicrobial Agents

It is generally frequently observed that bacteria present in a biofilm are less sensitive to chemical antimicrobials and antibiotics than their corresponding planktonic cells (Das *et al,* 1998; Russell, 1995; Brown & Gilbert, 1993). "A biofilm consists of functional consortia of microorganisms associated with a surface, and embedded within an often extensive exopolymer matrix of glycoproteins and polysaccharide" (Gilbert & Brown, 1995). Biofilms are widespread in nature, and problematic in industry creating many operational problems often resistant to aggressive treatment regimens (Wood *et al,* 1996). It is suggested that this observed increased resistance is associated with the presence of highly hydrated polyanionic matrices of extracellular polymeric substances, the glycocalyx (Wood *et al,* 1996). The cells growing at the surface of the biofilm are encased in the glycocalyx which act as a barrier protecting the cells from any antimicrobial agent (Wood *et al,* 1996). Three mechanisms are thought to provide the biofilm with enhanced microbial resistance. The first is the failure of the antimicrobial agent to penetrate the biofilm due to the presence of polymeric substances, which make up the biofilm matrix. (Costerton *et al,* 1999). The second hypothesis is related to nutrient limitation. It is known that some cells exhibit nutrient limitation and are, therefore, found

in a slow-growing or non-growing state. Cells in this state are less susceptible to antimicrobial agents (Costerton *et al,* 1999). The third mechanism suggests that certain cells display a distinctive and protective biofilm phenotype (Costerton *et al,* 1999). This mechanism does not infer a transport limitation like the other two mechanisms, but implies the reduced susceptibility is genetically programmed (Cochran *et al,* 2000). A great diversity of microorganisms are found in naturally occurring biofilms. Interactions between microbes during the formation of the biofilm can affect its formation, often resulting in a thicker and more stable structure. This can, in turn, result in greater resistance to antimicrobial agents (Skillman *et al,* 1999).

Hydrophobicity Differences in Relation to the Passage of Antimicrobial Agents across the Outer Membrane

The cell surface of smooth Gram negative bacteria has a hydrophilic nature, which is responsible for the observed resistance to hydrophobic antimicrobials. Wild-type rough mutants, which have an absence of heptose tend to be more hydrophobic and therefore, more permeable to hydrophobic antimicrobials (Rocchetta *et al,* 1999).

Investigations into mutants of *Escherichia coli* and *Salmonella typhimurium*, which were defective in lipopolysaccharides (LPS) and / or outer membranes, illustrated increased sensitivity to hydrophobic drugs and biocides. They also demonstrate enhanced uptake of gentian violet, a hydrophobic dye, and greater susceptibility to EDTA (Hancock, 1984). It is thought that the heptose-less mutant forms are much more hydrophobic due to phospholipid bilayer patches in the outer member (Smit *et al,* 1975). In these mutant strains the O-specific side chain and the majority of the core polysaccharides were absent, hence the phospholipid patches at the cell surface were observed to have their heads orientated towards the exterior. This suggests that hydrophobic molecules can gain entry

into the cell interior through the usually selectively permeable outer membrane (Russell, 1995). The absence of an O-chain in members of the Enterobacteriaceae is responsible for the formation of rough mutants (Jann & Jann, 1999).

Antimicrobial agents which demonstrated increased activity towards deep rough mutants in comparison to wild type cells, were generally found to be of hydrophobic in nature (Nikaido, 1976; Russell, 1992a). Agents which were mainly small hydrophilic molecules (molecular weight <600Da) were unaffected by any alterations in the LPS layer (Nikaido, 1976; Hancock, 1984). Nikaido (1976) proposed that the changes were not a direct result of the alterations in the LPS structure but were the result of an extensive reorganisation of the outer membrane (Russell, 1992a). The exposed phospholipid bilayer allows the penetration of hydrophobic molecules, for example, phenol (Nikaido & Nakae, 1979). The OMP mutants of *S. typhimurium* which have a normal wild-type LPS composition, but reduced levels of outer membrane proteins, exhibit sensitivity to crystal violet (Ames *et al,* 1974) and increased penetration to hydrophobic antimicrobials (Nikaido, 1976). This illustrates that membrane reorganisation, replacing proteins with phospholipids rather than altering the LPS structure, causes an increased hydrophobic permeability in the rough mutants (Nikaido & Vaara, 1985). Evans *et al,* (1991) demonstrated that mucoid strains of *P. aeruginosa* were associated with decreased sensitivity towards the quinolone antibiotic ciprofloxacin. *P. aeruginosa* mutant Z61 demonstrates a 6 fold increased permeability to the ß-lactam nitrocefin, accompanied by this observation is a 4 to 10,000 fold increase in susceptibility to 30 different antimicrobial agents, although there are no apparent alterations in the major OMP profiles (Angus *et al,* 1982). However, an alteration in the Lipid A was observed, which is thought to favour an increased proportion of open functional pores (Kronpinski *et al,* 1982) .

Efflux pumps

A variety of transport systems have been demonstrated to play an important role in both bacteria and eukaryotes by conferring resistance to toxic compounds (Paulsen *et al,* 1996). Active efflux proteins of wide specificity are common in wild types of bacteria and contribute significantly to the intrinsic resistance observed in *P. aeruginosa* (Ma *et al,* 1994). Although it is not possible to provide definite answers to physiological roles of the majority of multidrug efflux systems, it has been concluded that some native efflux systems have a role in extruding toxic compounds from the cell. Some appear to extrude exogenous or endogenous toxins from the cell, whilst others are involved in unrelated metabolic functions, for example, iron metabolism (Paulsen *et al,* 1996). Multiple drug resistant pumps (MDR's) function to effectively protect the cells from commonly used drugs, which are numerous in type and function (Ouellette *et al,* 1997). The majority of efflux systems typically deal with a narrow range of structurally related substances, for example, the exporter in *E. coli* responsible for extruding tetracycline along with a narrow range of structurally related analogues (Paulsen *et al,* 1996). However, some multidrug efflux pumps have been identified which are capable of extruding structurally dissimilar compounds (Paulsen *et al,* 1996).

The multidrug resistance phenomenon is connected to the over expression of transporters (Zgurskaya & Nikaido, 2000). Drug efflux transporters constitute between 6 and 18% of all bacterial membrane transporters (Paulsen *et al,* 1998). It is suggested that resistance to almost any antimicrobial may be achieved through efflux pump mechanisms (Zgurskaya & Nikaido, 2000).

The major facilitator superfamily (MFS) consists of membrane transport proteins ranging from bacteria to higher eukaryotes involved in the symport, antiport or uniport of various substrates (Paulsen *et al,* 1996). There has been greater than 300 individual

proteins identified belonging to the superfamily (Paulsen *et al,* 1996). Examples of such proteins include the lactose permease LacY of *E. coli* and the human GLUT glucose transporter (Paulsen *et al,* 1996). Marger and Saier (1993) identified five distinct clusters of families in the membrane transporter proteins of the MFS. These clusters are classified into (1) drug resistance; (2) sugar uptake; (3) uptake of Krebs cycle intermediates; (4) phosphate ester / phosphate antiport; and (5) oligosaccharide uptake (Paulsen *et al,* 1996). Proteins involved in drug resistance consist of PMF-dependent drug efflux proteins (Paulsen *et al,* 1996). This cluster includes a number of multidrug efflux proteins and other substrate-specific drug efflux proteins (Paulsen *et al,* 1996). One such example, is the tetracycline exporter TetB, which has been found to function as an electroneutral antiport system catalyses the exchange of a tetracycline divalent-metal-cation complex for a proton (Paulsen *et al,* 1996).

The small multidrug resistance (SMR) family are efflux transporters which prefer cationic, hydrophobic molecules. This family is conserved and found as membrane embedded proteins in otherwise unchallenged bacterial cells. The SMR and MFS families possess several structural and functional glutamate and aspartate residues, which are critical in the transport of cationic substances.

The resitance-nodulation-cell division (RND) superfamily of efflux pumps is the largest single family. This group transports mainly lipophilic and amphiphilic molecules and may be exampled by the AcrB and MexB proteins in *E. coli* and *P. aeruginosa* respectively (Zgurskaya & Nikaido, 2000).

The ATP-binding cassette (ABC) family of efflux pumps is the only major group to find its energy supply in ATP, rather than proton antiport. Transporters within this family have been reported to be involved in drug resistance to antimalarial agents in

Plasmodium falciparum and to leptomycin B in *Schizosaccharomyces pombe* (Paulsen *et al,* 1996).

The first evidence of the MDR in the Gram negative bacteria was reported by Lomovskaya and Lewis (1992). They observed that the MDR protein *Emr*AB in *Escherichia coli* protected the cells against nalidixic acid and thiolactomycin (Lewis *et al,* 1997). Poole *et al,* (1993) demonstrated an efflux system involving three proteins Mex A, Mex B and OMP M, which are critical to the intrinsic resistance mechanism in *P. aeruginosa*. It has been established that a knockout mutation involving genes of any of the three proteins results in a four-fold to ten-fold increase in susceptibility towards the quinolones, ß-lactams (except imipenem), tetracycline and chloramphenicol.

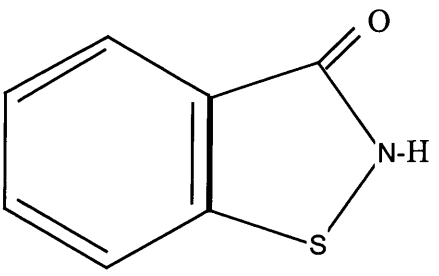
Gram-negative multidrug efflux transporters are composed of a three-component system. The transporter is located in the inner membrane, which has a functional association with a periplasmic accessory protein and an OMP (Zgurskaya & Nikaido, 2000). This formation aids the transport of toxins from the cytoplasm to the external environment. This system works synergistically with the intrinsically low permeability of the outer membrane towards antimicrobial agents (Zgurskaya & Nikaido, 2000). It has been shown that inner membrane transport proteins recognise antimicrobials (Zgurskaya & Nikaido, 2000) and are able to expel such agents, which would otherwise affect the cytoplasm and periplasm. Studies into AcrB and MexB transporters of *E. coli* and *P. aeruginosa* receptively, indicate the only requirement for the drug to be a substrate is the presence of a hydrophobic domain capable of insertion into the phospholipid bilayer (Nikaido, 1996). It is thought that the substrate interacts with the bilayer, gaining access to the transporter core whilst still in lipid phase (Zgurskaya & Nikaido, 2000). The drug binds in the periplasmic space and therefore, the large periplasmic domain may play a role in the efflux process (Zgurskaya & Nikaido, 2000). The periplasmic components belong to

the main fusion protein (MFP) family (Dinh *et al,* 1994) and they export various proteins, oligosaccharides, small molecules and divalent metal cations. Hydrophobic components at the N- and C-termini are thought to interact with the inner and outer membranes, allowing the transfer of substrates (Zgurskaya & Nikaido, 2000). The outer membrane component of the efflux system is interchangeable between systems and various systems may utilise OMPs with otherwise normal functions (Yoneyama *et al,* 1990).

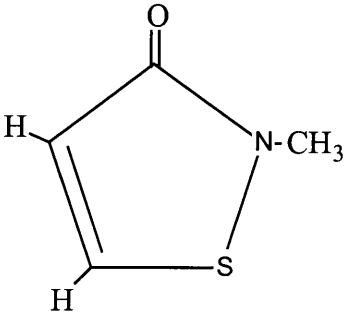
The Isothiazolone Biocides and their Mode of Action

The isothiazolone biocides include 1,2-benzisothiazolone (BIT), N-methylisothiazolone (MIT) and 5-chloro-N-methyl-isothiazolone (CMIT) (Figure 3). They are widely used as industrial and environmental biocides in the preservation of cosmetics, toiletries and paint products (Collier *et al,* 1991; Waigh & Gilbert, 1991; Sondossi *et al,* 1993). The isothiazolone biocides are most commonly used as commercial products where a mixture of the biocides may be applied. For example, Kathon™ (Rohm and Haas Inc.) comprises of a mixture of MIT and CMIT and Proxel™ (Zeneca plc.) is mainly composed of BIT. BIT effectively controls microbial growth in metal working fluids, were bacterial counts are reduced to zero after a four day contact period (Singer, 1976). The commercial products can be used as preservatives in a variety of products, for example paints, adhesives and detergents, and rapidly degrade in the environment.

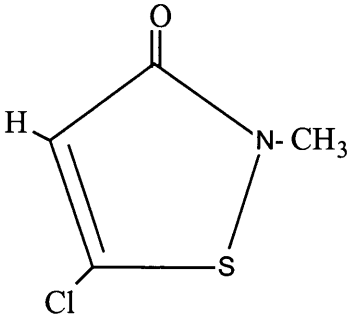
The biocides are known to react with accessible thiol groups within the cell, for example glutathione, mixed protein thiols and cysteine. Therefore, the antimicrobial activity of the biocides will be greatly reduced by the presence of extracellular thiol-containing intracellular agents (Fuller *et al,* 1985; Collier *et al,* 1990a) (Figure 4). Glutathione is the most abundant thiol-containing target in microbial cells at concentrations greater than 10mM (Owens & Hartman, 1986). Growth may be arrested



Benzylisothiazolone



N-Methylisothiazolone



5-Chloro-N-Methylisothiazolone

Figure 3: The structures of the Isothiazolone Biocides (Benzisothiazolone, BIT; N-Methylisothiazolone, MIT; 5-Chloro-N-Methylisothiazolone, CMIT).

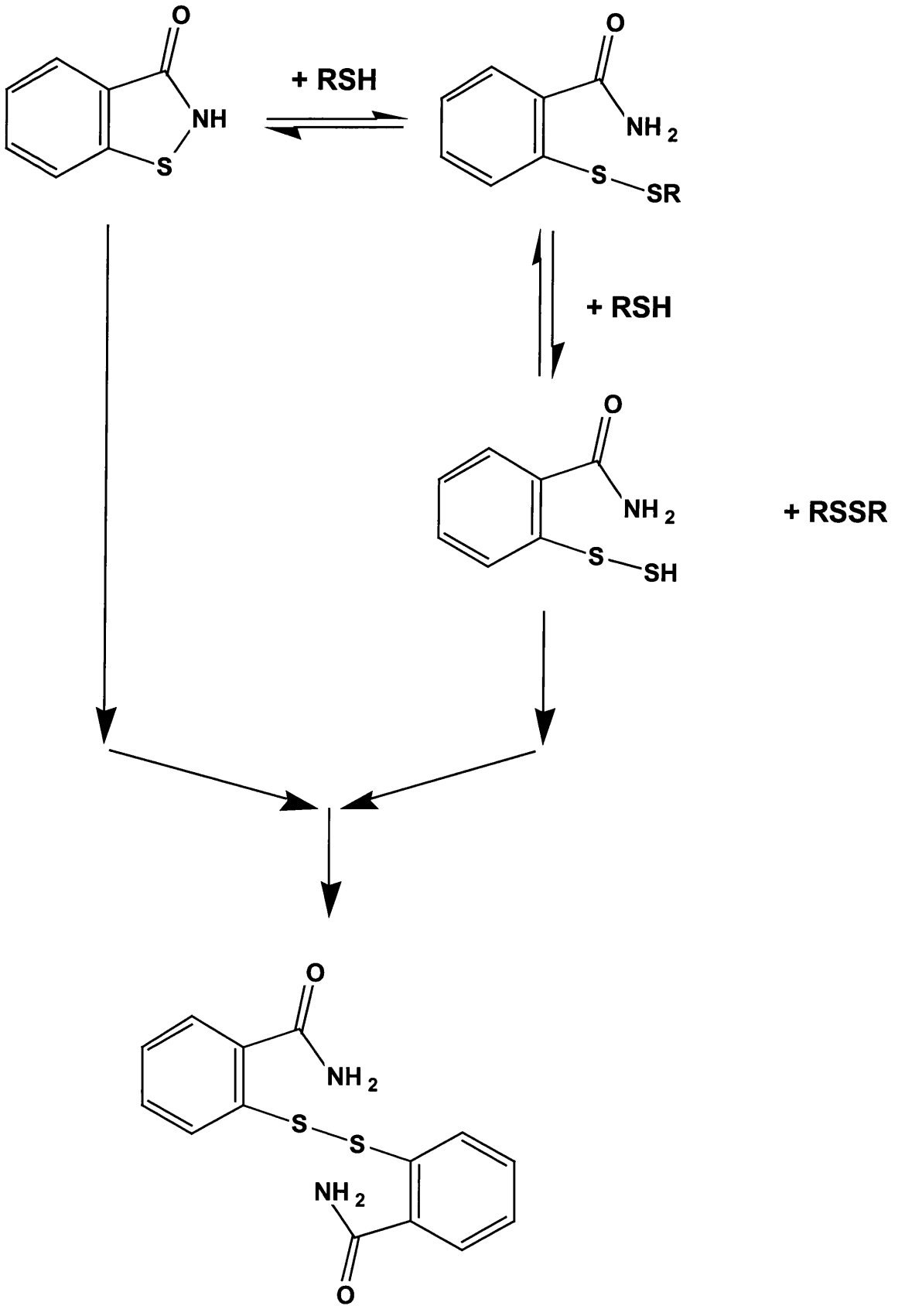


Figure 4: Proposed Thiol-Interactive Pathway for the Isothiazolone Biocide (after Fuller *et al,* 1996).

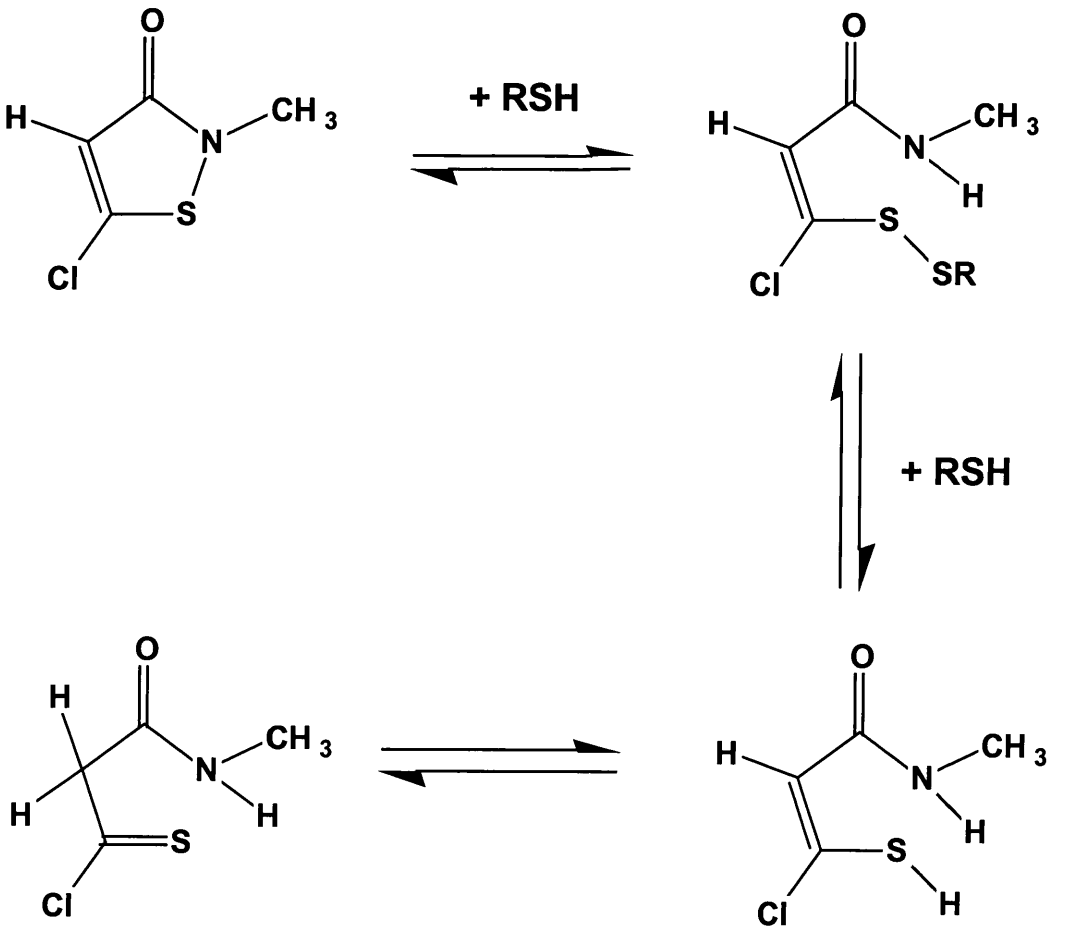


Figure 5: Thio-Acyl Chloride Tautomer Formation (after Collier *et al,* 1990b).

due to the disruption of the intracellular redox balance (Collier *et al,* 1991; Fuller *et al,* 1985). Alternatively, other thiol-containing agents may be the active sites of enzymes (Collier *et al,* 1990a). Thiol interactive agents are not commonly noted for biocidal properties, however, it has been demonstrated that CMIT shows fungicidal properties against *Schizosaccharomyces pombe* (Collier *et al,* 1990b; Figure 5). This observed difference in reactivity of the isothiazolone biocides can be related to the precise modes of action. The initial reaction for BIT, MIT and CMIT is to interact oxidatively with accessible thiol-groups forming mixed disulphide adjuncts (Collier *et al,* 1990a). Further interaction with thiols results in the formation and subsequent release of oxidised thiol dimers (e.g. glutathione disulphide) and reduced open-ring forms of the isothiazolones (e.g. mercaptoacrylamide; Collier *et al,* 1991). The mercaptoacrylamides can react further with the biocides resulting in the formation of biocide dimers (Collier *et al,* 1990b). The CMIT mercaptoacrylamide tautomerises producing a highly reactive thio-acyl (TAC) which has the ability to rapidly react with thiol-groups, amines and water (Collier *et al,* 1990b). This property is thought to account for the excellent biocidal properties of CMIT. However, it is not clear why this biocidal activity is limited to fungal cells and not demonstrated towards bacteria. A possible explanation may be physiological differences in eukaryotic and prokaryotic cells, and the permeability of the biocides into the cells (Collier *et al,* 1990b). The major interactive sites are within the cytosol, therefore, entry into the cell may be limited, particularly in Gram negative bacteria (Collier *et al,* 1990b). Although CMIT is not bactericidal it exhibits greater reactivity to bacterial cells than either BIT or MIT (Collier *et al,* 1990a). No specific binding proteins for any of the biocides have been observed (Collier *et al,* 1990b). However, differences occur when chlorinated and non-chlorinated compounds bind to the cell envelopes and cytosol (Collier *et al,* 1990b). Therefore, non-thiol interactions may be occurring for CMIT which will have greater

consequences for bacterial cells than fungal cells because in the former, many essential functions are membrane bound (Collier *et al,* 1990b). However, upon removal of LPS following treatment with EDTA, Kathon™ was observed to be bactericidal towards *P. aeruginosa* (Brözel & Cloete, 1994). A possible explanation for this is that entry into the cell *via* the OMPs is a rate limiting step. Therefore, removal of the LPS, which would increase the entry rate, permits a higher concentration of biocide into the cell and hence, results in bactericidal activity (Brözel & Cloete, 1994).

Upon exposure of *Legionella pneumophila* to Kathon™ CG (the active ingredient being CMIT) bactericidal activity was observed towards iron-restricted cells. Microorganisms have been observed to exhibit different phenotypes when grown in iron-deprived and iron-sufficient conditions (Barker *et al,* 1992; Brown & Williams, 1985; Domenico *et al,* 1996; Pettersson *et al,* 1997). The phenotypes expressed during iron-deprived growth were found to differ significantly in their susceptibility to chemical inactivation (Barker *et al,* 1992).

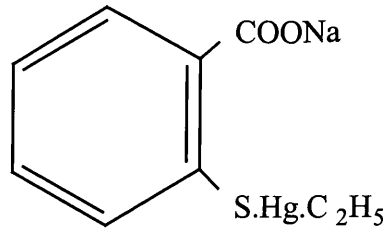
Other Thiol-Interactive Agents

The primary mode of action of the isothiazolone is to interact oxidatively with accessible thiol groups. Bronopol bronidox and thiomersal are also thiol-interactive agents. Bronopol (Figure 7; 2-bromo-2-nitro-propan-1,3-diol), demonstrates a broad spectrum of antibacterial action (Waigh & Gilbert,1991). It is widely used as a preservative in pharmaceutical and cosmetic products at concentrations of up to 0.1% (Waigh & Gilbert, 1991; Shepherd *et al,* 1985), but only demonstrates selective action against *P. aeruginosa.* (Collier, 1997). The mechanism of action of bronopol is to interact with essential thiol groups within the cell, resulting in oxidation through a radical anion intermediate. In the presence of bronopol cysteine is rapidly converted to cystine (Shepherd *et al,* 1987).

Bronopol demonstrates significant bactericidal activity, which cannot be explained solely by the oxidation of thiol groups, but is the result of by-product formation of superoxide and peroxide during the thiol-oxidation process (Waigh & Gilbert, 1991). During aerobic conditions bronopol catalyses the oxidation of thiol-groups to their disulphide adjuncts, this process continues in the presence of oxygen and bronopol. At this point bacteriostatic action by the biocide will be demonstrated, with the production of peroxide and superoxide by-products. It is the by-products, which are directly responsible for the bactericidal action demonstrated by bronopol (Waigh & Gilbert, 1991).

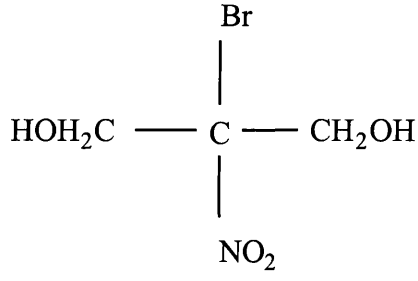
Bronidox (5-Bromo-5-nitro-1,3-dioxane; Figure 6) has a similar broad spectrum of antimicrobial activity to bronopol, however, it is considered to have better stability in basic media (Ghannoum *et al,* 1986). It is used as a preservative to prevent microbial spoilage in cosmetics and toiletries (Ghannoum *et al,* 1986). The mode of action of bronidox is similar to the structurally related nitro compound bronopol, that is the oxidation of essential protein thiols (Ghannoum *et al,* 1986).

Thiomersal (merthiolate; sodium-o-(ethylmercurithio)-benzoate; Figure 6) was originally used as a skin disinfectant, but is now more commonly used as a fungicidal and preservative agent for biological products in the range of 0.01 to 0.02% (Russell 1992b). Thiomersal oxidatively reacts with thiol-groups demonstrating bacteriostatic and bactericidal action (Waigh & Gilbert, 1991).



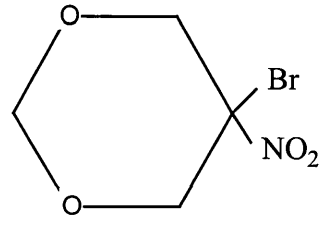
Thiomersal

(merthiolate; sodium-o-(ethylmercurithio)-benzoate)



Bronopol

(2-bromo-2-nitropropan-1,3-diol)



Bronidox

(5-Bromo-5-nitro-1,3-dioxane)

Figure 6: The Structure of some Thiol-Interactive Biocides.

Resistance of *Pseudomonas aeruginosa* to Isothiazolone as Observed by Brözel and Cloete

Brözel and Cloete (1994) exposed *P. aeruginosa* PAO1 to Kathon™ (a mixture of 1.15% CMIT and 0.35% MIT). They observed a gradual increase in minimum inhibitory concentrations of biocide to increasing concentrations of Kathon™, and concluded this was due to an adaptive or phenotypic mechanism.

The minimum inhibitory concentration (MIC) of Kathon™ was determined for wild type *P. aeruginosa*. *P. aeruginosa* was cultured in the presence of a quarter the concentration of the previously determined MIC. The MIC value was redetermined following each growth period and the bacteria was then re-exposed to a quarter of the redetermined MIC. This procedure was repeated until 10 subsequent passages in the presence of biocide had been achieved, at which point the cells were deemed to be 'resistant' to the biocide. A gradual increase in the MIC from an initial value of 300μL L^{-1} to 607μL L^{-1} was observed in three cultures tested in parallel over a 15 day exposure period. The resistant cells were then cultured for three consecutive passages in the absence of Kathon™ and the development of resistance was observed to follow the same pattern. This was thought to be indicative of a phenotypic adaptation. A phenotypic adaptation is an alteration which demonstrates a change in response to environmental stimuli, but does not necessarily require a genome alteration.

SDS-PAGE analysis of wild-type or sensitive and resistant cells of *P. aeruginosa* revealed two differences, indicating an adaptive resistance mechanism to the Kathon™ biocide.

(1) Sensitive cells of *P. aeruginosa* exposed to 300μL L-1 of biocide for 24h produced copious amounts of extracellular polysaccharide (EPS). A 54 kDa OMP was also

detected and correlated with the EPS production (Grabert *et al,* 1990). The production of EPS was not observed in sensitive (non-exposed) and resistant cells and was therefore, thought to be an initial stress response which is not maintained following extended exposure to the biocide. The 54 kDa protein was only present in reduced levels. The induced culture (first exposure cells) exhibited a wider stress response producing minor OMPs, which were not detected in sensitive, resistant or non-exposed resistant cells. Hence, these minor proteins were not thought to contribute to the improved resistance.

(2) A 35 kDa OMP detected in sensitive cells was not present in resistant or induced cultures. This was called the T-OMP (sometimes referred to as Opr T, OMP T or protein T). The protein was also undetected in resistant cells cultured for three times in the absence of biocide. This indicates the resistance mechanism is a more permanent mechanism or mutation.

Brözel and Cloete (1994) concluded that the isothiazolone biocides entered into the bacterial cell via T-OMP because removal of the LPS rendered the resistant cells as susceptible to the biocide as sensitive cells and that the resistance was acquired by an adaptive mechanism where T-OMP is suppressed.

Aims

The aims of this study are to investigate the induction of resistance in passaged cells of *Pseudomonas aeruginosa* PAO1, towards the isothiazolone biocides (BIT, MIT & CMIT) and the thiol-interactive agent thiomersal. The examination of the growth characteristics of *P. aeruginosa* PAO1, and establishment of the most suitable method of minimum inhibitory concentration (MIC) determination must be achieved in order to quantify the progressive development of resistance. A suitable method must be determined in order to induce resistance towards the three isothiazolones and thiol-interactive agent thiomersal, whilst maintaining sufficient cell numbers and adopting a quantifiable method to estimate the MIC of the resistant cultures. The resistant and control passages of cells will then be observed for a comparison of their outer membrane protein (OMP) profiles *via* sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis. This will be undertaken with the aim of identifying OMP profile changes with particular reference to the presence or absence of T-OMP. The elimination of other possible physiological alterations in the cell components, which may account for any observed resistance towards the four test biocides, will be undertaken. This may include investigations into the lipopolysaccharide (LPS) component of the cell and possible alterations in the cell surface hydrophobicity between observed mucoid and non-mucoid forms of the resistant cultures.

Chapter Two

General Experimental Methods

Protocols for Bacterial Growth and Maintenance of Cultures

Maintenance and Growth of Cultures

A culture of *Pseudomonas aeruginosa* PAO1 NCIMB 10548 was obtained from the National Collections 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 from 1 to 4. Slope number 1 was used to inoculate overnight cultures for experimental purposes and slope number two was the backup slope in case of contamination. Slope number three was used for further subculturing and slope number four was kept as part of a stock culture collection. The maintenance of cultures in quadruplicate ensured that the stock bacterial cultures were not ruined by a single occurrence of contamination. Inoculated agar slopes were incubated overnight at 37°C. After the incubation period, stock cultures were maintained at room temperature in a darkened cupboard. Culture maintenance was observed by subculturing onto fresh appropriate agar slopes at fortnightly intervals. Liquid cultures were grown in R2A and CDM for 24h at 37°C in an orbital incubator (Gallenkamp, INA-305) at 200rpm. Culture identification was determined using the API 20 NE system (Biomerieux, France). The cultures were stored on slopes at room temperature in a dark cupboard.

Reagents

Sigma, Poole, Dorset: trizma hydrochloride; ethylenediaminetetra-acetic acid; pyruvic acid; tetraethylammoniumchloride; dithiothreitol; glutathione;5,5-dithio-bis (2 nitrobenzoic acid); casein; N-laurylsarcosine; protease peptone number 3; sodium carbonate; sodium thiosulphate; soluble starch; TEMED; TRIZMA base; yeast extract, thiomersal.

BDH Chemicals, Poole, Dorset: Disodium hydrogen phosphate (Na_2HPO_4); potassium dihydrogen phosphate (KH_2PO_4); dipotassium orthophosphate (K_2HPO_4); ferric ammonium citrate; ammonium chloride (NH_4Cl); glycerol; ethanol; sodium chloride (NaCl); D-glucose; sodium dithionite; calcium chloride ($CaCl_2$); magnesium sulphate heptahydrate ($MgSO_4.7H_2O$); hydrochloric acid (HCl); sodium hydroxide (NaOH); sodium nitrate ($NaNO_3$).

The isothiazolone (MIT and CMIT) biocides were kind gifts from Zeneca Specialities plc, (Biocides Research, Blackley, Manchester) and Nipa Ltd (BIT). Cetrimide was supplied by Rhone-Poulenc (Cheshire)

Sterilisation and Preparation of Media

R2A Medium

R2A medium was prepared according to protocol 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 required 1.5% technical agar (Oxoid number 3) was added as a gelling agent. The medium was sterilised by autoclaving at 121°C, 15psi for 15 minutes.

Chemically Defined Medium (CDM)

CDM was prepared according to Dinning (1995), replacing 0.5% w/v succinic acid with glycerol. Solution A was prepared by dissolving K_2HPO_4 (2.56 g), KH_2PO_4 (2.08 g) and NH_4Cl (1.00 g) in order, in 900 mL of deionised water. The pH was adjusted to pH 6.8 with the addition of either 0.1M HCl or 0.1M NaOH, the volume made up to 1 L with deionised water. The solution was sterilised by autoclaving at 121°C, 15psi for 15 minutes. Solution B was prepared by dissolving ferric ammonium citrate (1.0 g) and $CaCl_2$ (0.1 g) in 100mL of deionised water. This was sterilised by filtration through a 0.22μm pore size cellulose acetate (Whatman, England) filter under vacuum. Solution C, a 1M glycerol solution was prepared by dissolving glycerol (46.45 g) in 400 mL of deionised 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 500 mL with deionised water. Solution C was sterilised by autoclaving at 121°C, 15 psi for 15 minutes. Solution D was prepared by dissolving $MgSO_4.7H_2O$ (0.5 g) in 900 mL deionised water. The total volume was made up to 1 L using deionised water. Solution D was sterilised by autoclaving at 121°C, 15 psi for 15 minutes. The CDM was completed with the aseptic addition of 5 mL of solution B, 15 mL of solution C and 10 mL of solution D to 1 L of solution A.

Preliminary Identification Techniques

Gram Reaction

The Gram stain was used to distinguish between Gram positive and Gram negative bacterial cells. Bacterial cells were placed onto a glass slide and made into a suspension with distilled water, allowed to air dry and then gently heat-fixed. Crystal violet solution was added to the slide for 1 minute. The crystal violet was decanted from the slide and iodine solution was added to the slide for 1 minute. A 1:1 (v/v) solution of acetone / alcohol was used to decolouirse the slide and immediately washed off with water. The final step was to counterstain with safranin solution for 10 minutes. The cells staining

purple were classified as Gram positive cells and the cells staining pink were classified as Gram negative cells (Brock & Madigan, 1991).

Motility Test

An aliquot (5 mL) of nutrient broth (Oxoid, England) containing 0.5% technical agar (Oxoid number 3) were added to a bijou bottle to produce sloppy agar. A piece of glass tube (5 mm diameter) extending above the level of the broth / agar solution was added to bottle. The bottles were autoclave at 121°C for 15 minutes. The sloppy agar was inoculated with the test bacteria by inoculating the bacteria inside the glass tube. If bacterial growth was observed on the surface of the agar outside the tube, this was evidence of passage through the sloppy agar and it was presumed that the bacteria were motile.

Catalase Test

A sample of the test bacteria was place in a clean petri-dish. A drop of hydrogen peroxide (100 volume) was added to the test bacteria. If bubbles were observed immediately it was assumed that the bacteria possessed the enzyme catalase, which converts hydrogen peroxide to water and oxygen.

Oxidase Test

The test bacteria were placed on a clean filter paper using a wooden stick. A drop of the oxidase reagent (BioMéreux, France) was added to the test bacteria. If a black coloration occurred within 10 seconds it was presumed that the bacteria were oxidase positive.

Preparation of Cell-Free Extracts

The bacterial cells were fractionated using a homogeniser 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 (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 the absorbance (E_{260nm}).

Determination of Homogenising Time

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

Preparation of Cells

An aliquot (5 mL) of overnight culture of *P. aeruginosa* ($1x10^9$ CFU mL^{-1}) grown in R2A medium was centrifuged at 5000g (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 (2 mL). Ht was determined by homogenising 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 10 second intervals up to 1 minute, and every minute up to 5 minutes, until the optical density readings ceased increasing. The Ht determined for *P. aeruginosa* was 2.5 minutes, this was when the E_{260nm} ceased increasing.

Bicinchoninic Acid (BCA) Assay for Protein Quantification

The bicinchoninic acid (BCA) assay was originally described by Smith *et al,* (1985). It is dependent on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions and is therefore, similar to the traditionally used Lowry method (Lowry *et al,* 1951). However, BCA is stable under alkaline conditions and therefore, the assay can be performed in a one-step process (Walker, 1984a). The assay is also considered to be more tolerant to a range of detergents and denaturing agents that are known to interfere with the Lowry assay, although it is more sensitive to reducing sugars (Walker, 1984b).

Bicinchoninic acid, sodium salt (BCA-Na) reacts with cuprous ion (Cu^{+}) in alkaline conditions forming an intense purple complex, this purple coloration can be monitored at 562nm in order to determine the protein concentration, (Smith *et al,* 1985). A standard assay (detects in the range of 0.1 to 1.0 mg protein $mL^{-1}$and a microassay (0.5 to 10 μg protein mL^{-1}) have been described (Walker, 1984a).

Preparation of Solutions for the Standard Assay

A stock of reagent A was prepared, (BCA-Na, 1% (w/v); sodium carbonate, 2% (w/v); sodium tartrate, 0.16% (w/v); sodium hydroxide, 0.4% (w/v); sodium bicarbonate, 0.95% (w/v) dissolved in 50 mL of distilled water), the pH was adjusted to pH 11.25 using 1M NaOH. The stock of reagent B comprises of copper sulphate (4% w/v). Directly prior to protein estimation reagent C was prepared by adding 100 volumes of solution A to 2 volumes of solution B, ensuring an apple green coloration developed.

Preparation of Standard Curve

Aliquots were withdrawn from a 1mg mL^{-1} stock of Bovine serum albumin (BSA) and diluted with dH_2O to give a total volume of 50 μL. Final BSA concentrations ranged from 0 mg mL^{-1} to 1 mg mL^{-1} of protein. An aliquot 1 mL of reagent C was added to each tube and incubated at 37°C for 30 minutes. The absorbancy was measured at 562nm

(Novaspec II, LKB) and plotted against known protein estimations to produce a standard curve (Figure 7).

Analysis of Samples

Aliquots (5 μL or 10 μL) of the test samples were diluted to a total volume of 50 μL with dH_2O. An aliquot (1 mL) of reagent C was added and samples were incubated at 37°C for 30 minutes. The absorbancy was measured at 562nm and compared against the standard curve (Figure 7) in order to estimate protein concentration.

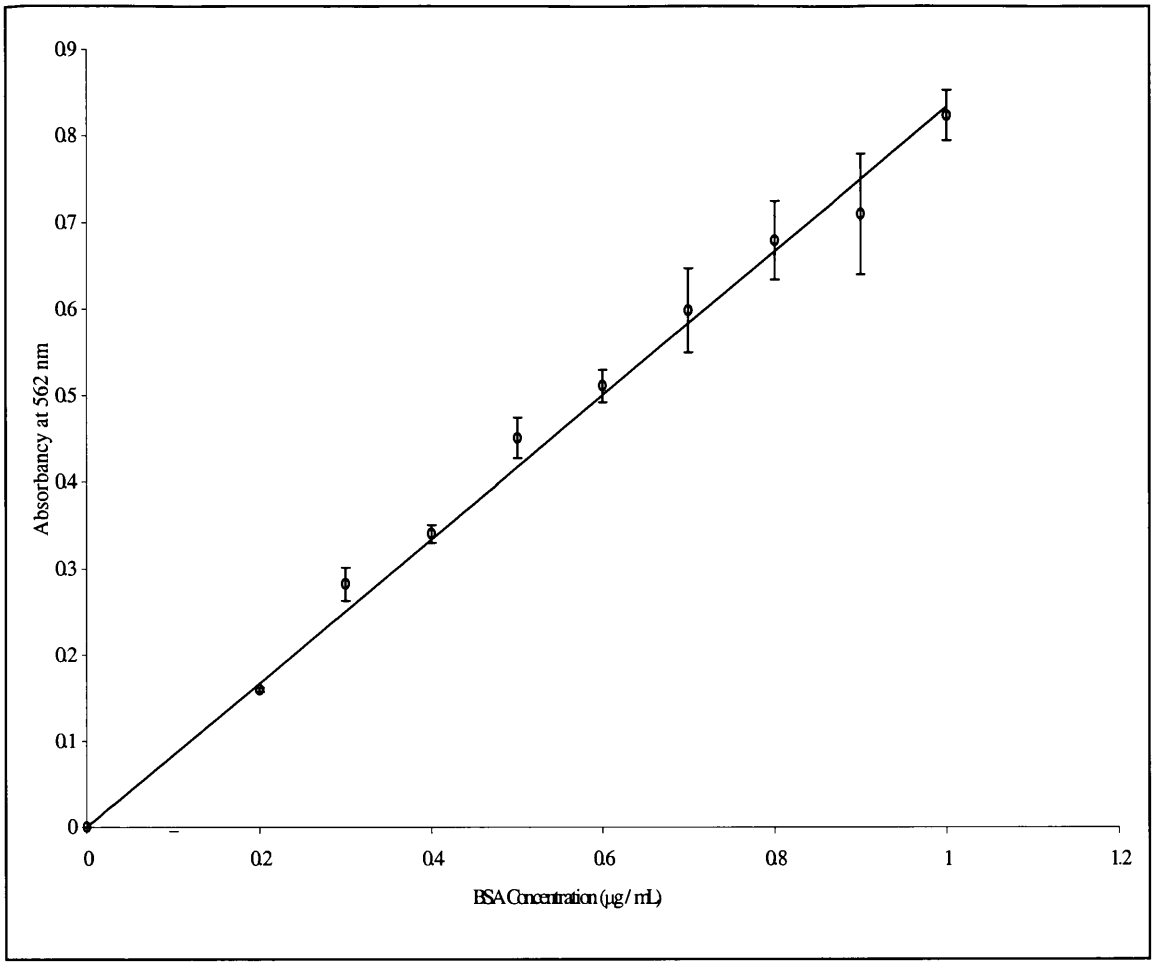


Figure 7: Standard curve for the estimation of protein (Bovine Serum Albumin (BSA)) concentration using the Bicinchoninic Acid (BCA) assay. Error bars are calculated as the standard deviation of the individual data points.

Protocols for the Preparation of SDS-Polyacrylamide Gels for Protein Separation

Preparation of Separating Gel Mixture

Stock acrylamide solution (10 mL; acrylamide, 73 g; bis-acrylamide, 2 g; dissolved in distilled water, 250 mL), distilled water, 12 mL; and stock separating buffer (7.5 mL; SDS, 1 g; "Tris" buffer, 45.5 g; made up to 250 mL with deionised water, pH 8.0) were added together and degassed. Stock ammonium persulphate solution (45 μL; 10% w/v) and N,N,N',N'-tetramethyl-ethylenediamine (TEMED; 15 μL) were added to begin the polymerisation process. The mixture was mixed gently to avoid re-gassing, and poured into a prepared gel cast (0.5mm thick: mini-PROTEAN II, Biorad). The gel was overlayed with water-saturated butan-2-ol, and left to set for approximately 1h.

Preparation of Stacking Gel Mixture

Stock acrylamide solution, 750 μL; distilled water, 3 mL; stock stacking gel buffer, (1.25 mL; SDS, 1 g; and "Tris" buffer, 15.1 g; made up to 250 mL with deionised water, pH 6.8) were mixed together and degassed. Stock ammonium persulphate solution (15 μL) and TEMED (5 μL) were added and the contents were gently mixed to avoid any re-gassing. The butan-2-ol overlay was removed from the polymerised 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 and the wells were washed with distilled water to remove any traces of buffer. The gel was transferred to a mini-PROTEAN II system, Biorad, the upper and lower buffer chambers were filled with reservoir buffer (glycine, 0.192M; "Tris", 0.025M; and SDS (0.1% w/w), ensuring the wells were filled with buffer.

Protocols for the Staining of Polyacrylamide Gels following the Separation of Proteins

Coomassie Brilliant Blue R250 Staining

The gels were immersed in coomassie brilliant blue R250 (0.25g in methanol, 125 mL; glacial acetic acid, 25 mL; water, 100 mL) and gently agitated using an orbital shaker (Stuart Scientific, UK) for between 2 and 12 hours. The gels were destained by immersing in destaining solution (methanol, 450 mL; glacial acetic acid, 100 mL; water, 450 mL) with gentle agitation using an orbital shaker (Stuart Scientific, UK) for between 3 and 4 hours or until the background colour had disappeared and a clear banding pattern was observed (Walker, 1984b)

Silver Staining

Silver staining can detect 0.1 to 1 ng of protein per band and it is therefore, a useful technique when the protein concentration is low. The gels were immersed in fixing solution (50% methanol, 100 mL; 12% glacial acetic acid, 24 mL; 37% formaldehyde, 100 μL; made up to 200 mL with distilled water) for a minimum of 60 minutes but may be left overnight. The gels were transferred to wash A (50% ethanol, 250 mL; made up to 500 mL with distilled water) for 20 minutes, this step was repeated twice. The gels were placed in pretreat solution (sodium thiosulphate.$5H_2O$, 0.04g; made up to 200 mL with distilled water) 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, 0.4g; 37% formaldehyde, 150 μL; made up to 200 mL with distilled water), and then washed twice in distilled water for 20 seconds. The gels were placed in develop solution (anhydrous sodium carbonate, 12g; 37% formaldehyde, 100 μL; sodium thiosulphate.5 H_2O, 0.0008g; made up to 200 mL with distilled water) for as long as required until the bands were clearly visible. The gels were washed twice in distilled water for 20 seconds and then transferred to stop solution (50% methanol, 100 mL; 12% glacial acetic acid, 24 mL; made up to 200 mL with distilled water) for 10 minutes. The gels were finally placed

in wash B (50% methanol, 250 mL; made up to 500 mL with distilled water) for 20 minutes. The gels may be stored in wash B for 3 to 4 weeks at 4°C (Walker, 1984c).

Protocols for the Staining of Lipopolysaccharide Gels

The lipopolysaccharide gels were stained according to Preston & Penner, 1987. The gels were immersed in fixing solution (ethanol, 400 mL; glacial acetic acid, 50 mL; made up to 1 L with distilled water). The gels were transferred to oxidising solution (periodic acid, 3.5 g; ethanol, 200 mL; glacial acetic acid, 25 mL; made up to 500 mL with distilled water) for five minutes and then washed for 15 minutes in distilled water, this was repeated twice. The gels were immersed in freshly prepared staining agent (concentrated NH_4OH, 2 mL; 0.1M NaOH, 28 mL; 20% w/v $AgNO_3$, 5 mL; distilled water, 115 mL) which was prepared by adding the NH_4OH to the NaOH, the $AgNO_3$ was added whilst stirring, the transient brown colour should rapidly disappear and then the water was added. The gels were agitated vigorously on an orbital shaker (Stuart Scientific, UK) for 10 minutes, and then twice washed in distilled water for 10 minutes. The gels were transferred to developer solution (citric acid, 0.05 g; 37% formaldehyde, 0.5 mL; made up to 1 L with distilled water) for 2 to 5 minutes. The developer solution was decanted from the gels at the first sign of discoloration and washed three times in distilled water. The gels were stored in distilled water at 4°C for up to one week.

Recovery of an Active Form of the 5-Chloro-N-Methylisothiazolone (CMIT)

The isothiazolone biocides readily react with thiol groups (Collier *et al,* 1990b). It was necessary to recover un-reacted CMIT from the sample provided. The reacted CMIT exhibited a peak at a wavelength of 260 nm, whilst un-reacted CMIT was observed at a peak of 280 nm (Figure 8). The reacted and un-reacted CMIT were separated using flash chromatography and the pure fractions identified using uv / vis spectrometry.

Preparation of Column

A loose slurry of silica gel (the bed length was approximately 50 mm) and petroleum ether (fraction) was prepared and gently poured down the side of a clamped chromatography column (length, 240 mm; diameter, 40 mm; porosity, 3 sinter). Any excess slurry was washed down the side of the column with petroleum ether. The column was rolled between the hands to remove any air bubble as these would result in the column cracking. Two pieces of filter paper were gently placed on top of the column. The petrol was pumped out of the column under pressure until the top filter was dry, the column was carefully pressed down using a glass rod, ensuring a level surface. This process was repeated until the excess petroleum ether was removed. A layer of toluene was overlayed to a depth of 1 mm and allowed to run through the column under pressure. This ensured the column ran evenly because tolulene appeared as a grey colour on the column.

Preparation of Sample

CMIT (1 g) was added to distilled water (20 mL) and the pH was adjusted to pH 7 using solid sodium bicarbonate. The solution was added to a separating funnel and 4 x 30 mL aliquots of chloroform were added. The separating funnel was shaken and vented three times after each addition of chloroform and the solution was allowed to settle into two layers. NaCl (approximately 0.5 g) was added and the solution was shaken and vented again and then allowed to settle into two layers. The two layers were run out of the separating funnel, the top layer from the top of the funnel and the bottom layer from the

bottom. The sample is contained in the solvent layer. Anhydrous magnesium sulphate was added to the solvent layer to dry off any excess water and then removed from the solution by filtration. The solvent layer was rotor-evaporated (Buchi, Switzerland) to produce a solid which was redissolved in 1 mL of chloroform.

Recovery of Active CMIT from sample

Active CMIT was recovered from the sample using flash-chromatography. The sample was added to the surface of the column using a Pasteur pipette and overlayered with toluene. The first elution fraction of chloroform and n-hexane (Table 1) was added to the column. The fractions were eluted from the column in approximately 5 mL aliquots under pressure. The remaining elution fractions were added to the column ensuring the column was not allowed to dry out. Ultra-violet visible spectrometer (Perkins Elmer, Model Lambda 2, England) studies using, were performed on all the fractions in order to establish which fraction contained the active form of the biocide.

Table 1 : Elution system used to extract the active form of the biocide CMIT from the sample (All fractions were in 50 mL aliquots).

Fraction	Chloroform (%)	n-hexane (%)
1	0	100
2	10	90
3	20	80
4	30	70
5	40	60
6	50	50
7	40	60
8	30	70
9	20	80
10	10	90
11	0	100

The fraction which contained the most reactive CMIT was fraction number eight (Figure 9).

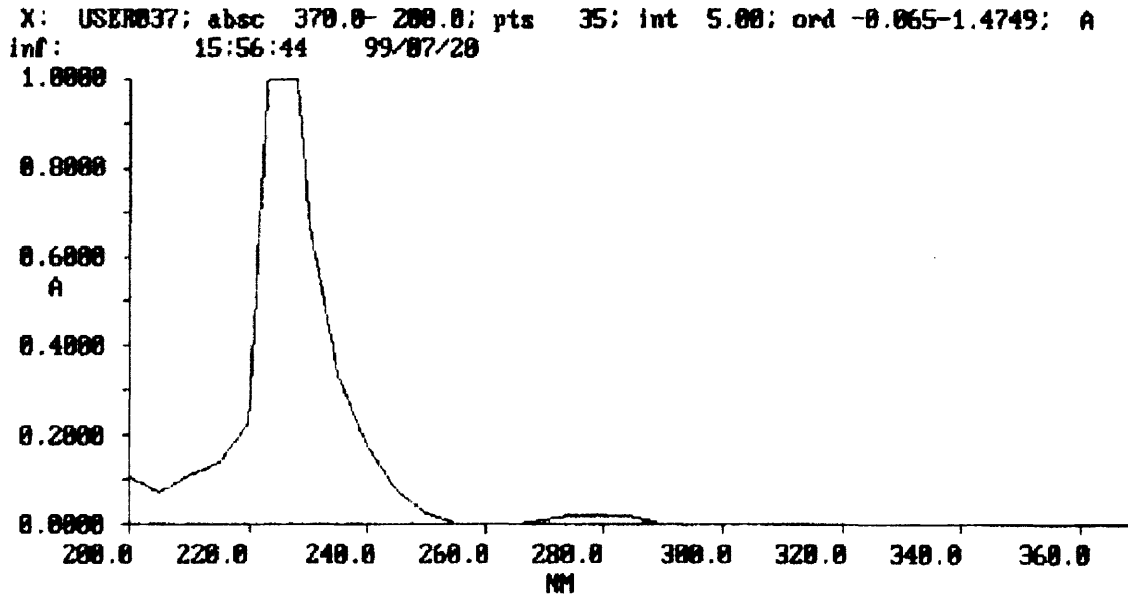


Figure 8: Profile of the reacted and unreacted forms of CMIT from the U/V vis spectrometer. The peak at 225 nm is the reacted form and the peak at 280 nm is the unreacted form.

Statistical Methods

All experiments in this project were designed to allow for statistical analysis and were performed in triplicate. Experimental data presented in this thesis represent the mean of those triplicate data sets. Where Standard Error bars are shown these were calculated *via* the methods of Jarrell (1994) Where Standard deviation. Error bars are shown these were calculated using the standard deviation of the data set.

Chapter Three

The Growth Inhibitory and Biocidal Activity of the Isothiazolone Biocides

The aim of *in vitro* antimicrobial susceptibility testing is to evaluate growth in the presence of various antimicrobial agents in order to determine the activity of such agents and determine if biocidal or biostatic activity is demonstrated. In order to assess the inhibitory activity of the isothiazolone biocides, the growth characteristics of *Pseudomonas aeruginosa* PAO1 and biocidal activity of the four biocides used in the investigation must be demonstrated. Initially the growth characteristics of *P. aeruginosa* in the appropriate media were evaluated, before exposing the bacteria to the biocides.

When studying the mechanism of antibacterial action a quantitative assessment of the activity of the biocides must be carried out under relevant growth conditions (Bloomfield, 1991). The biocidal agent is assessed according to static or biocidal activity; static action results in the prevention (biocidal activity) or inhibition (static activity) of growth and is reversible if the biocide is removed or neutralised, whereas biocidal action is a non-reversible and lethal action which kills the cell (Bloomfield, 1991). When examining a population of cells, it is difficult to determine whether the action of the biocide is biocidal or biostatic. This is because some cells may be dying whilst others are actively dividing (Bloomfield, 1991). In addition, the concentration of the biocide may in part dictate the action of the biocide, at high concentrations biocidal action may be demonstrated whilst at low concentrations the action may be biostatic (Bloomfield, 1991). The action of antibacterial agents may occur on a particular site or numerous sites within the cell. In order to effectively assess the action of the biocide it is important to understand the factors which may affect the activity of the agent in relation to biochemical effects (Bloomfield, 1991).

Traditional microbiologists use total and viable count methods in order to assess the growth of bacteria. These techniques are still used today, however, concern has arisen

as to the accuracy by which they assess bacterial growth, and in turn predict the effectiveness of an antimicrobial agent. Magee *et al,* (1997) postulated that data spanning the past 50 years indicated that the plate count method is an invalid predictor of the viable count. It was suggested that although data should be expressed in colony forming units per mL of overnight culture (CFU mL^{-1}), the dogma of one colony being equivalent to one single microorganism should be rejected. In addition estimations were inaccurate when high serial dilutions were required. The plate method is thought to have survived throughout this period due to its practical simplicity and inexpense, and not due to its scientific validity. Although it works relatively effectively in the clinical situation, whereby the cells are often estimated in terms of life or death. However, a new method is required in order to assess microbial numbers in relation to antimicrobial susceptibility.

The Growth Characteristics of Pseudomonas aeruginosa PAO1.

The growth of *P. aeruginosa* can be assessed by three different methods, these are total count, viable count and measures of turbidity (optical density). Bacteria grow by cell division (binary fission) and, therefore, growth can be assessed by the total number of bacteria present at one time (Fuchs & Kröger, 1999). This can be achieved by a direct count (total count). However, this method does not distinguish between living and dead cells. It is, therefore, an inappropriate method to use to assess the activity of an antimicrobial agent. A more useful method is to assess the viable count, whereby a sample is diluted in order to determine the number of colonies on an agar plate. This enables the colony forming units per mL of culture to be calculated (CFU mL^{-1}) for any given time. The size of the bacterial cell lies within the order of wavelength of visible light and can be measured using a spectrophotometer to measure the turbidity (Fuchs & Kröger, 1999). The intensity of light passing through a given culture is related to the absorbancy (Fuchs & Kröger, 1999). This technique is only accurate when the absorbancy at 470 nm is less than 0.5. Therefore, above this point the sample must be diluted (Lawrence & Maier, 1977).

The Growth of Pseudomonas aeruginosa PAO1

An aliquot (25 mL) of R2A medium was inoculated with a pure culture of *P. aeruginosa* PAO1 from a streak plate, in 100 mL erlenmeyer flask. The flask was incubated overnight at 37°C, 200 osc min^{-1} in an orbital incubator (Gallenkamp INA-305). A fresh aliquot of R2A medium was inoculated with 250 μL of an overnight culture of *P. aeruginosa*. This was performed in duplicate. The cultures were incubated at 37°C, 200 osc min^{-1} in an orbital incubator (Gallenkamp INA-305).

Assay for Growth

At hourly intervals an aliquot (1 mL) of culture was removed and the optical density was measured at 470nm using a spectrophotometer (Novaspec II LKB town). The sample was aseptically replaced in the respective flasks, so that the volume:surface area of the flask did not significantly alter and hence, the aeration rate remained constant throughout the duration of the experiment. If the optical density reading was greater than 0.5 at E_{470nm}, a 1 in 10 dilution of the culture was performed in sterile medium (Lawrence & Maier, 1977). This was not returned to the flask but discarded. The $E_{470\,nm}$ of this dilution was observed and the volume multiplied by 10 to give the actual accurate, corrected optical density.

Viable Count

An aliquot (100 μL) of culture was aseptically removed and serially diluted in the range of 1 x 10^{-2} to 1 x 10^{-10} CFU in 0.9% sterile saline. Aliquots (100 μL) of the dilutions were placed on R2A or CDM agar plates in duplicate and spread over the surface of the plate using a sterile glass spreader. The plates were incubated overnight at 37°C, colonies were counted and viable counts were calculated according to the formula (1).

(1) $$CFU\ mL^{-1} = N(1/DF)$$

Figures 9 and 10 show the growth curves for *P. aeruginosa* obtained in R2A and CDM media respectively. In R2A medium the lag phase was short, lasting only 2 hours, before cells entered the exponential phase. The exponential phase lasted from the second hour to the ninth hour, therefore mid-exponential phase was at 5.5 hour after inoculation. The doubling time of *P. aeruginosa* in R2A medium was calculated to be 48 minutes.

Growth in the CDM media was considerably slower. The cells continued in lag phase up to four hours. Exponential phase lasted from between the fourth and twelve hours, therefore, mid-exponential phase was not observed until the eighth hour after inoculation. The doubling time was considerably longer in CDM medium at 132 minutes. The bacterial growth is considerably slower in CDM because the supply of nutrients is lower than in the nutrient-enriched R2A medium.

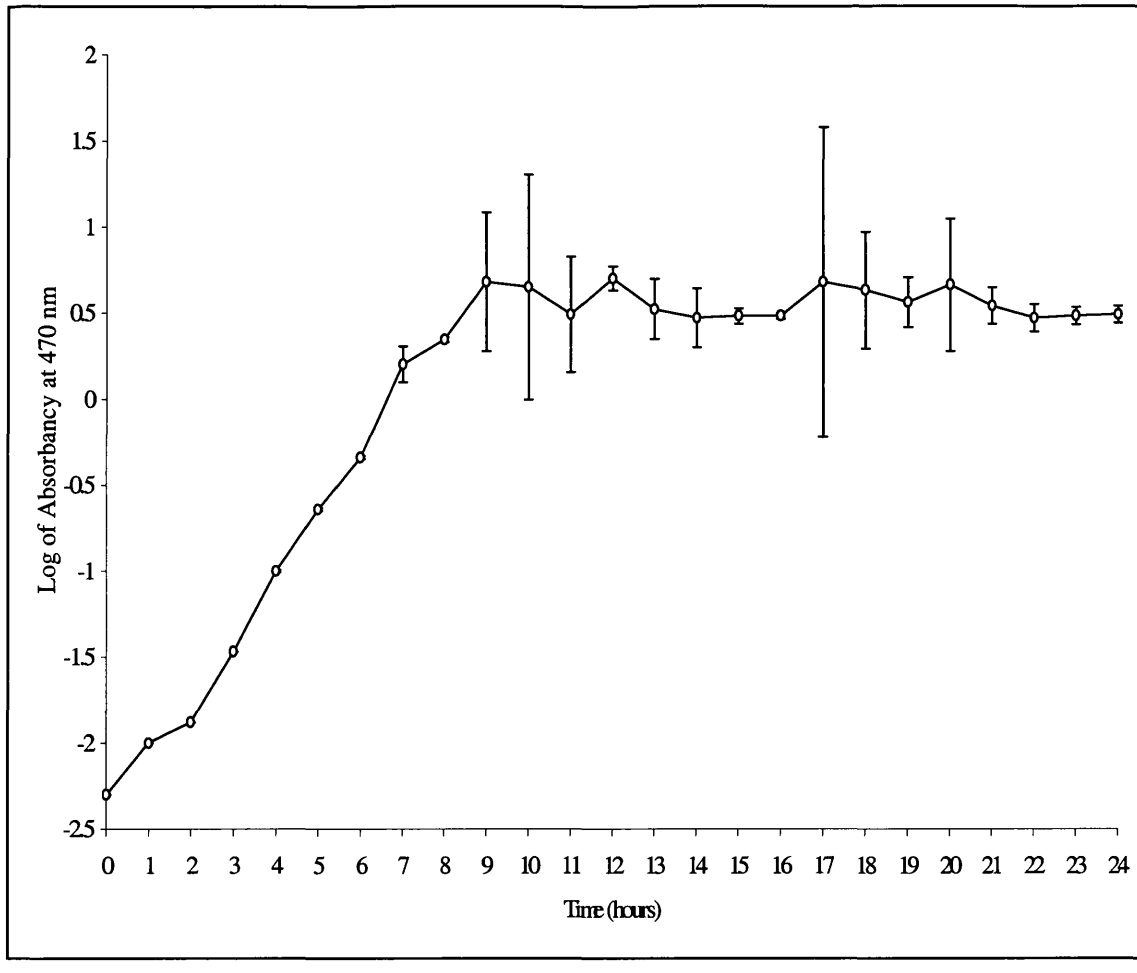


Figure 9: The $\log_{10}$ of the increase in absorbancy (470_{nm}) of *Pseudomonas aeruginosa* PAO1 in R2A medium with time. Error bars are calculated as the standard deviation of the individual data points.

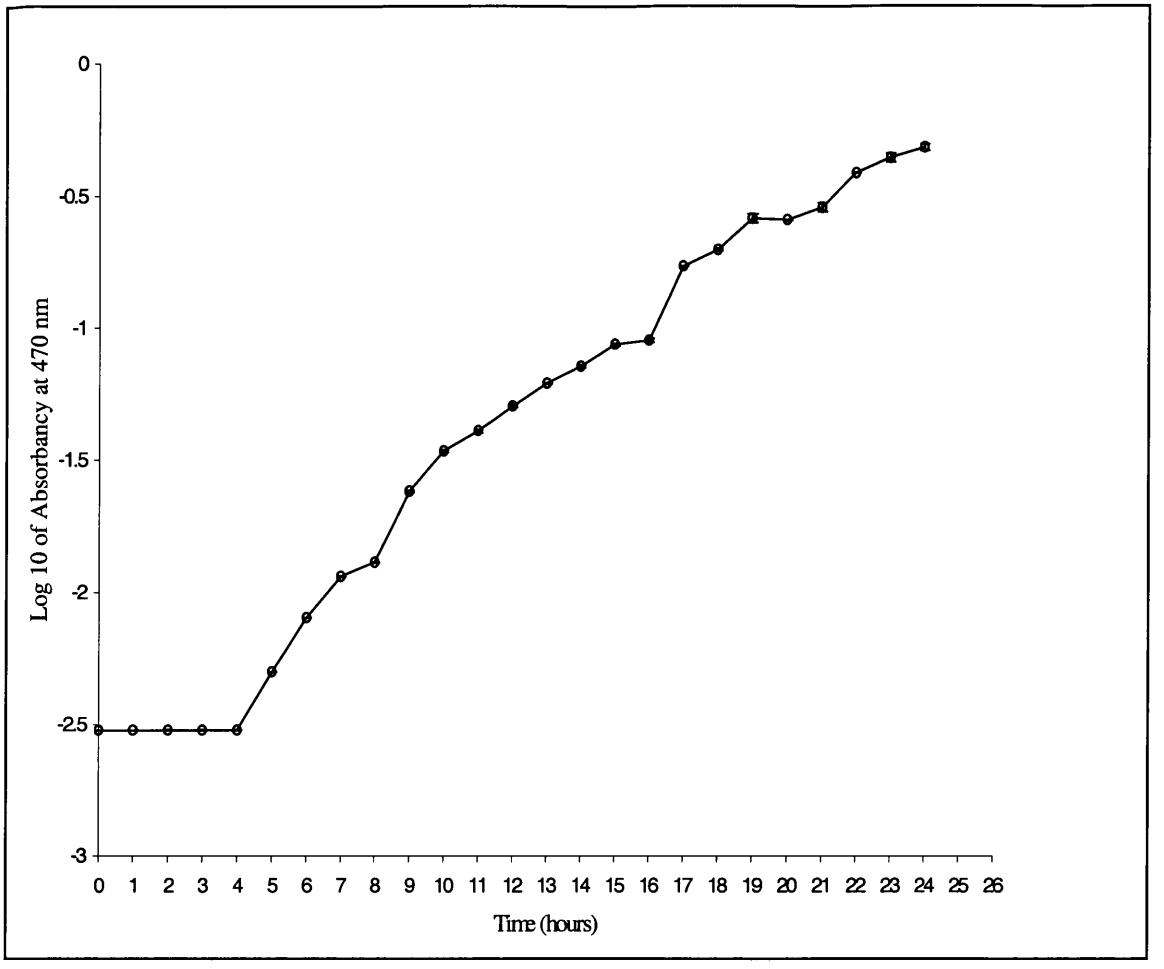


Figure 10: The $\log_{10}$ of the increase in absorbancy (470_{nm}) of *Pseudomonas aeruginosa* PAO1 in CDM with time. Error bars are calculated as the standard deviation of the individual data points.

Assessment of Antimicrobial Activity of the Isothiazolone Biocides and Thiomersal

A quantitative and reproducible method was required in order to assess the antibacterial action of the isothiazolone biocides. The general principle in determining the minimum inhibitory concentration (MIC) of biocide required to inhibit bacterial growth, is to introduce the organism into varying concentrations of the antimicrobial agent with optimum nutrients and growth conditions. However, this may promote criticism because in the natural environment the bacteria may not have a plentiful supply of nutrients available. It is thought that nutrients are limited in the natural environment and, therefore, the traditional media used in the culture of bacteria in order to maximise their growth does not provide realistic conditions. The artificial conditions created by commercial media contains a greater supply of nutrients than found in the natural environment. Following an incubation period, generally between 18 to 24h, the culture is visually examined in order to determine the concentration of biocide at which growth is inhibited (Bloomfield, 1991). One important point to note during the estimation of the MIC is the nature of the inoculum. The MIC will vary if the inoculum size is not standardised between experiments and, therefore, it is advisable to use a standard inoculum size. The preparation of the cells in order to achieve a standard inoculum may also affect the MIC determined. Washed cells are routinely prepared by successive centrifugation and resuspension in order to achieve a reproducible cell density. However, when subjected to centrifugation forces, a significant reduction in the viability of logarithmic phase *P. aeruginosa* has been observed (Gilbert *et al,* 1995). The subsequent growth of sub-lethally injured cells is significantly reduced when the cells are exposed to environmental conditions (Gilbert *et al,* 1995), for example; sub-MIC of antimicrobial agents. In order that the MIC determination is accurate and reproducible cultures should be used immediately and not stored for periods of time in a refrigerator (Gilbert *et al,* 1995). Two techniques were utilised in order to determine the most suitable method of MIC determination. These were the tube dilution method and the growth rate method.

Tube Dilution Method

The tube dilution or serial dilution method according to Bloomfield (1991), provides a quantitative assessment of active concentrations of antimicrobials. However, it must be remembered that the actual MIC value lies between the observed maximum inhibitory concentration and the minimum inhibitory concentration (Bloomfield, 1991). Initially a screening for the MIC point is performed, this involves either a 10-fold increase or a doubling of antimicrobial concentration (Bloomfield, 1991). Once the approximate value is determined an arithmetical series of not less than eight dilutions is employed, at which point the choice of dilutions takes into account the actual concentrations in use (Bloomfield, 1991).

Determination of MIC

Aliquots (9 mL) of media (R2A or CDM) were prepared and doubling concentrations of biocide were added in 1 mL aliquots, (Table 2) in order to determine the initial screening point for the MIC. Tubes were inoculated with 100 μL of a 19h culture of *P. aeruginosa* PAO1 grown at 37°C, 200 osc min^{-1} and vortexed (Rotamixer, Hook and Tucker). The tubes were incubated at 37°C and observed for growth at 19h and 48h. Once the initial screening point had been determined the procedure was repeated in order to establish the exact MIC. In determining the exact MIC the dilutions ranged between the two concentrations where growth was visible and absent (Table 3).

Table 2: Observation of growth in dilution tubes of *Pseudomonas aeruginosa* PAO1 in R2A medium in order to determine the screening MIC of BIT.

Biocide concentration ($\mu g\ mL^{-1}$)	Growth	
	19h	48h
0	+	+
1	+	+
2	+	+
4	+	+
8	+	+
16	+	+
32	+	+
64	-	-
128	-	-
256	-	-

+ = Presence of Growth

- = Absence of Growth

Table 3: Observation of growth in dilution tubes of *Pseudomonas aeruginosa* PAO1 in R2A medium in order to determine the exact MIC of BIT.

Biocide concentration ($\mu g\ mL^{-1}$)	Growth	
	19h	48h
0	+	+
30	+	+
35	+	+
40	+	+
45	+	+
50	+	+
55	-	-
60	-	-
65	-	-

+ = Presence of growth

- = Absence of growth

Table 4: Summary of MIC results obtained in R2A medium.

Biocide	Screening MIC ($\mu g/mL^{-1}$)		Exact MIC ($\mu g/mL^{-1}$)	
	19h	48h	19h	48h
BIT	64	64	60	60
MIT	32	32	20	20
CMIT	2	2	1.3	1.3
Thiomersal	16	16	9.5	9.5

Table 5: Summary of MIC results obtained in CDM medium.

Biocide	Screening MIC ($\mu g/mL^{-1}$)		Exact MIC ($\mu g/mL^{-1}$)	
	19h	48h	19h	48h
BIT	8	8	5.8	5.8
MIT	4	4	2.4	2.4
CMIT	1	1	0.25	0.25
Thiomersal	nd	nd	nd	nd

nd = not done

The MIC values determined by the tube dilution method vary considerably between the two different types of media. This is due to quenching of the biocides by the presence of accessible thiol groups in R2A medium. The mode of action of the isothiazolone biocides and thiomersal is to react oxidatively with accessible thiol groups. Hence, the presence of thiol groups in the R2A medium will result in a greater MIC of biocide to inhibit the growth of the bacteria (Fuller *et al,* 1985; Collier *et al,* 1990b; Chapter 2: Ellmans assay).

Growth Rate Method

Cultures of *P. aeruginosa* were grown in the presence of varying concentrations of biocide. An aliquot (250 μL) of *P. aeruginosa* was inoculated into R2A media. Cultures were incubated at 37°C for 8h in an orbital incubator (Gallenkamp INA-305) at 200 osc min^{-1}. After 1h varying concentrations of biocide were added to the flasks in 1 mL aliquots. The optical density was measured at hourly intervals using a spectrophotometer (Novaspec II LKB, Surrey) optical density was plotted against time (Figure 11). The growth rate was calculated as a percentage of the control slope and plotted against biocide concentration. The curve was extrapolated down to the x-axis in order to determine the MIC (Figure 12).

An example of the growth rate curve and gradient curve is shown in Figures 11 and 12. The results are summarised in Table 6.

Table 6: Minimum inhibitory concentrations obtained by the growth rate method in different media for *Pseudomonas aeruginosa* PAO1.

Biocide	MIC ($\mu g\ mL^{-1}$)	
	R2A	CDM
BIT	44	nd
MIT	39.5	nd
CMIT	2.0	nd
Thiomersal	nd	nd

nd = not done

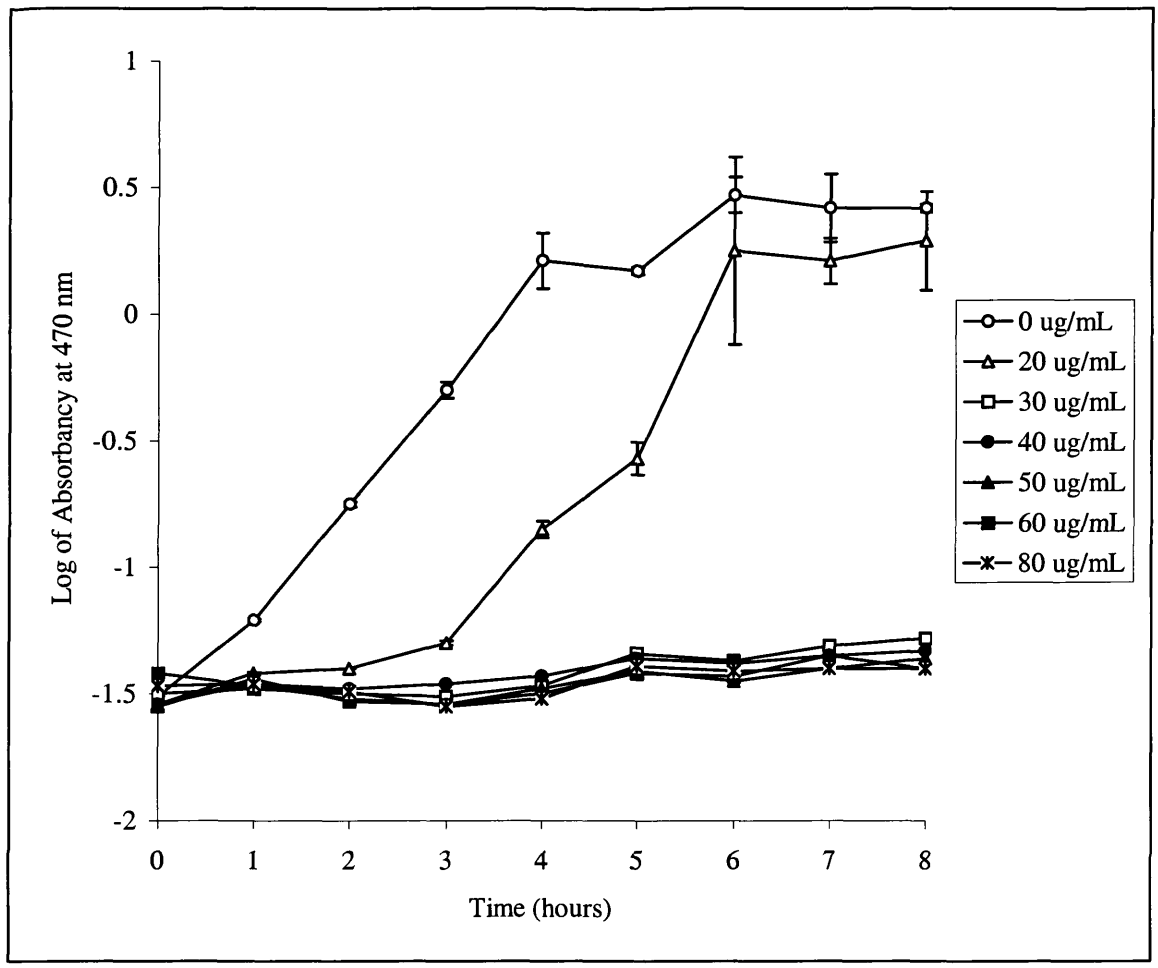


Figure 11: The effects of varying concentrations of BIT upon the rate of growth of *Pseudomonas aeruginosa* PAO1, when added to early logarithmic phase cultures in R2A media.

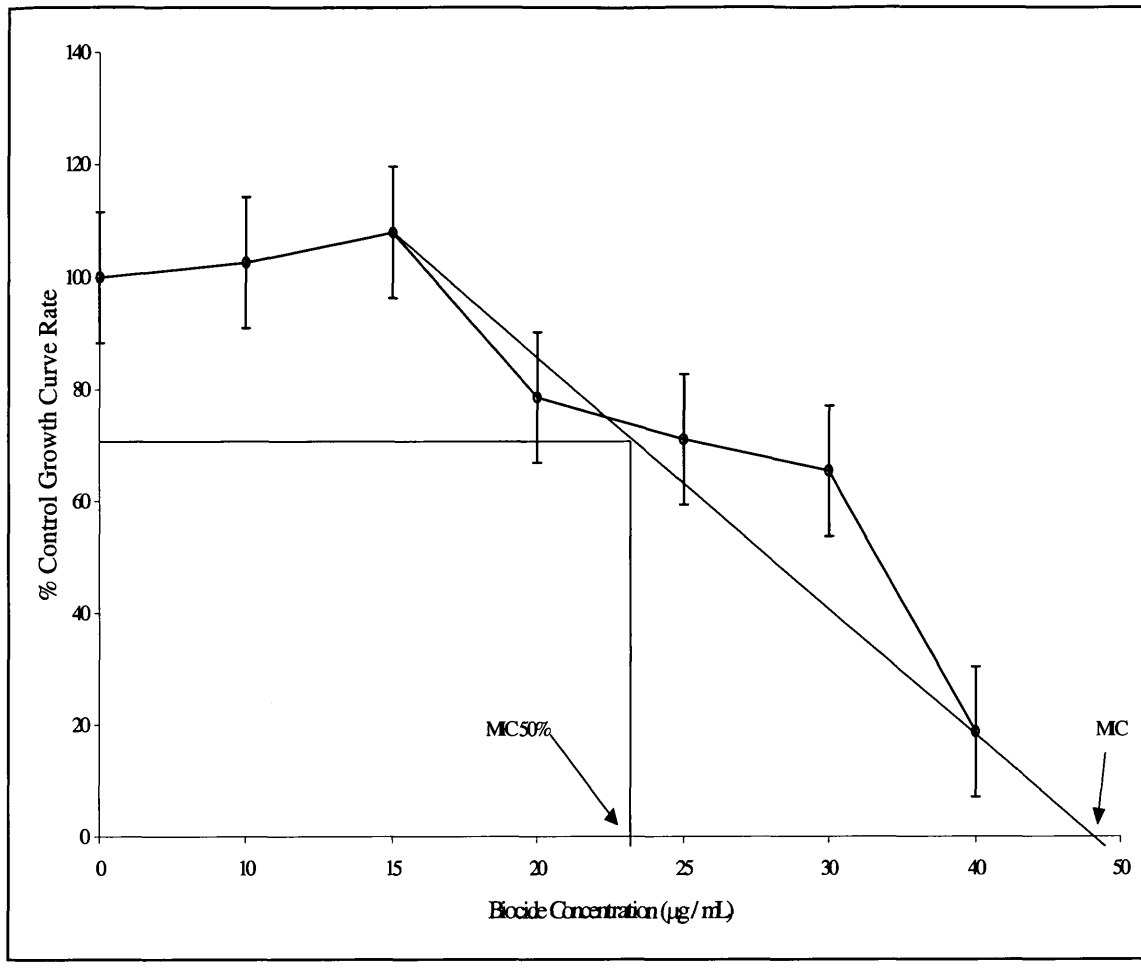


Figure 12: The Gradient Slope of *Pseudomonas aeruginosa* PAO1 calculated as a percentage of the growth rate when exposed to varying concentrations of BIT cultured in R2A media (MIC 50 %: the concentration of biocide giving 50% inhibition of the control growth rate; MIC: predicted MIC for the growth rate method).

It was not possible to obtain MIC results for the growth rate method in CDM medium. This was due to the poor growth rate during the eight hours growth period throughout the experiment. It was decided that it would not be accurate to extend the growth period because the isothiazolone biocides react with accessible thiols as soon as they are solubilised and, therefore, a true MIC value would not be obtained. The MIC value for the growth rate method can be calculated by two methods. The MIC value can be read directly from the interception point on the x-axis, this gives a value of 49 μg mL^{-1}(Figure 12). Alternatively the MIC 50% (the concentration of biocide giving 50% inhibition of the culture growth rate) can be determined and then doubled to give an estimated MIC (83 μg mL^{-1}). In comparison to the tube dilution method (60 μg mL^{-1}) the first method gives a closer estimation of MIC.

Table 7: Comparison of MIC values determined for the tube dilution and growth rate methods.

Biocide	Media	MIC Determination ($\mu g\ mL^{-1}$)	
		Tube Dilution	Growth Rate
BIT	R2A	60	49
MIT	R2A	20	39.5
CMIT	R2A	1.3	2.0
Thiomersal	R2A	9.5	nd
BIT	CDM	5.8	nd
MIT	CDM	2.4	nd
CMIT	CDM	0.25	nd
Thiomersal	CDM	0.8	nd

nd = not done

Ellman's Assay for the presence of Thiol Groups

The mode of action of the isothiazolone biocides is to react oxidatively with thiol groups, therefore the presence of thiols in media quenches the activity of the biocides. The Ellman's assay (Ellman, 1964) can be performed in order to estimate the concentration of thiols in the medium and hence, determine the magnitude by which the biocide activity may be quenched.

Ellman's reagent (5,5-dithiobis(2-nitrobenzoic acid) or DTNB) reacts with thiol containing agents as detailed below (Figure 13). The reaction results in a yellow coloration, which can be assayed at E_{412nm}. The assay determines the cysteine equivalency of the reading that is proportional to the total thiol content. A standard curve is constructed with known concentrations of cysteine standard and the test samples are compared against these standards.

Preparation of Reagent

Tubes were prepared as outline in Table 8 adding sodium phosphate buffer (0.1M NaH_2PO_4 (5.5mL) and 0.1M Na_2HPO_4 (94.5 mL), pH 8.0), distilled water and cysteine stock (0.05 g L^{-1}) or test sample (table 8). The stock DTNB (30.8 mg in 100 mL deionised water) was the final component to be added. The tubes were vortexed (Rotamix, Hook & Tucker) and incubated at room temperature for 15 minutes and the optical density (E_{412nm}) was determined. Tube 11 (Table 8) was used as a reagent blank.

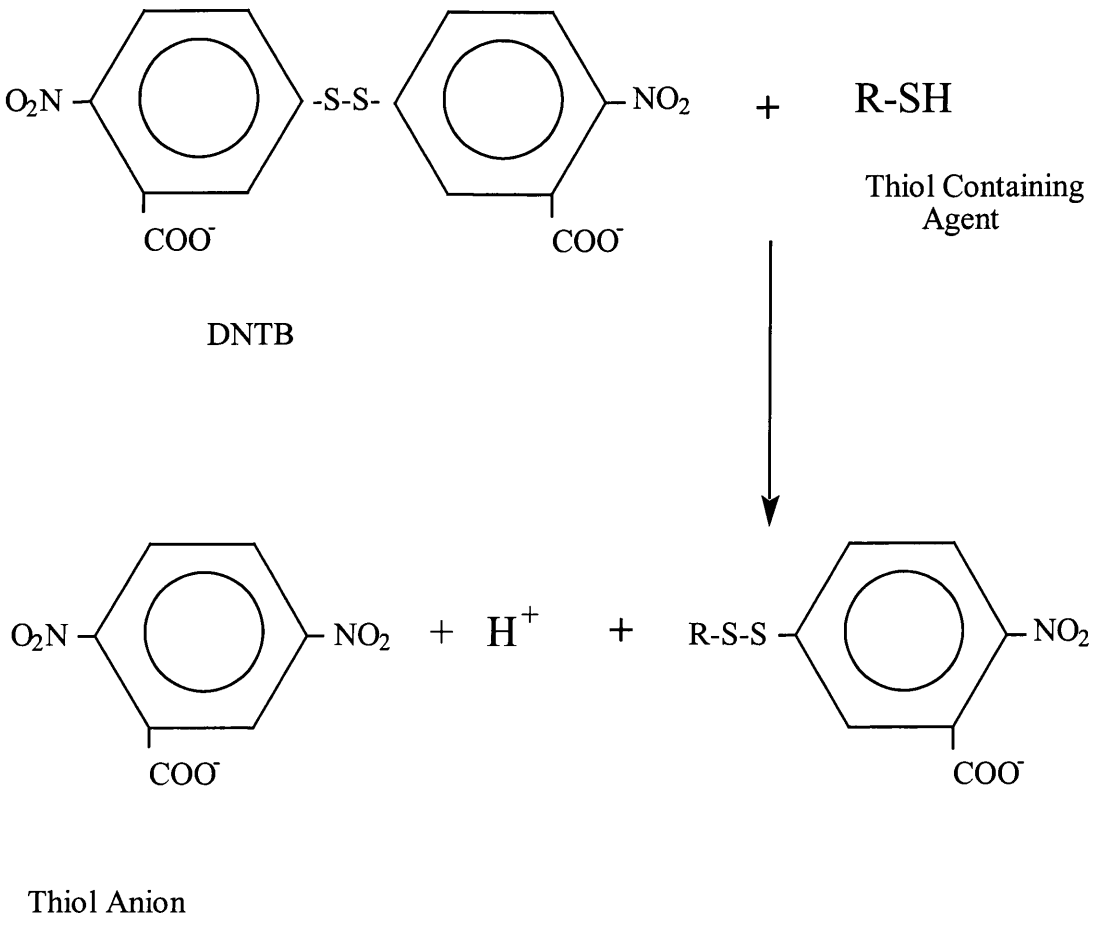


Figure 13: Schematic diagram of the reaction of Ellmans Reagent with Thiol groups.

Table 8: Preparation of tubes in the Ellman's assay.

Tube	Vol. of Buffer (mL)	Vol. of DTNB (mL)	Vol. Of Cysteine Stock (0.05 g L^{-1}) (mL)	Vol. Of Water (mL)	Vol. Of R2A Media (mL)	Vol. Of CDM Media (mL)	Average Optical Density (470nm)
1	1	1	1000	2.0	-	-	0.073
2	1	1	900	2.1	-	-	0.057
3	1	1	800	2.2	-	-	0.043
4	1	1	700	2.3	-	-	0.037
5	1	1	600	2.4	-	-	0.030
6	1	1	500	2.5	-	-	0.021
7	1	1	400	2.6	-	-	0.022
8	1	1	300	2.7	-	-	0.007
9	1	1	200	2.8	-	-	0.007
10	1	1	100	2.9	-	-	0.007
11	1	1	0	3.0	-	-	0
12	1	1	-	-	3	-	0.0175
13	1	1	-	2.7	0.3	-	0
14	1	1	-	2.97	0.03	-	0
15	1	1	-	-	-	3	0
16	1	1	-	2.7	-	0.3	0
17	1	1	-	2.97	-	0.03	0

- = no addition.
Tubes 1 to 11 = standard curve
Tubes 12 to 17 = test samples.

A standard curve was prepared (Figure 14). The accessible thiol-groups (as cysteine equivalency) contained in the medium can then be estimated from the graph. The absorbancy of tube 12 is extrapolated on the graph and it is possible to estimate the concentration of accessible thiol-groups present in the R2A is 0.05 μg mL^{-1}. Therefore, the activity of the biocides in the R2A medium is quenched by a factor of 0.05 μg mL^{-1}. Hence, this must be taken into account when estimating the MIC values in the R2A medium. There were no detectable thiol-groups present in the CDM, hence the effect of the biocides is not quenched by this medium. The absorbancy (E_{412nm}) of tube 12 was 0.0175. Therefore, from the standard curve the cysteine concentration equals 0.00029 g L^{-1}. Taking into account the dilution factor this is equal to 0.00145 g L^{-1}, which is equivalent to 1.45 μg mL^{-1}.

Table 9: Comparison of tube dilution MICs determined for the four biocides in R2A and CDM media, taking into account quenching by accessible thiol-groups.

Biocide	MIC ($\mu g\ mL^{-1}$)			
	R2A	R2A (minus quenching factor)	R2A (% difference)	CDM
BIT	60	58.55	1.8	5.8
MIT	20	18.55	7.25	2.4
CMIT	1.3	-0.15	-	0.25
Thiomersal	9.5	8.05	15.2	0.8

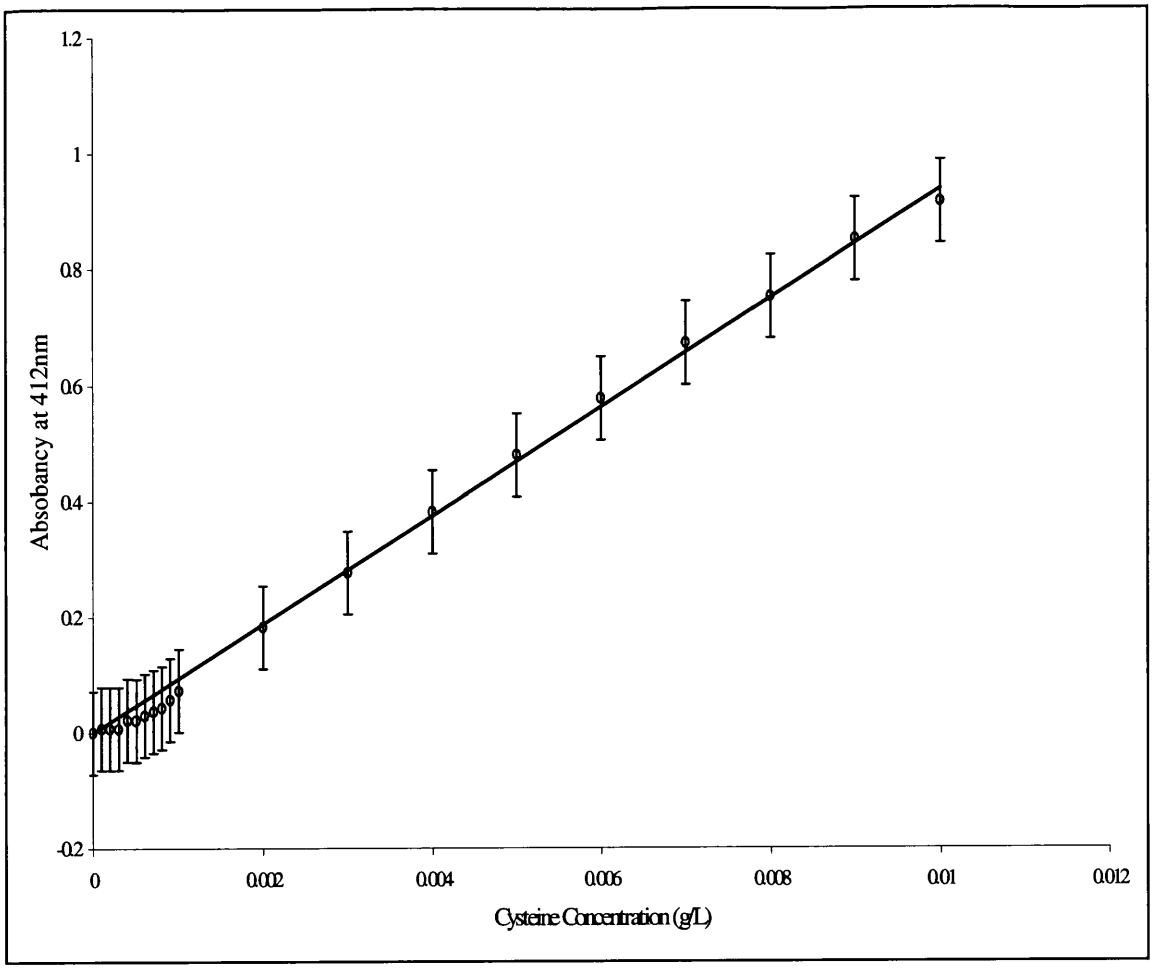


Figure 14: Standard Curve for the Estimation of accessible Thiol Groups (Ellmans Assay).

The Biocidal Activity of the Thiol-Interactive Isothiazolone Biocides and Thiomersal

The mode of action of biocides may be less specific than that observed with antibiotics (Sox, 1997). Biocidal products may damage many of the cell components and, therefore, it may be more difficult to determine whether biocidal or biostatic action is responsible (Sox, 1997). Whereas, the effects of an antibiotic usually result from an interaction with a single enzyme or macromolecule target within the cell, for example, the fluoroquinolones bind DNA gyrase, thus the target site is very specific and it is simpler to predict the mode of action (Sox, 1997). Strongly reactive compounds, for example, hypochlorite or hydrogen peroxide react with many sites within the bacterial cell, therefore, cell death will result from the combined effects upon the cell (Sox, 1997). It has been established that the mode of action of the isothiazolone (Collier *et al,* 1990c) and thiomersal biocides (Waigh & Gilbert, 1991) is to interact oxidatively with accessible thiol-groups. However, there is a marked variation in the antimicrobial activity of the biocides, which is demonstrated by the considerable differences in the observed MIC values previously determined. For example, the MIC for BIT in R2A medium is 60 μg mL^{-1}, whereas the MIC for CMIT under the same growth conditions is 1.3 μg mL^{-1}. This is thought to be due to an additional reaction that occurs due to the chlorination of the CMIT biocide (Collier *et al,* 1990a; Figure 5). CMIT is thought to demonstrate bactericidal activity (Lewis & Miller, 1974). It was demonstrated by Collier *et al,* (1990b) that CMIT exhibits fungicidal activity, however, this study did not observe bactericidal activity with this compound. This is thought to be due to the quenching of the biocide by accessible thiol groups in the medium and the bacterial cell structure. This is most notably in the Gram-negative outer membrane, which is impermeable to hydrophobic antimicrobials. The isothiazolone biocides MIT and BIT and the thiol-interactive agent thiomersal only demonstrate bacteriostatic activity (Collier *et al,* 1990b; Waigh & Gilbert, 1991). Upon establishment of the minimum inhibitory concentrations it was not possible to distinguish if the inhibitory effect is due to bacteriostatic or bactericidal action. Therefore, it was

necessary to determine the concentration of biocide required in order to kill the bacteria. Hence, this would establish whether biocidal or biostatic activity was demonstrated by the biocides.

Preparation of growth media

Five flasks with various concentrations of biocide (ranging from 0 μg mL^{-1} to the MIC value of the biocide, and double the MIC) were prepared aseptically in duplicate for each biocide (BIT, MIT, CMIT and thiomersal) in R2A and CDM media. The flasks were inoculated with 1mL of an overnight culture (16 hour) of *P. aeruginosa* PAO1 grown in the appropriate culture medium.

Preparation of Sodium Thioglycolate Stock

The sodium thioglycolate has an accessible thiol-group, which quenches the activity of the biocides. In order to ensure sufficient thiols were present to quench the activity of the biocides, the molarity of thiols in the highest concentration flask was determined. This concentration was doubled (resulting in a stoichiometric ration of 2:1, sodium thioglycollate to biocide) in order to determine the concentration of sodium thioglycolate to apply (Table 10). This procedure ensures that the biocides were quenched by excess thiols.

Table 10: Molarity of sodium thioglycolate required to quench the activity of the thiol interactive biocides.

Biocide	Media	Biocide (M)	Sodium Thioglycolate (M)
BIT	R2A	1.855×10^{-11}	3.71×10^{-11}
MIT	R2A	8.26×10^{-12}	1.65×10^{-11}
CMIT	R2A	4.35×10^{-13}	8.70×10^{-13}
Thiomersal	R2A	1.16×10^{-12}	2.32×10^{-12}
BIT	CDM	1.90×10^{-12}	3.80×10^{-12}
MIT	CDM	1.07×10^{-12}	2.14×10^{-12}
CMIT	CDM	8.35×10^{-14}	1.67×10^{-13}
Thiomersal	CDM	2.47×10^{-14}	4.94×10^{-14}

The Biocidal Assay

The flasks were incubated in an orbital incubator (Gallenkamp INA-305) at 37°C,180 osc min^{-1}. At time interval 0h an aliquot (0.1mL) of culture was removed and added to the sodium thioglycolate (9.9 mL) solution and incubated at room temperature for 30 minutes. An aliquot (1 mL) of the sodium thioglycolate / culture suspension was serially diluted in the range of 10^{-3} to 10^{-7} in sterile 0.9% saline. An aliquot (1 mL) of the dilutions was spread onto duplicate R2A or CDM agar plates using a glass spreader. The plates were incubated for 24 hours at 37°C. The procedure was repeated at time intervals 2, 4, 6 and 24 hours. Viable counts were determined and Lethal time 90% ($LT_{90\%}$;the time taken for a 90% or 1 log cycle reduction in viability) values estimated.

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Table 11: The LT_{90} and LT_{90}:MIC estimations from the biocidal assay for the isothiazolone biocides and thiomersal.

Biocide	$LT_{90\%}$ (hours)	Biocide conc ($\mu g\ mL^{-1}$)	LT_{90}:MIC ratio
BIT	2.5	8.7	1:3.5
MIT	5	3.6	1:0.7
MIT	4	4.8	1:1.2
CMIT	Nd	nd	nd
Thio	5.5	0.1	1:0.02
Thio	4.5	0.2	1:0.04
Thio	5.5	0.3	1:0.05

nd = not done

Discussion

A good bactericidal effect is normally exhibited when the $LT_{90\%}$ value is less than two hours. Table 11 demonstrates that this was not observed with any of the biocides used here. Therefore, it is suggested that the biocides BIT, MIT and thiomersal inhibit growth of bacteria by bacteriostatic action rather than bactericidal action. The LT_{90}:MIC ratio is normally considered to be indicative of bactericidal action when the ratio is less than 1:4,

this was observed with all the biocides used. These results indicate that all the biocides tested are bactericidal, at or near their MIC value, but that the activity is slow in onset and operation. Therefore, the time required, for the $LT_{90\%}$ would eliminate the biocides as possible bactericidal agents.

Chapter Four

Induction of Resistance in *Pseudomonas aeruginosa* PAO1 to the Isothiazolone Biocides.

Brözel and Cloete (1994) exposed *P. aeruginosa* to Kathon™ (a mixture of 1.15 % w/v CMIT and 0.35% w/v MIT). They observed an increase in the minimum inhibitory concentration in response to increasing concentrations of the Kathon™ biocide. In their experiment three cultures were tested in parallel over a 15 day exposure period. The MIC increased from an initial value of 300 $\mu L\ L^{-1}$ to 607 $\mu L\ L^{-1}$. They observed that the MIC of the cultures increased at similar rates and, therefore, they did not attribute the adaptation to a mutational event, but to a specific intracellular mechanism. The isothiazolone biocides are known to interact oxidatively with accessible thiol-groups (Collier *et al,* 1990a), however, it was observed that resistant cells did not produce any isothiazolone-quenching agents (Brözel & Cloete, 1994). Upon SDS-PAGE analysis it was observed that a 35 kDa outer membrane (designated T-OMP) was present in sensitive or unexposed cells, but was absent in resistant cells (Brözel & Cloete, 1994). It was noted that the absence of T-OMP occurred within 24 hours of exposure to the biocide and was still suppressed following 72 hours growth in biocide free medium (Brözel & Cloete, 1994). They concluded that this adaptation resulting in the suppression of the T-OMP, was a phenotypic adaptation.

The aim of this chapter was to induce resistance in *P. aeruginosa* PAO1 to the three pure isothiazolone biocides, BIT, MIT and CMIT and to thiomersal. Thiomersal is a thiol-interactive agent, but it is not a member of the isothiazolone group of compounds and is therefore used as a positive control. Brözel and Cloete induced resistance in *P. aeruginosa* (isolated from a cooling water system) against Kathon™. Kathon is a commercial product consisting of 1.15% CMIT and 0.35% MIT stabilised with $MgCl_2$ and $MgNO_3$ (Thor chemicals). Therefore, the MICs observed by Brözel and Cloete (1994) were a result of the combined effect of CMIT and MIT. It is, therefore, not possible to determine from the Brözel and Cloete investigation which biocide was responsible for the

development of resistance. The isothiazolone biocides are neutralised by accessible thiol groups. R2A medium is a complex medium with accessible thiol groups. It was, therefore, necessary to take into account the quenching effect of the medium and repeat the experiment with a medium, which does not contain accessible thiol-groups. The induction of resistance in this investigation was performed according to the Brözel and Cloete method with some variations. For example, Brözel and Cloete centrifuged the inoculum to concentrate the cells. This has previously been observed to stress the cells such that the MICs observed would be affected (Gilbert *et al,* 1995). Therefore, in this investigation the inoculum size was not adjusted by centrifugation. Brözel and Cloete concluded that the loss of T-OMP associated with an increase in resistance to Kathon™ was due to its role as a route of entry into the cell interior, and suggested that T-OMP is a specific OMP to the isothiazolone biocides.

Induction of Resistance

The induction of resistance in *P. aeruginosa* to the isothiazolone biocides and thiomersal was performed according to the method previously described by Brözel & Cloete (1994). Triplicate cultures of *P. aeruginosa* were incubated for 24h at 37°C, in an orbital incubator (Gallenkamp INA-305) at 200 osc min^{-1}. The MIC of the respective biocides was determined according to the tube dilution method (Bloomfield, 1991) using a standard inoculum (100 μL in 10 mL; the overnight culture was diluted to achieve an inoculum of 10^9 CFU mL^{-1}). An aliquot (250 μL) of the original cultures of *P. aeruginosa* was inoculated into fresh medium containing a quarter strength concentration of biocide of the previously established MIC (MIC/4). The new culture was incubated for 24h at 37°C in an orbital incubator at 200 osc min^{-1}. The MIC of the new bacterial culture was redetermined. Once the MIC value had been redetermined an aliquot (250 μL) of the previous culture was inoculated into fresh medium containing MIC/4 of the newly established MIC. This was repeated until ten successive passages had been performed in the presence of increasing MIC/4 of the biocide (passage number eleven). This was the point in the experiment when Brözel and Cloete deemed the cells to be resistant and due to

time limitations the cells were not exposed to biocide for any further passages, although the MICs may continue to increase with further exposure to the biocides. At this point an aliquot (250 μL) of the final cultures in the presence of biocide was inoculated into fresh medium and incubated as previously described. The MIC value was redetermined by the tube dilution method (Bloomfield, 1991). This was repeated until three successive passages had been performed in the absence of biocide. This was repeated for R2A medium against all four biocides (BIT, MIT, CMIT and thiomersal) and CDM against the three isothiazolone biocides (BIT, MIT and CMIT).

Figures 15 to 16 illustrate the observed increase in MIC for all four biocides in R2A medium and three biocides in CDM. It was not possible to establish results for cells exposed to thiomersal and grown in the CDM. This is thought to be due to the combined stress of growing the cells in a chemically defined medium and to biocide exposure. This absence of growth in CDM medium and combined exposure to thiomersal was possibly an early indication that the resistance mechanism for this compound differs from that of the isothiazolone biocides.

Exposure to BIT

The initial MIC against BIT when grown in R2A medium was 56 μg mL^{-1}. Within 24 hours of exposure to the biocide the MIC value had increased to a value of 60 μg mL^{-1} (Figure 15). The MIC value gradually increased with each progressive exposure to the biocide, until passage 11 at which point the MIC was 98.6 μg mL^{-1}. This represented an increase of 76% from the initial MIC. The following three passages were performed in biocide-free medium and immediately the MIC value decreased to 95 μg mL^{-1} (passage 12). With the two successive passages in biocide-free medium the MIC decreased in a step-wise fashion, and at passage 14 the MIC was 84 μg mL^{-1}. These results are summarised in Table 12. By comparison, the initial MIC for the cells exposed to BIT cultured in CDM was 5.73 μg mL^{-1}. This observed MIC in CDM was a 10-fold decrease in the MIC values when *P. aeruginosa* was cultured in R2A medium.

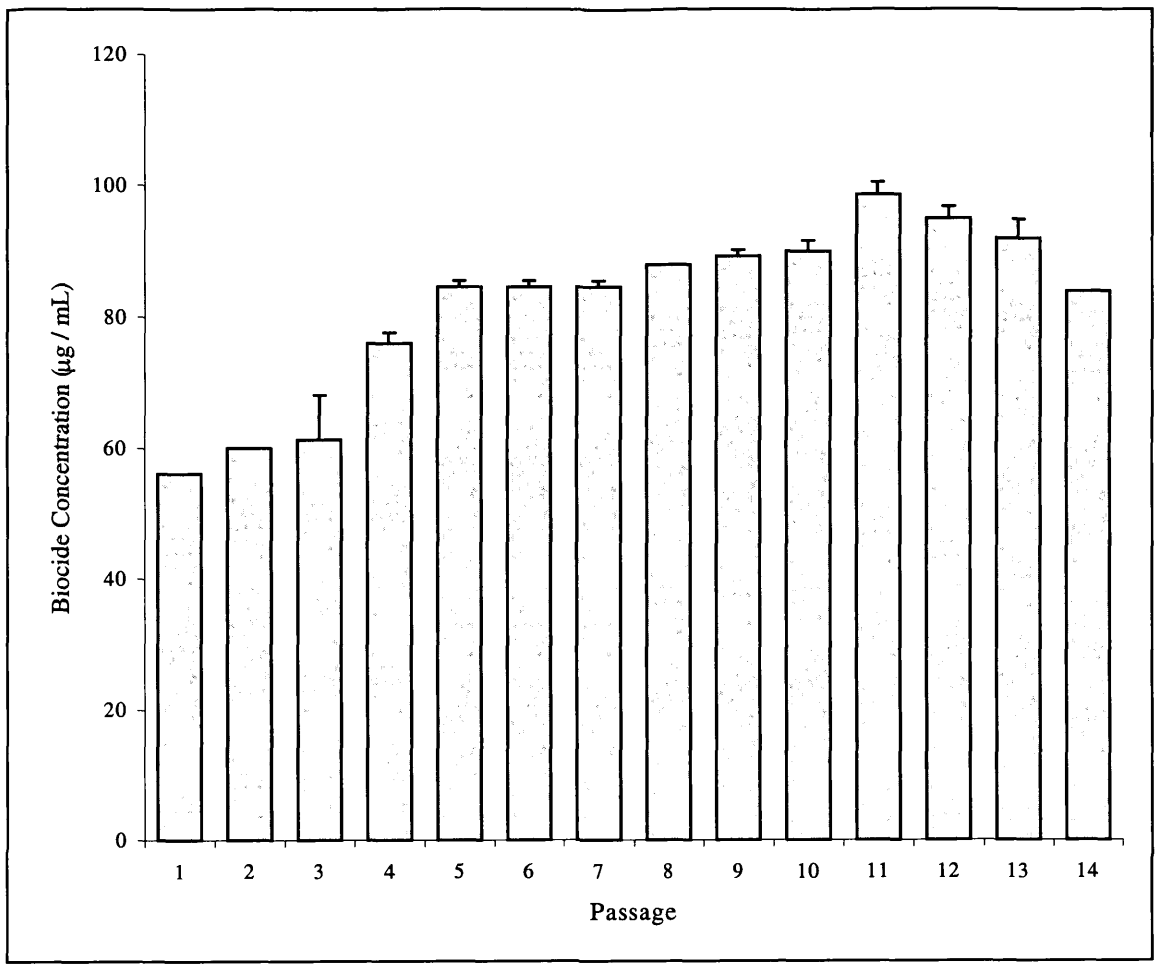


Figure 15: Increase in MIC for BIT against *Pseudomonas aeruginosa* PAO1 in R2A medium as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

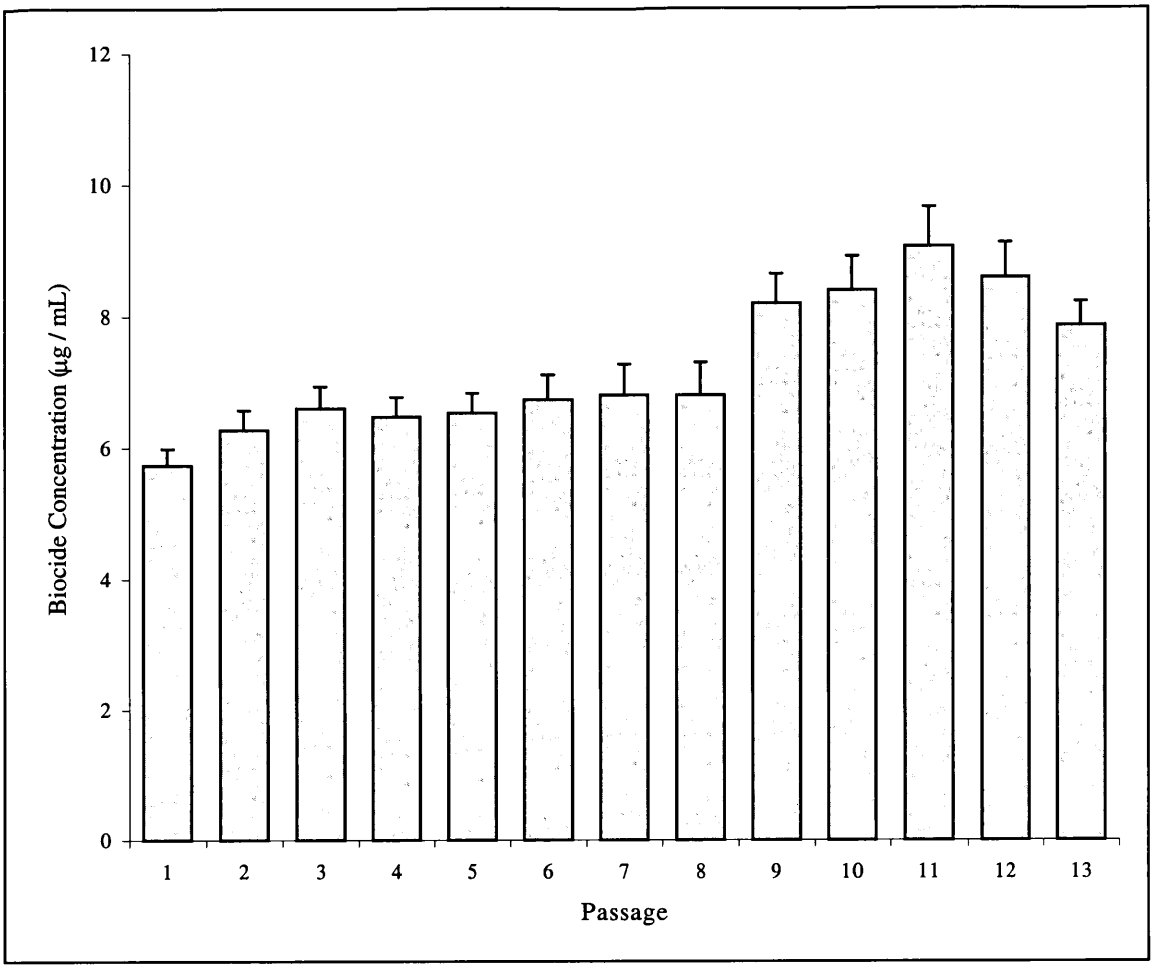


Figure 16: Increase in MIC for BIT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

The MIC decreased by 1.8% when the quenching activity of the thiol-groups were taken into account. Therefore, the effect of nutrient limitation had a marked effect on the MIC. The MIC values exposed to BIT when grown in CDM gradually increased (Figure 16), up to passage 11. The cells were then deemed 'resistant' and the corresponding MIC was 9.07 $\mu g\ mL^{-1}$. This was an increase of 58 %. Immediately following the biocide-exposure passages the cells were passaged in biocide-free medium. The MIC values immediately began to decrease, and at passage 14 the MIC had decreased to 6.4 $\mu g\ mL^{-1}$.

Exposure to MIT

The initial MIC against MIT when the cells were cultured in R2A medium was 19 $\mu g\ mL^{-1}$. This is considerably lower from the initial MIC against BIT (56 $\mu g\ mL^{-1}$), indicating that MIT demonstrates a greater activity towards *P. aeruginosa* than BIT. Within 24 hours exposure to the biocide the MIC had significantly increased. At passage 2 the MIC value was 28.6 $\mu g\ mL^{-1}$, an increase of almost 10 $\mu g\ mL^{-1}$ (Figure 17). The MICs continued to increase gradually with every successive passage in the presence of biocide. However, in the majority of instances, the increase in MIC between the passages appeared to be in the range of 2 to 5 $\mu g\ mL^{-1}$. At passage 11 the MIC had increased to 48.66 $\mu g\ mL^{-1}$, representing an increase of 156%. However, upon removing the cells from the presence of the biocide the MIC began to decrease. At passage 14 the MIC had decreased to 39.3 $\mu g\ mL^{-1}$. Upon exposure of the cells to MIT grown in CDM (Figure 18) the initial MIC was 10-fold lower than that observed in R2A medium, at 2.46 $\mu g\ mL^{-1}$. At passage 2 the MIC had increased by 10 $\mu g\ mL^{-1}$ to 3.4 $\mu g\ mL^{-1}$. The MIC continued to increase up to passage 11, to a value of 9.8 $\mu g\ mL^{-1}$, this was an increase of 298%. When the cells were cultured in the biocide-free medium, at passage 14 the MIC had decreased to 9.0 $\mu g\ mL^{-1}$.

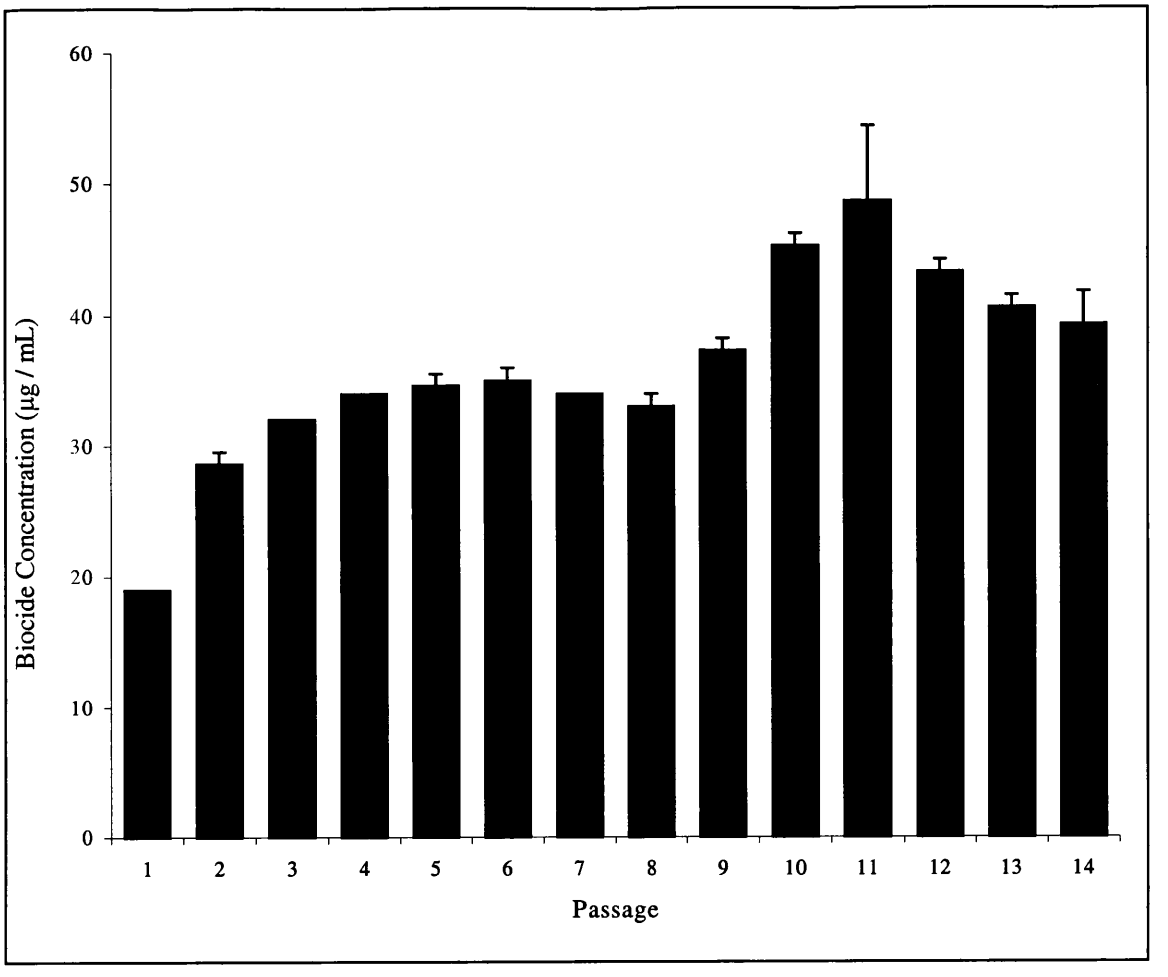


Figure 17: Increase in MIC for MIT against *Pseudomonas aeruginosa* PAO1 in R2A medium as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

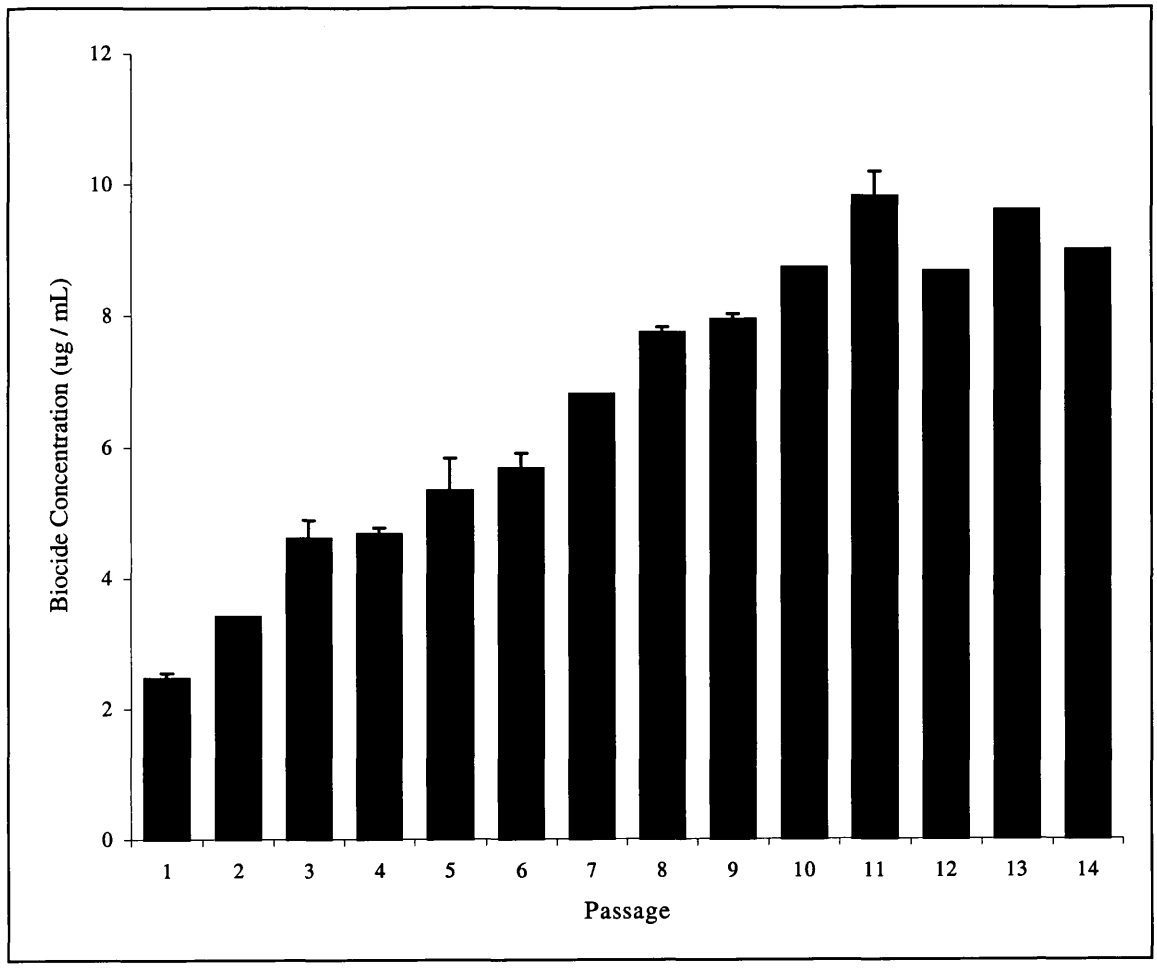


Figure 18: Increase in MIC for MIT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

Exposure to CMIT

The initial MIC value for the cells exposed to CMIT when grown in the R2A medium was 1.3 $\mu g\ mL^{-1}$ (Figure 19). This is considerably lower than the MICs obtained for the other isothiazolone biocides indicating that CMIT demonstrates the greatest reactivity of the isothiazolone biocides towards *P. aeruginosa* PAO1. This is thought to result from the different mode of action of CMIT (Figure 5). The MIC did not increase until passage five (to 1.76 $\mu g\ mL^{-1}$) when the cells were exposed to CMIT. Therefore, it was not until the fourth successive passage with biocide that a marked MIC increase was observed. The MICs then continued to increase in a step-wise fashion, and at passage 11 the MIC had increased to 2.5 $\mu g\ mL^{-1}$, an increase from the initial MIC of 92%. The cells were then passaged in biocide-free medium and within the first 24 hours a significant decrease in MIC was observed at 2.0 $\mu g\ mL^{-1}$. This value did not decrease any further, but at passage 13, had fallen to 1.8 $\mu g\ mL^{-1}$ by passage 14. Upon exposure to CMIT in CDM, the initial MIC was 0.25 $\mu g\ mL^{-1}$ (Figure 20). A marked increase in MIC to 0.3 $\mu g\ mL^{-1}$was observed within the first passage to the biocide when the cells were grown in the chemically defined medium. However, the two proceeding passages did not demonstrate any significant increase. This delay in the development of resistance is possibly due to the stress of growth in the CDM media and exposure to CMIT (the most reactive isothiazolone biocide). At passage 5 (that is following the fourth biocide-exposure) the MIC began to increase again. At passage 11 the MIC of the 'resistant' cells was 0.6 $\mu g\ mL^{-1}$, this is an increase of 140% from the original MIC. When the cells were grown in biocide-free medium the MIC values began to decrease, by passage 14 the MIC was 0.15 $\mu g\ mL^{-1}$. This value is lower than the original MIC obtained for the sensitive cells. Therefore, this may imply that the development of resistance towards CMIT varies from that of the other biocides. Alternatively, it may be connected to the greater reactivity of the CMIT biocide.

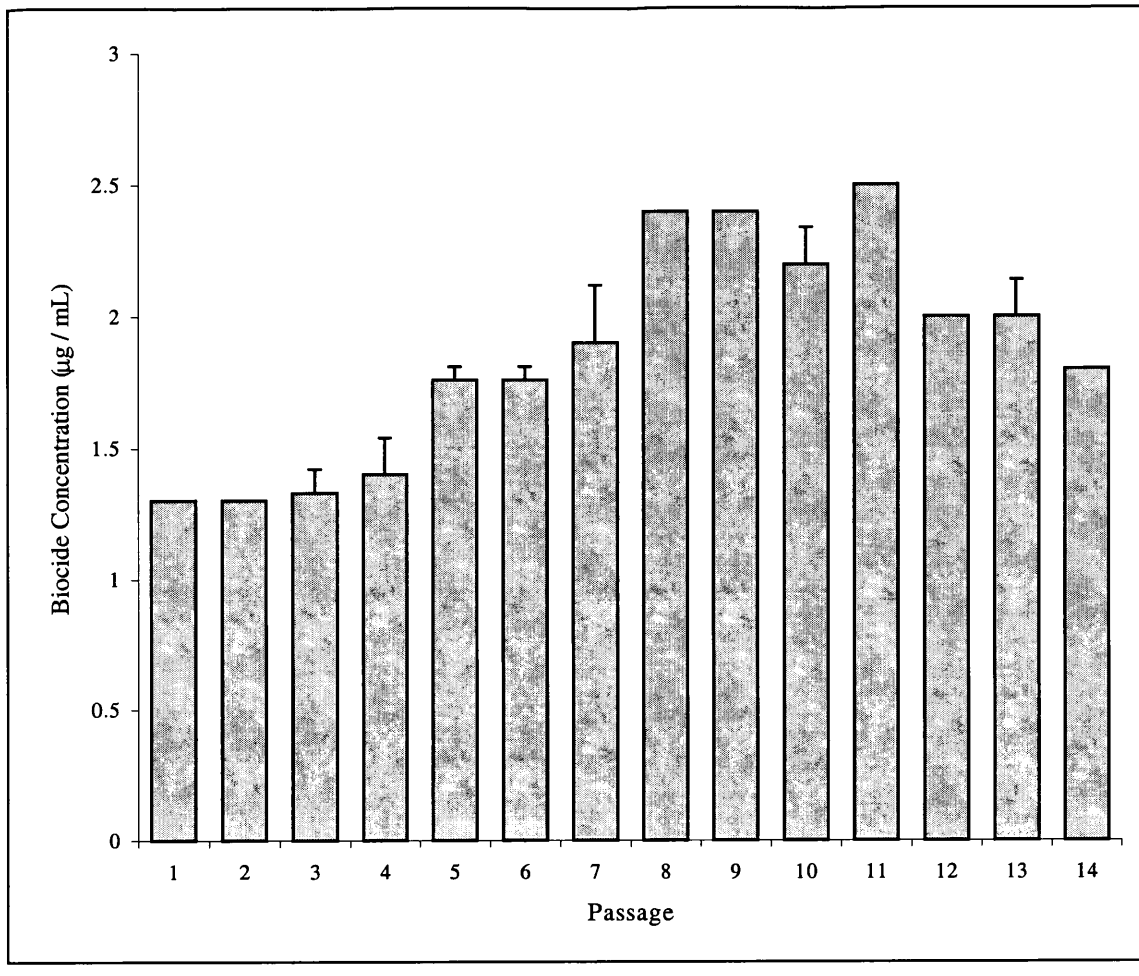


Figure 19: Increase in MIC for CMIT against *Pseudomonas aeruginosa* PAO1 in R2A medium as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

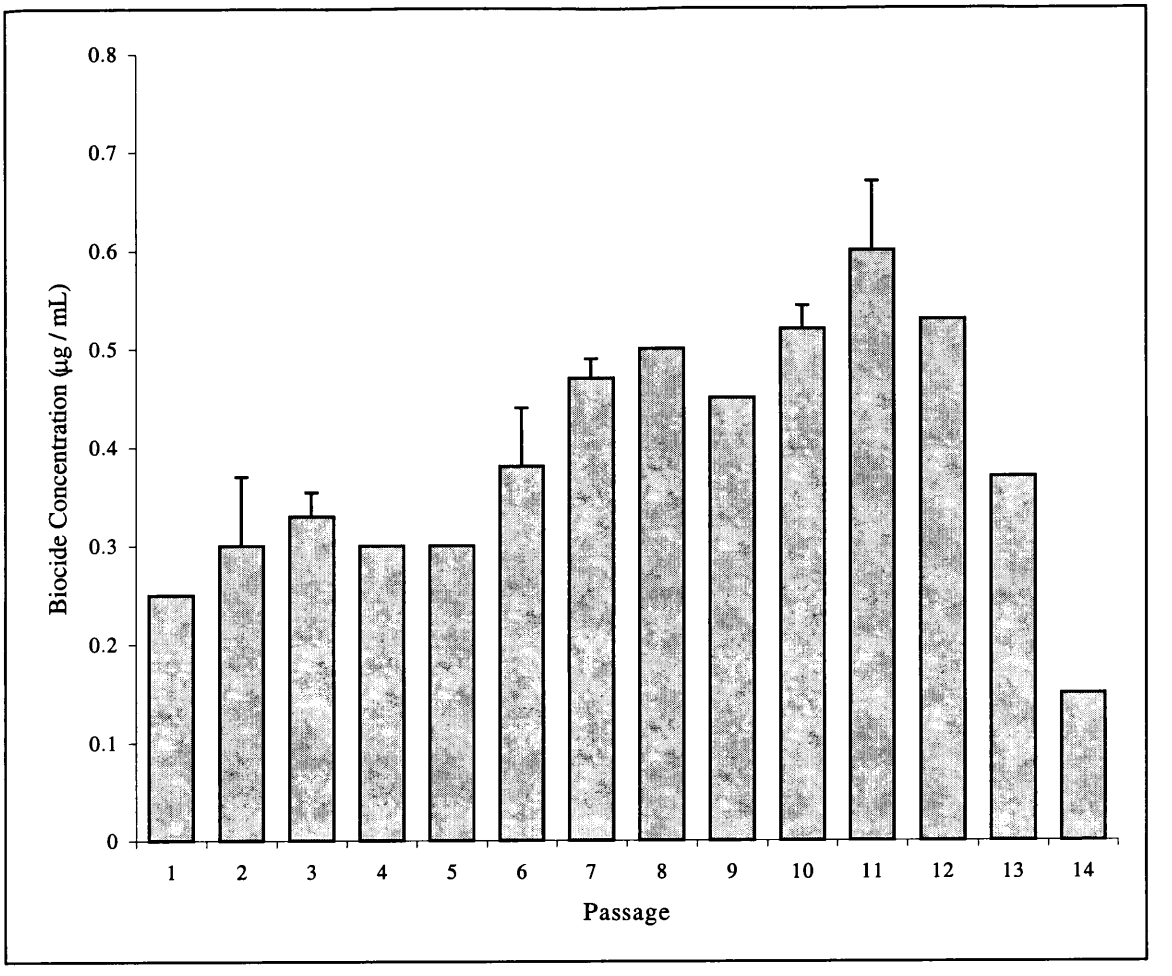


Figure 20: Increase in MIC for CMIT against *Pseudomonas aeruginosa* PAO1 in CDM as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

Exposure to Thiomersal

The initial MIC for the cells exposed to thiomersal was 9.4 $\mu g\ mL^{-1}$ (Figure 21). Within the first passage to the biocide the MIC had increased by just 0.6 $\mu g\ mL^{-1}$ to 10.0 $\mu g\ mL^{-1}$. Within the following 9 exposures to the biocide the MIC continued to increase in steps of between 0.3 and 0.6 $\mu g\ mL^{-1}$. By passage 11 the MIC had increased to 17.93 $\mu g\ mL^{-1}$, an increase of 91%. Upon the initial passage in the biocide-free medium the MIC continued to increase to 18.0 $\mu g\ mL^{-1}$. This value remained the same following the second 24 passage in biocide-free medium. It was not until passage 14 that a decrease in MIC was observed at 16.8 $\mu g\ mL^{-1}$.

The Induction of Resistance

The induction of resistance to all four biocides was observed to occur in a step-wise fashion. This would indicate, as do the findings of Brözel and Cloete (1994), that the process of adaptation to the isothiazolone biocides and other thiol-interactive agents is a phenotypic process. This can be supported by the observation that upon removal of the presence of the biocide the MIC values began to decrease. This was, therefore, indicative of a phenotypic adaptation to the external environment and not a specific genetic change. However, slight differences in the process of adaptation may indicate that variations in the exact mechanisms of resistance development are occurring between biocide treatments. For example, an immediate response was observed in all inductions of resistance except with exposure to CMIT. It has already been observed that CMIT demonstrates a differing mode of action to the other two isothiazolone biocides (Collier *et al,* 1990c). Therefore, a different mechanism of resistance development may occur. Although an immediate increase in MIC was observed with CMIT when the cells were grown in CDM, no significant increase in MIC was observed in the following two biocide-passages. In addition, the cells exposed to thiomersal grown in R2A medium did not immediately begin to exhibit a decrease in MIC when the resistant cells were passaged in the biocide-free medium. This may indicate that a different mechanism may occur during the development of resistance to thiomersal.

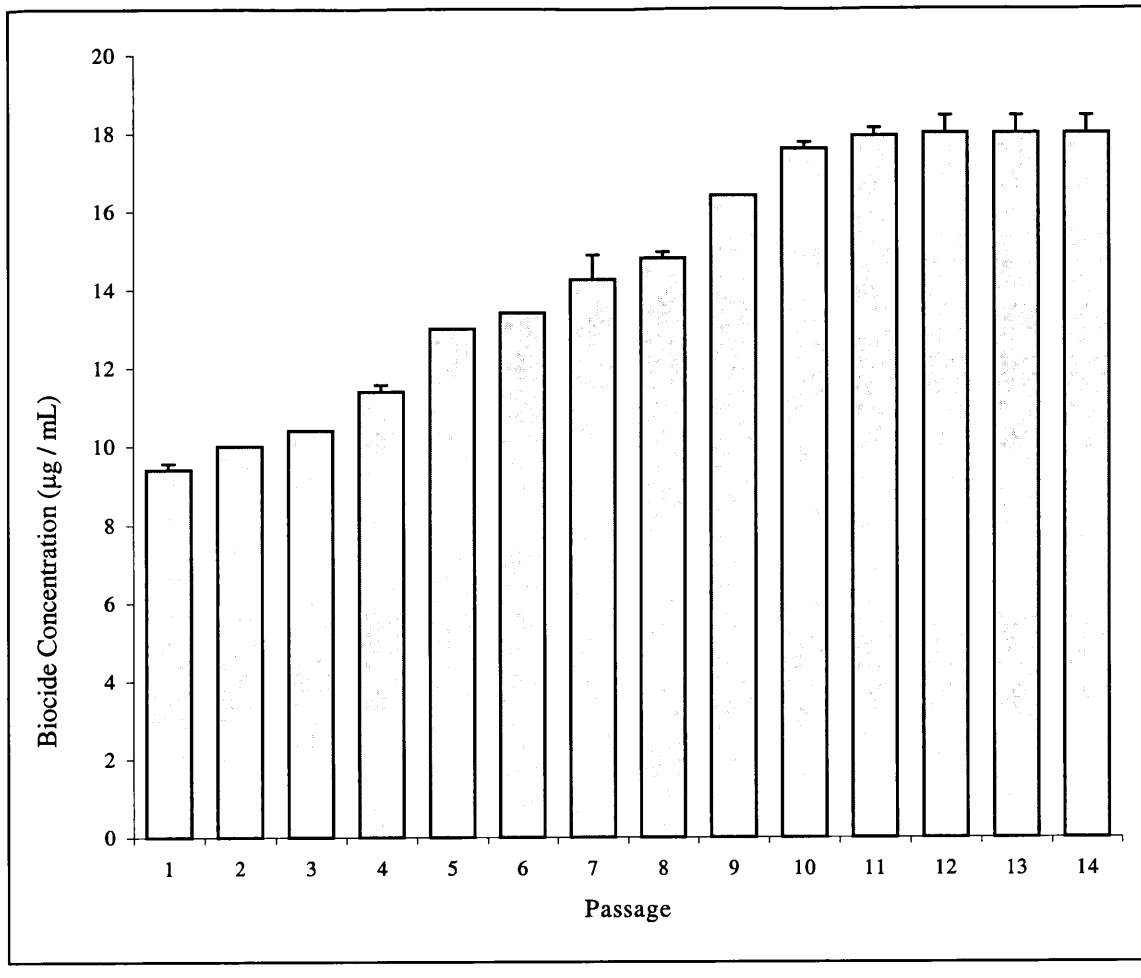


Figure 21: Increase in MIC for Thiomersal against *Pseudomonas aeruginosa* PAO1 in R2A as a function of passage number. Error bars are calculated as the standard deviation of the individual data points.

In support of this suggestion, the MIC remained unchanged when the cells were exposed to thiomersal and cultured in CDM. This may be due to the effect of nutrient limitation and biocide stress. Whereas, the R2A medium quenched the activity of thiomersal due to the presence of accessible thiol-groups allowing the bacteria to recover from biocide and nutrient stresses in order to grow and develop resistance to the biocide. However, growth was observed when exposing *P. aeruginosa* to the isothiazolones, even with CMIT, which demonstrated greater activity towards *P. aeruginosa* than BIT, MIT or thiomersal. The pattern of resistance was observed to be a definite step-wise increase when resistance was induced towards thiomersal. However, the pattern of resistance with the three isothiazolone biocides was less definite, and thus may indicate a different pattern in the development of resistance.

The process of induction of resistance is comparable to that observed by Brözel and Cloete (1994). However, the reactivity of the pure forms of the isothiazolone biocides was observed to be greater than that of Kathon™ used by Brözel and Cloete (1994). They observed an increase in MIC from 300 $\mu L\ L^{-1}$ to 607 $\mu L\ L^{-1}$. Which, in effect, is an increase of 0.3 $\mu L\ mL^{-1}$ to 0.67 $\mu L\ mL^{-1}$, which is significantly lower than the MICs observed in this study. When taking into account that the active ingredients of the commercial product (1.15% CMIT and 0.35% MIT), the increase in MIC towards CMIT is 0.00345 $\mu L\ mL^{-1}$ to 0.00698 $\mu L\ mL^{-1}$ and towards MIT is 0.00105 $\mu L\ mL^{-1}$ to 0.00212 $\mu L\ mL^{-1}$. Therefore, Brözel and Cloete (1994) observed approximately 100% increase in MIC towards the two active components of Kathon (CMIT and MIT). However, the active components of the commercial product only constitute 1.45% of the total product. Therefore, the effect of the induced resistance seems less pronounced than when using the pure forms of the biocide. This may be due to loss of reactivity of the biocide during the storage of the commercial product, in comparison to the pure forms of the biocides, which are stored in a dry, cold environment, in a solid state.

Table 12: Summary of the average MICs for R2A and CDM media when exposed to the four biocides. The figures in the brackets indicate the percentage increase in MICs for passage 11 over passage 1 and the percentage decrease in MIC for passage 14 over passage 14.

Biocide	Medium	MIC ($\mu g\ mL^{-1}$) of passage number:		
		1	11	14
BIT	R2A	56	98.6 (76)	84 (14.8)
MIT	R2A	19	48.7 (156)	39.3 (19.3)
CMIT	R2A	1.3	2.5 (92)	1.8 (28)
Thiomersal	R2A	9.4	17.93 (91)	16.8 (6.3)
BIT	CDM	5.73	9.07 (58)	6.4 (29)
MIT	CDM	2.46	9.8 (298)	9.0 (8.2)
CMIT	CDM	0.25	0.6 (140)	0.15 (75)
Thiomersal	CDM	nd	nd	nd

nd = not done.

Investigation of Cross-Resistance

Cross-resistance to unrelated antibiotics is a property of some mutants. For example, multiple-antibiotic-resistant *E. coli* also show cross-resistance to fluoroquinolone. Cross-resistance to chemically unrelated antibiotics can be associated with fluoroquinolone resistance (Poole *et al,* 1993). The extensive use of cationic biocides may possibly result in the selection of staphylococcal strains demonstrating resistance to antibiotics and biocides (Russell *et al,* 1998). It was proposed by Sticker *et al,* 1983 that the widespread use of chlorohexidine was responsible for selecting multiple antibiotic-resistant strains in Gram-negative bacteria (Russell 1995).

The MIC of the previously induced resistant cultures was obtained in order to determine whether cross-resistance occurred between the three isothiazolone biocides, and the isothiazolone biocides and the thiol-interactive agent Thiomersal (Table 13).

An aliquot (250 μL) of the resistant culture was inoculated in triplicate into fresh medium containing MIC/4 concentration of biocide (MIC determined from the eleventh passage). Cultures were incubated for 24h at 37°C in an orbital incubator at 200 osc min^{-1}. The MIC was determined against the other three biocides (Tables 13 and 14) by the tube dilution method (Bloomfield, 1991).

Table 13: MIC of the resistant cultures of *Pseudomonas aeruginosa* PAO1 (grown in R2A medium) at MIC/4 of biocide when examined against the other three biocides.

Resistant cultures	MIC of original biocide ($\mu g\ mL^{-1}$) at passage		MIC to test biocides cultures ($\mu g\ mL^{-1}$)			
	1	11	BIT	MIT	CMIT	Thiomersal
BIT	56	98.6	nd	40	2.2	10
MIT	19	48.6	70	nd	2.0	20
CMIT	1.3	2.5	80	50	nd	18
Thiomersal	9.4	18.0	70	40	2.2	nd

nd = not done

Table 14: MIC of the resistant cultures of *Pseudomonas aeruginosa* PAO1 (grown in CDM medium) at MIC/4 of biocide when examined against the other three biocides.

Resistant cultures	MIC of original biocide ($\mu g\ mL^{-1}$) at passage		MIC to test biocides cultures ($\mu g\ mL^{-1}$)			
	1	11	BIT	MIT	CMIT	Thiomersal
BIT	5.73	9.1	nd	11	0.3	0.05
MIT	2.46	9.8	6	nd	0.5	0.15
CMIT	0.25	0.6	12	12	nd	0.25
Thiomersal	nd	nd	nd	nd	nd	nd

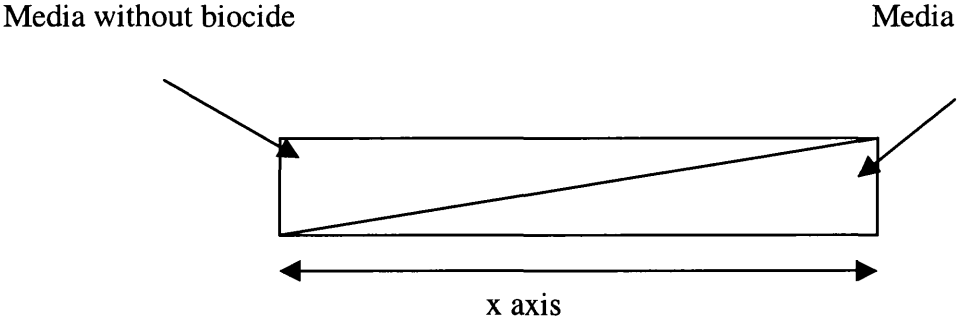
nd = not done

The results indicate that once resistance has been induced in a culture towards one of the biocides, that resistance is to some extent exhibited with other members of the same group and other thiol-interactive agents. A marked increase was observed in the MICs against the cross-resistance test strains over the original MICs prior to biocide exposure. The passaging of cells in CDM exposed to thiomersal was not done due to the extreme sensitivity of the cells used. This is due to the double challenge of chemically defined medium and biocide presence. However, once resistance had been induced to the isothiazolones it was possible to determine the MICs against thiomersal. This indicated that the induced resistance was transferable to other thiol-interactive agents.

Maintenance of Resistance Induced Cultures on Gradient Plates

The gradient plates employed were composed of two layers of agar, the bottom layer contained 1.5 x the MIC of biocide of the culture to be inoculated, which was allowed to set at an angle in a plastic square petri dish (BDH; 20 cm x 20 cm). The top layer contained the appropriate biocide-free medium, which was allowed to set on the level. The biocide diffused through the biocide-free upper layer producing a biocide gradient over the length of the plate. The surface of the plate was inoculated with cultures of *Pseudomonas aeruginosa.* Growth of the bacteria was observed to cease at the MIC position of the plate.

Figure 22: Schematic diagram of the cross section of a gradient plate.



An aliquot (25 mL) of R2A agar or CDM agar with 1.5 x MIC of biocide was allowed to set at an angle to produce a diagonal slope. An aliquot (25 mL) of the same agar without the addition of biocide was poured onto the biocide agar and allowed to set on the level. The biocide diffused through the top layer of agar producing a biocide gradient over the length of the plate. The surface of the plate was then inoculated with biocide resistant *P. aeruginosa* from MIC/4 exposed passages. The plates were incubated at 37°C for 2 to 3 days for the R2A medium and 5 to 6 days for the CDM medium. The point at which growth ceased on the surface of the agar is to be used to indicate the approximate MIC.

Two colonial morphologies were observed when the resistant cultures were grown on the gradient plates, a mucoid and non-mucoid form. It was thought that this may be

related to alterations in the lipopolysaccharide of the outer membrane. he two colonial morphologies were cultured and any subsequent alterations in the LPS structure were observed and results reported in Chapter 6.

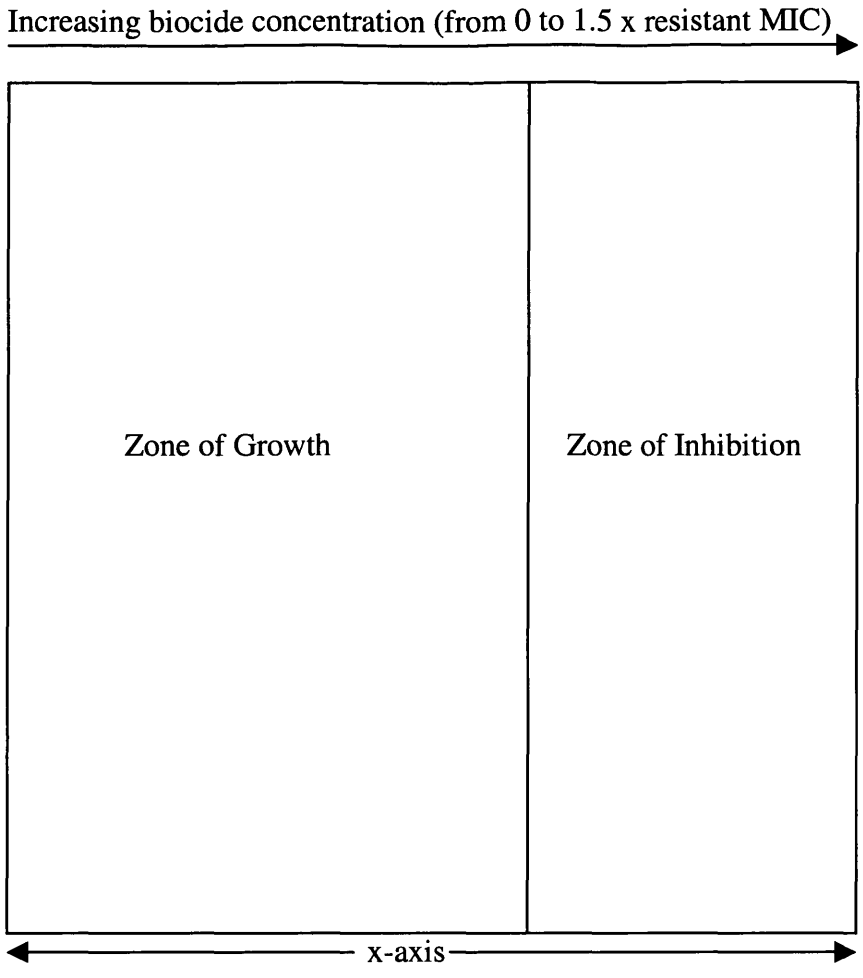


Figure 23: Schematic representation of the growth pattern of resistant cultures of *Pseudomonas aeruginosa* PAO1 towards the isothiazolone biocides and thiomersal on a gradient plate.

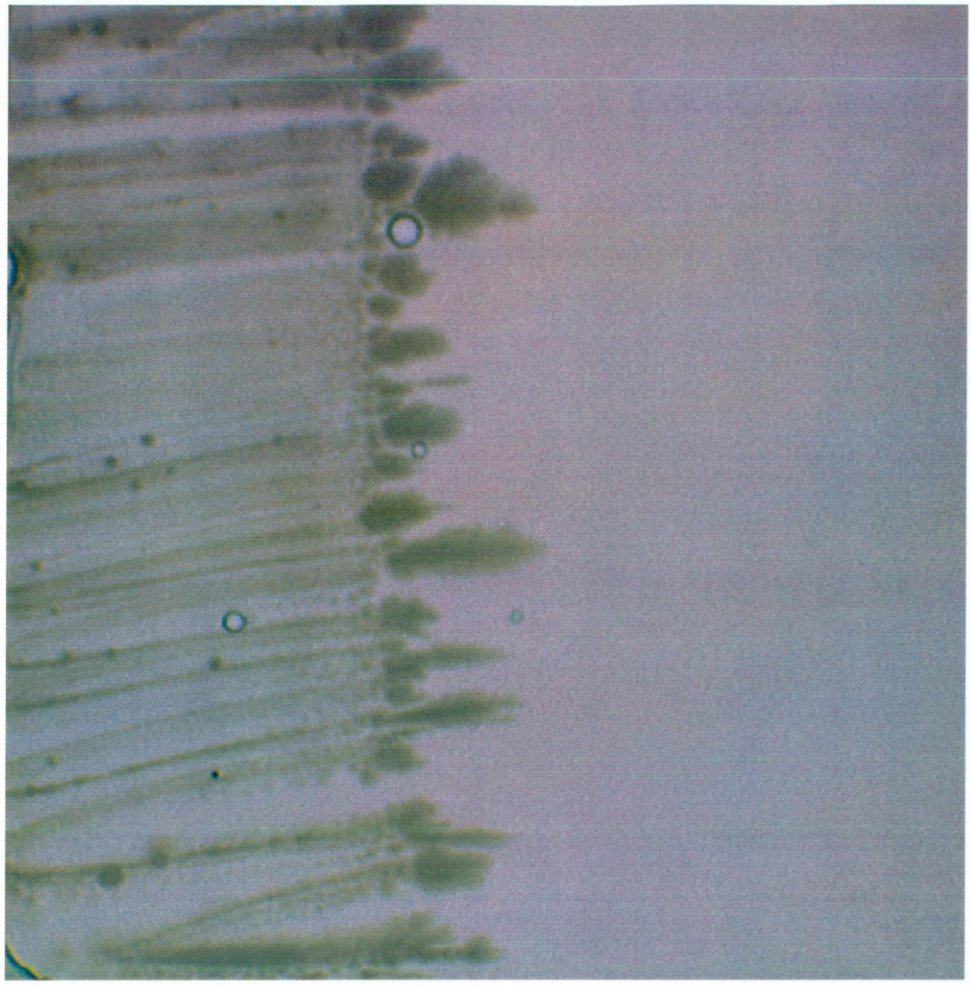


Figure 24: Growth of *Pseudomonas aeruginosa* PAO1 on a gradient plate with increasing concentrations of the biocide BIT. The MIC point is clearly visible on the gradient plate. The colonies growing in the region with the greatest concentration of biocide will be the most resistant, therefore inoculation into the next passage should be taken from this point.

Chapter Five

The Analysis of the Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 following Exposure to Biocidal Agents and Nutrient Limitation

The outer membrane of *P. aeruginosa* contains a number of proteins, some of which form diffusion channels which allow the transport of ions and small molecules into the cell interior (Jann & Jann, 1999). These channel forming proteins are referred to in this investigation as porins or outer membrane proteins (OMPs). OMPs are also present in other species of bacteria for example, *Escherichia coli*, *Salmonella spp.* and *Neisseria spp.* (Jann & Jann, 1999). Other proteins are present in the outer membrane, which have a structural role and participate in the assembly and maintenance of the outer membrane or as receptor sites for siderophores, bacteriophages and bacteriocins (Jann & Jann, 1999). Alterations in the OMPs and the integrity of the lipopolysaccharide (LPS) influence the permeability of antimicrobial agents across the outer membrane of Gram negative bacteria (Giordano *et al,* 1993). It is thought that modification of the LPS structure plays an important role in the regulation of protein channels. Therefore, increased tolerance to antimicrobial agents may only be associated with observable changes in either the OMPs or LPS, whilst the mechanism of resistance is associated with both (Giordano *et al,* 1993).

P. aeruginosa contains a limited number of OMPs present in very high numbers. For example, OMP F is present at 10^5 copies per cell (Kragelund *et al,* 1996). OMP F is constitutively expressed and contains surface-exposed epitopes (Kragelund *et al,* 1996). The majority of OMPs are highly conserved between different serotypes of *P. aeruginosa* (Kragelund *et al,* 1996). *P. aeruginosa* is thought to contain only one non-specific OMP (OMP F), whereas *E. coli* contains a number of non-specific OMPs (Nikaido, 1992), for example, OMP A which is classed as a major OMP in *E. coli* (Sugawara *et al,* 1996). OMP F is thought to allow the passage of polysaccharides of at least 2000 Da. However, it is estimated that only 1 to 2 % of the channel functions as a transport channel and therefore, it demonstrates low permeability (Nikaido, 1992). Therefore, it is suggested that the majority

of solutes cross the outer membrane *via* specific OMPs. A number of specific OMPs have been identified from *P. aeruginosa* and associated with resistance to a variety of antimicrobial agents.

Various reports have associated resistance to antimicrobials with alterations in the outer membrane proteins of Gram negative bacteria. A reduction in the intensity of OMP F (38 kDa) and a prominent increase in a 41 kDa protein was associated with resistance towards erythromycin in *P. aeruginosa* S-6 (Tateda *et al,* 1994). The role of the 41 kDa protein is unknown, but it is possibly an altered form of the OMP F (Tateda *et al,* 1994). This is because the total amount of the 38 kDa and 41 kDa protein in the treated cells is equivalent to the total amount of 38 kDa in the pre-treated cells (Tateda *et al,* 1994). However, this was not observed in the PAO1 strain. Following sub-MIC treatment with aztreonam (a β-lactam antibiotic) an increase in copy number was observed in a 38 kDa protein in *P. aeruginosa* (OMP F). Alterations in the integrity of the LPS profile were also observed with the same exposure (Magni *et al,* 1994; Cipriani *et al,* 1991). A reduction in the expression of 35 kDa OMP of *P. aeruginosa* was associated with exposure to norfloxcin (Hostacka & Karelova, 1997b).

Imipenem is a broad-spectrum carbapenem ß-lactam antibiotic, which is highly active against *P. aeruginosa.* It is of particular interest because of its high potency, broad spectrum and the general lack of microbial cross-resistance, found with other ß-lactams during it's use (Ochs *et al,* 1999). Resistance towards the antibiotic developed at a significant rate, which was associated with the loss of OMP D (Huang *et al,* 1992). *P. aeruginosa* cells demonstrating resistance towards imipenem have recently been discovered to lack the protein D2 (Yoshihara *et al,* 1996). In addition to the loss of the OMP D2, Yamano *et al,* (1996) observed a decrease in the production in three other outer membrane proteins (C, E1 and E2) associated with resistance towards ß-lactams. They also demonstrated resistance towards the newer quinolones and chloramphenicol. The loss of

OMP D expression is thought to result from deletions in the OMP D coding region and the upstream promoter region (Ochs *et al,* 1999). The mutants isolated in the Huang *et al,* (1992) study also demonstrate resistance towards zwitterion carbapenem antibiotics. However, there is no apparent cross-resistance demonstrated towards other ß-lactam antibiotics. It is thought that OMP D is an imipenem and basic amino-acid-specific, channel-forming protein, due to the presence of a specific binding site in its channel (Huang *et al,* 1992). It has been suggested that D2 is composed of a 27 kDa domain which is responsible for channel formation and a 19 kDa domain which is responsible for gate formation (Yoshihara *et al,* 1996). The protein also demonstrates binding affinity towards imipenem and a similar basic amino acid structure, implying it forms a pore with specificity towards this structure (Yoshihara *et al,* 1996).

It was originally believed that many Gram-negative bacteria lacked a hydrophobic uptake pathway across their outer membranes. It is now thought that there may be appreciable trans-outer-membrane permeation of hydrophobic and amphipathic molecules (Hancock, 1998). Therfore, the reason for the resistance may be active efflux (Hancock, 1998). Active efflux proteins of wide specificity are common in wild-type bacteria and contribute significantly to intrinsic resistance of *P. aeruginosa* (Ma *et al,* 1994). The hydrophobic fluorescent probe (NPN) is only taken up by *P. aeruginosa* when treated with a polycation, such as an aminoglycoside, which breaks down the OM permeability barrier (Hancock, 1998). The NPN is rapidly effluxed unless an energy inhibitor is present (Hancock, 1998). Poole *et al,* (1993) demonstrated an efflux system involving three proteins, Mex A, Mex B and Opr M, which are critical for intrinsic resistance of *P. aeruginosa.* A knockout mutation involving the genes of any of these proteins results in a fourfold to tenfold increase in susceptibility to quinolones, ß-lactams (except imipenem), tetracycline and chloramphenicol. Resistance to quaternary ammonium compounds is mediated by efflux pumps such as *qac*A, *qac*E and *smr* in Gram-positive bacteria

(Chapman, personal communication) The *qac*A pump is a membrane bound proton-motive force dependent cation export protein, belonging to the major facilitator superfamily (MFS) of transport proteins (Chapman, personal communication).

Multipledrug resistance pumps (MDRs) were originally discovered in eukaryotic cells conferring drug resistance from tumour cells to a variety of pathogens (Ouellette *et al,* 1997). The eukaryotic MDRs differ from the prokaryotic ones in that they use ATP as their energy source instead of the proton motive force. However, they are all alike in that they function to effectively protect the cells from many commonly used drugs and are numerous in type and function (Ouellette *et al,* 1997). MDRs are common in eukaryotic microorganisms where they appear to be responsible for conferring drug resistance in pathogenic yeasts including *Cryptococcus neoformans* and *Candida albicans* (Ouellette *et al,* 1997). Two distinctive proteins are clearly involved in conferring drug resistance in *C. albicans*, one of which BEN^R belongs to the major facilitator superfamily and is involved in inferring resistance to benomyl, methotrexate and fluconazole (Ouellette *et al,* 1997). The second protein CDR1 is an ABC transporter and is associated with resistance towards fluconazole and other azole drugs (Ouellette *et al,* 1997).

The first evidence of MDRs in Gram-negative bacteria was reported in 1992 by Lomovskaya & Lewis, who discovered that an *E. coli* MDR protein (Emr AB) protects the cells against antimicrobials such as nalidixic acid and thiolactomycin (an antibiotic which inhibits fatty acid synthesis) (Lewis *et al,* 1997). Köhler *et al,* (1996) discovered that the overexpression of OMP M and OMP J was associated with resistance towards trimethoprim and sulfamethoxazole and demonstrated that the *mexABoprM* efflux system is the major reason for the resistance.

The aims of this chapter were to observe any OMP shifts during the induction of resistance to the isothiazolone biocides and thiomersal.

Outer Membrane Protein (OMP) Preparations

OMPs were prepared for SDS-PAGE analysis by the method of Pugsley *et al*, (1986). An overnight culture of *P. aeruginosa* ($1x10^9$ CFU mL^{-1}) was centrifuged (IEC Centra-4B) at 5000 *g* for 15 minutes. Cells were washed three times in 25 mM Tris buffer (pH 7.4) containing 1 mM L^{-1} $MgCl_2$. Cells were homogenised for 2.5 minutes on ice. Sarcosine (Sigma) was added to give a final concentration of 2% w/v, and samples were kept on ice for 20 minutes. The insoluble outer membranes were sedimented out at 11,500 g for 1h at 4°C. The supernatants were removed and the pellets were washed in deionised water (1 mL). The pellet was resedimented out at 11,500 g for 1h at 4°C. Samples were stored in the freezer until required.

Prior to SDS-PAGE analysis, the pellet was resuspended in 100 μL of Tris-HCl buffer (pH 7.4). 50 μL of sample was mixed with an equal volume of cracking buffer (Tris-HCl, 0.5M, pH 6.8, 3.75 mL; mercaptoethanol, 1.5 mL; SDS, 0.6 g; glycerol, 3 g; bromophenol blue stock, 0.1% w/v, 1 mL; pH 6.8 made up to 10 mL with distilled water). Samples were heated in boiling water bath (Gallenkamp) for 5 minutes immediately prior to loading of the gels. The remainder of the samples were stored at -18°C for protein estimation.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Preparation of Separating Gel Mixture

Stock acrylamide solution (10 mL; acrylamide, 73 g; bis-acrylamide, 2 g; dissolved in distilled water, 250 mL), distilled water (12 mL), and stock separating buffer (7.5 mL; SDS, 1 g; Tris buffer, 45.5 g; made up to 250 mL with deionised water, pH 8.0) were added together and degassed. Stock ammonium persulphate solution (10% w/v; 45 μL), and N,N,N',N'-tetramethyl-ethylenediamine (TEMED; 15 μL), were added to begin the polymerisation process. The mixture was mixed gently to avoid re-gassing, and poured into a prepared gel cast (0.5mm thick: mini-PROTEAN II, Biorad). The gel was overlayed with water-saturated butan-2-ol, and left to set for approximately 1h.

Preparation of Stacking Gel Mixture

Stock acrylamide solution (750 μL), distilled water (3 mL) and stock stacking gel buffer (1.25 mL; SDS, 1 g; Tris buffer, 15.1 g; made up to 250 mL with deionised water, pH 6.8) were mixed together and degassed. Stock ammonium persulphate solution (15 μL) and TEMED (5 μL) were added and the contents were gently mixed to avoid any re-gassing. The butan-2-ol overlay was removed from the polymerised 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 (0.5mm, 10 wells, Biorad) in place. Once the stacking gel had set the combs were gently removed and the wells were washed with distilled water to remove any traces of buffer.

The gel was transferred to a mini-PROTEAN II system, (Biorad) the upper and lower buffer chambers were filled with reservoir buffer (glycine, 0.192M; Tris, 0.025M; SDS, 0.1% w/w), ensuring the wells were filled with buffer.

Preparation of OMP Samples

An aliquot (250 μL) of Tris-buffer (0.5M, pH6.8) was added to the OMP pellets, an aliquot (100 μL) of this preparation was added to 100 μL of cracking buffer (Tris-HCl, 0.5M pH 6.8, 3.75 mL; ß-mercaptoethanol, 1.5 mL; SDS, 0.6 g; glycerol, 3 g; bromophenol blue, 0.1 %, 1 mL; made up to a total volume of 10 mL with distilled water, pH adjusted to pH 6.8). Samples were heated in a boiling water bath (Gallenkamp, England) for 10 minutes.

Preparation of Outer Membrane Protein Profiles

Molecular weight marker (SDS-7, Sigma or low molecular weight calibration kit for SDS electrophoresis, Amersham Pharmacia Biotech; 10 to 20 μL) and test samples (35 to 45 μL) were loaded into the wells using a pipette (Sealpipette, Jencons). A constant current (35 mA) was applied to the gels by a powerpack 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 Coomassie brilliant blue R250 stain or silver stained (Chapter 2). The protein concentrations for the test samples were estimated using the BCA assay (Chapter 2). The gels were analysed using Phoretix imaging analysis software (non-Linear Dynamics Ltd, Newcastle upon Tyne, England).

The Phoretix imaging system was used to estimate the molecular weights and Rf values of all the outer membrane proteins in the samples so that it was possible to establish any differences between the sensitive and resistant cultures.

Figure 30 shows the OMPs of the sensitive and resistant cultures to the biocide MIT. In order for the Phoretix imaging 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 marker lanes on the gel and any grimaces (this is the name designated by Phoretix for straightening the bands) on the gel were identified and corrected. The background of the gel was determined such that the system recognised the gel background and distinguished the protein bands. Once the protein peaks were detected the edges of the protein could then be identified, such that the system knew when the protein began and ended on the image. Figures 26 and 27 show the peak analysis of the molecular weight markers and OMPs isolated from passage 1 cells exposed to MIT. Once these preliminary stages of analysis had been established the molecular weights and Rf values were determined. In order to estimate the molecular weights the marker lanes were initially identified and the molecular weights assigned to the individual proteins (Figure 25). The system then computed the curve to relate the molecular weight to pixel position on the gel (Figure 28). The molecular weights were then be propagated on the gel. This is shown as the white lines on Figure 25. This was achieved by either propagating the molecular weights by the position of the molecular weights on the gel or the position of the molecular weights between two standard lanes. The Rf values were then computed and propagated along the gel, where (0) indicates the beginnings of the gel and (1) indicates the end of the gel. The measurement data propagated for Figure 25 is given in Table 15. The band number, position on the gel (given as pixel position), molecular weight and Rf value of the protein bands are also given.

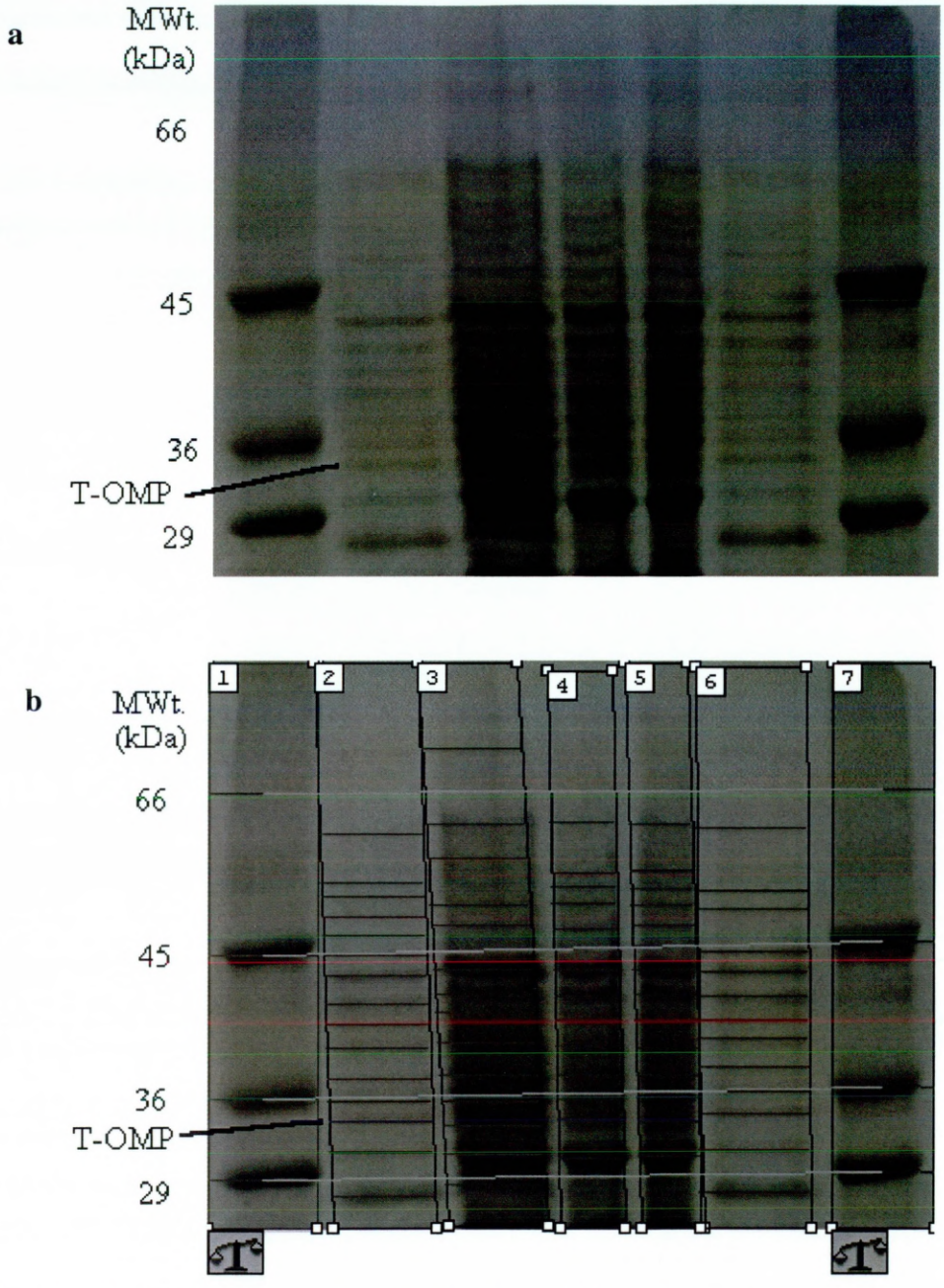


Figure 25 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to MIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 1 cells; Lane 4: passage 11 cells; Lane 5: passage 14 cells; Lane 6: control cells; Lane 7: molecular weight markers. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 24 μg mL^{-1}; Lane 4: 22μg mL^{-1}; Lane 5: 6 μg mL^{-1}.

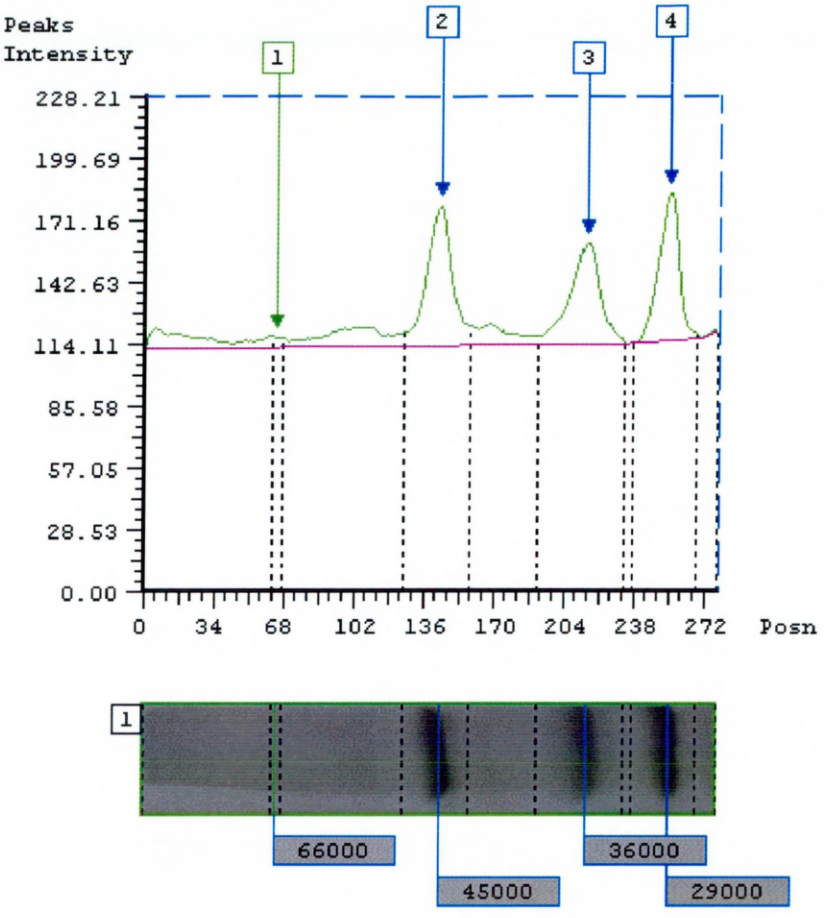


Figure 26: Peak Analysis of Molecular Weight Marker Lane. Upper graph indicates Peak Intensity in comparison to Pixel position (Posn), which is related to band presence in lower band image. Numbers in boxes on the graph indicate peak and band position. Numbers on the band image box indicate the molecular weights of the individual bands.

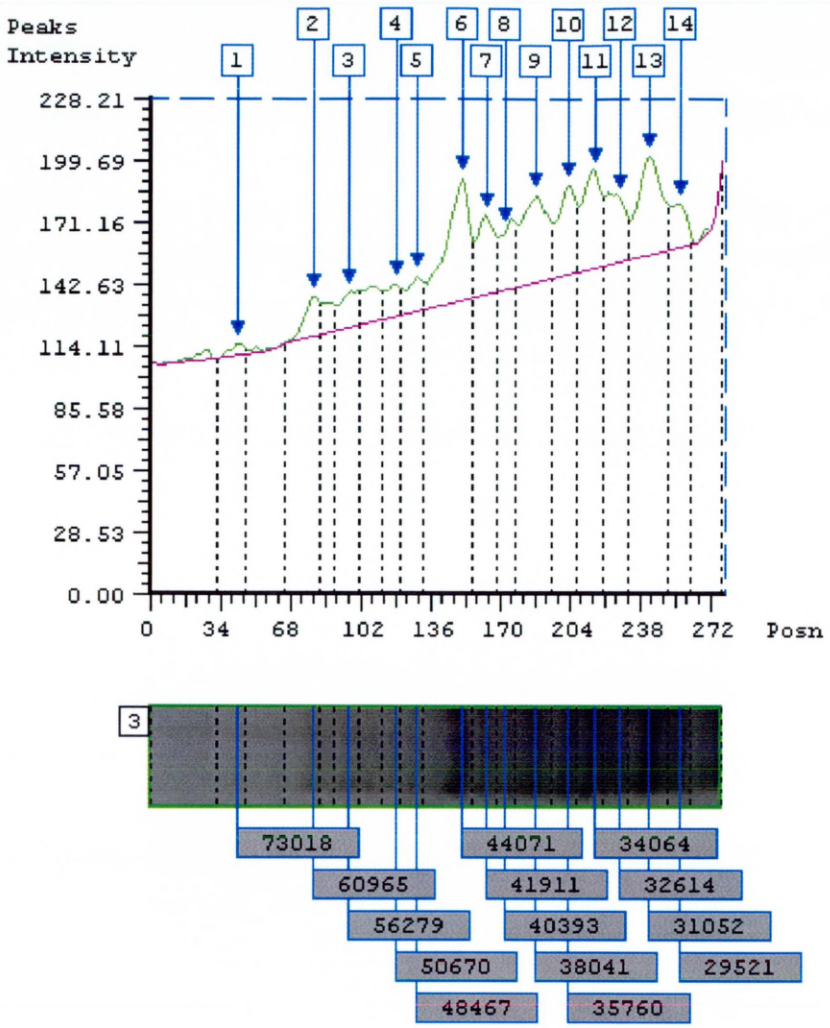


Figure 27: Peak Analysis of Outer Membrane Proteins of Passage One cells of *Pseudomonas aeruginosa* PAO1 exposed to MIT. Upper graph indicates Peak Intensity in comparison to Pixel position, which is related to band presence in the lower band image. Numbers in boxes on the graph indicate band position; numbers in boxes on the image indicate the molecular weights of the individual bands.

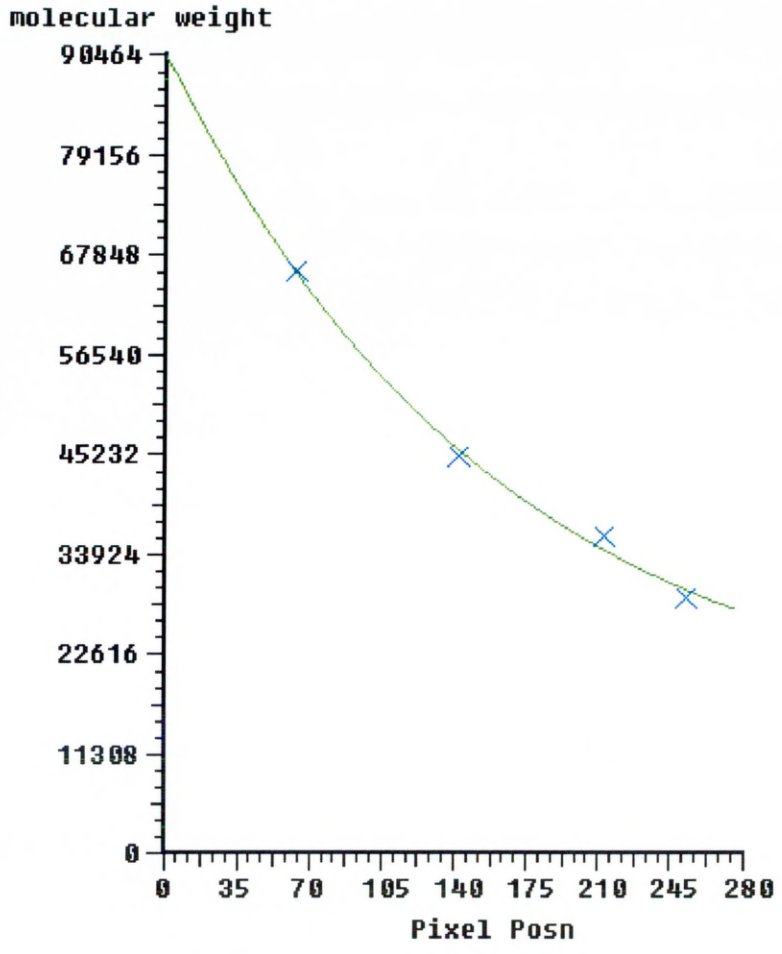


Figure 28: Calibration of molecular weight in Relation to Pixel Position on the gel in order to propagate the molecular weight across the gel and therefore, estimate the molecular weights of all the observed protein bands in the gel.

Table 15: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 exposed to MIT and cultured in R2A media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane 2; N: novel protein).

Lane 1 (molecular weight markers)					Lane 2 (control cells)					Lane 3 (passage 1 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	64	66000	0.23	-	1	84	59624	0.30	-	1	42	73018	0.15	N
2	144	45000	0.52	-	2	108	53405	0.39	-	2	79	60965	0.28	1
3	215	36000	0.77	-	3	115	51752	0.41	-	3	96	56279	0.35	N
4	255	29000	0.91	-	4	125	49508	0.45	-	4	119	50670	0.43	3
					5	134	47599	0.48	-	5	129	48467	0.46	4
					6	144	45595	0.52	-	6	151	44071	0.54	6
					7	154	43706	0.55	-	7	163	41911	0.59	N
					8	168	41245	0.60	-	8	172	40393	0.62	8
					9	178	39609	0.64	-	9	187	38041	0.67	10
					10	190	37769	0.68	-	10	203	35760	0.73	11
					11	205	35647	0.73	-	11	216	34064	0.78	12
					12	216	34208	0.77	-	12	228	32614	0.82	13
					13	226	32978	0.81	-	13	242	31052	0.87	14
					14	241	31266	0.86	-	14	257	29521	0.92	15
					15	263	29017	0.94	-					

Lane 5 (passage 11 cells)					Lane 6 (passage 14 cells)					Lane 7 (molecular weight markers)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	74	61100	0.27	N	1	79	60503	0.28	N	1	79	59913	0.29	1
2	99	54178	0.36	N	2	102	54178	0.37	N	2	110	51702	0.40	3
3	106	52436	0.39	2	3	111	51953	0.40	3	3	119	49617	0.43	4
4	114	50542	0.41	3	4	119	50084	0.43	4	4	140	45205	0.51	6
5	127	47670	0.46	5	5	129	47882	0.46	5	5	150	43310	0.54	7
6	147	43705	0.53	7	6	139	45821	0.50	6	6	162	41195	0.58	8
7	160	41395	0.58	8	7	151	43520	0.54	7	7	174	39240	0.63	9
8	172	39432	0.63	9	8	163	41395	0.58	8	8	183	37873	0.66	10
9	185	37474	0.67	10	9	176	39276	0.63	9	9	197	35901	0.71	11
10	199	35546	0.72	11	10	190	37188	0.68	10	10	209	34349	0.75	12
11	211	34029	0.77	12	11	203	35415	0.73	11	11	220	33031	0.79	13
12	221	32853	0.80	12	12	214	34029	0.77	12	12	238	31070	0.86	14
13	240	30823	0.87	N	13	226	32627	0.81	13	13	259	29058	0.94	15
					14	243	30823	0.87	N					

An outer membrane protein in the range of between 31 and 36 kDa was observed to have disappeared following exposure to all four biocides (Table 16). There were slight variations in the observed molecular weight of this OMP, which may be due to variations in scanning and exact estimation of the Rf values estimated by the computer software (Table 17). However, observation of the gel image indicates that T-OMP was positioned above a more prominent OMP at 31266 Da (Figure 25). The mean of the estimated values for T-OMP in this investigation was 33 kDa, with a standard deviation of 1.1718 kDa. Brözel and Cloete (1994) observed T-OMP to be at 35 kDa, however, they did not use an imaging software package to estimate the molecular weights of the proteins. The T-OMP disappeared after the first passage in the presence of the biocides MIT and Thiomersal in R2A medium and BIT and MIT in CDM medium (Table 17). The T-OMP disappeared after the third passage in the presence of the biocide BIT when cultured in R2A medium. However, when exposed to the biocide CMIT, the OMP did not disappear until the third passage when cultured in R2A and it was not definitely absent until after the sixth passage in the presence of biocide. CMIT is the most reactive of the four biocides used in the study and a significant increase in the MIC was not observed until the third or fourth passage in the presence of biocide (Figures 19 and 20). Thus, this may account for T-OMPs failure to disappear completely until the seventh passage. OMP profiles of the cells exposed to MIT, BIT and Thiomersal are given if Figures 25, 29 and 30 respectively and the measurement data propagated by the Phoretix software is given in Tables 15, 16 and 17 respectively. There were some variations in the number of outer membrane proteins in the control cells and resistant passages, however, the most prominent difference was the absence of T-OMP. All of the passages from 1 to 12 of the cells exposed to CMIT cultured in CDM are shown in Figures 25 to 33 and the measurement data is shown in Tables 21 to 24. It was not evident from the gels that T-OMP disappears until passage four, after which T-OMP is clearly absent until passage 11.

Once the cells were cultured in biocide free medium, T-OMP reappeared. This was clearly observed in Figures 25 and 29 to 33. T-OMP was clearly visible in passage 12 cells exposed to CMIT cultured in CDM (Figure 33). The protein was also clearly present in the

passage 14 cells exposed to BIT and MIT cultured in R2A (Figures 25 and 29). This implies the loss of T-OMP is a phenotypic adaptation related to the direct presence of the biocide and not a genotypic adaptation.

Table 16: Observation of the presence of T-OMP following exposure to the isothiazolone biocides and thiomersal in R2A media and CDM medium.

Passage	R2A media				CDM		
	BIT	MIT	CMIT	Thio.	BIT	MIT	CMIT
1	+	+	+	+	+	+	+
2	+	-	+	-	-	-	+
3	+	-	-	-	-	-	+
4	-	-	-	-	-	-	+/-
5	-	-	-	-	-	-	+/-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	+	-	+	+/-	-	+	+
13	+	+	+	+	-	+	+
14	+	+	+	+	+	+	+

+ = presence of T-OMP

- = absence of T-OMP

Table 17: Estimation of the Molecular Weight of T-OMP by the Phoretix Software.

Biocide	Media	Passage Number	Molecular Weight (Da)
BIT	R2A	1, 11, 14	32249
MIT	R2A	1, 11 ,14	32978
CMIT	R2A	1, 11 ,14	35803
Thiomersal	R2A	1, 11 ,14	31224
BIT	CDM	4, 5	36577
MIT	CDM	1, 11 , 14	32978
CMIT	CDM	1 – 4	33297
CMIT	CDM	5 – 8	33138
CMIT	CDM	9 – 12	32310
CMIT	CDM	13, 14	31581

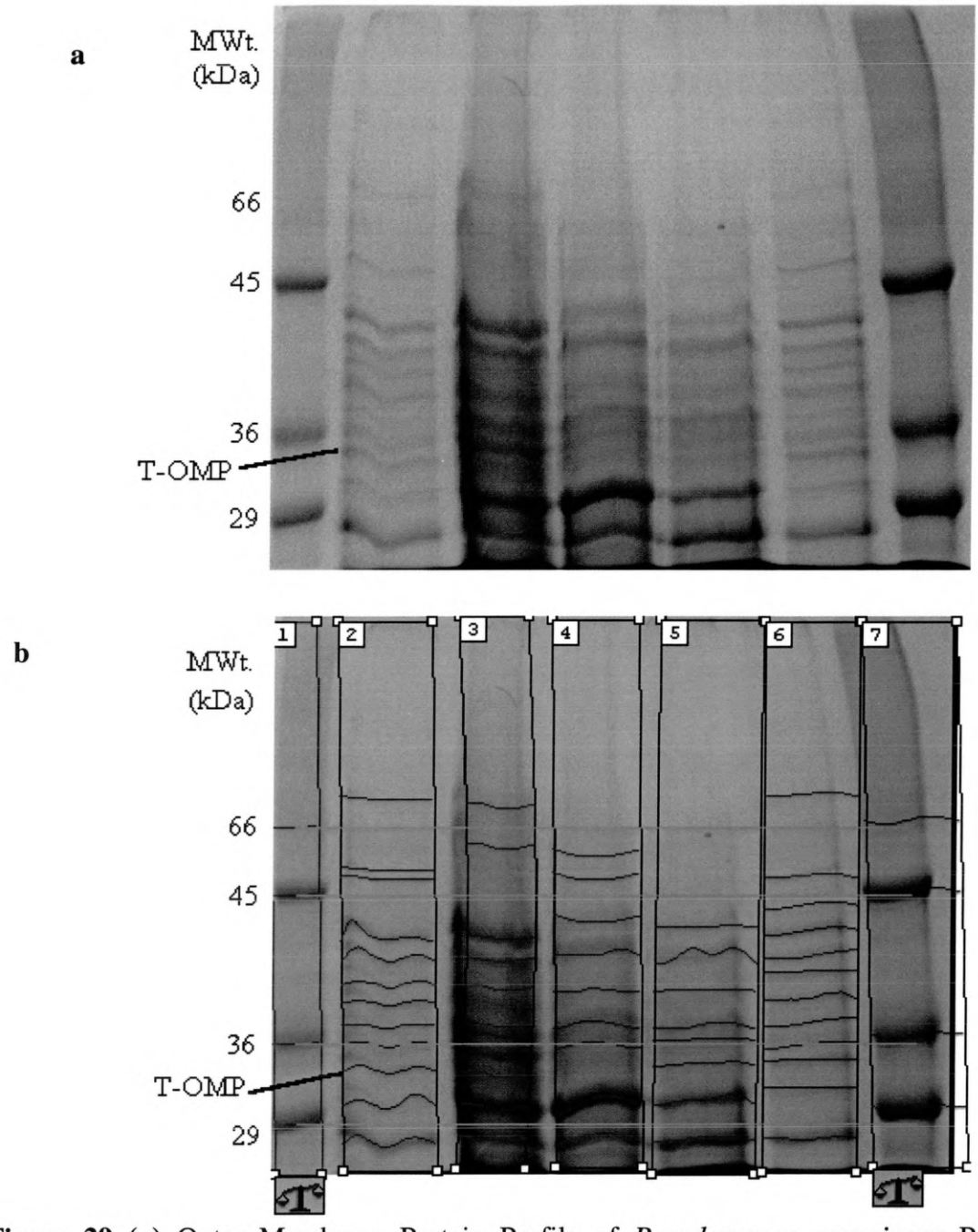


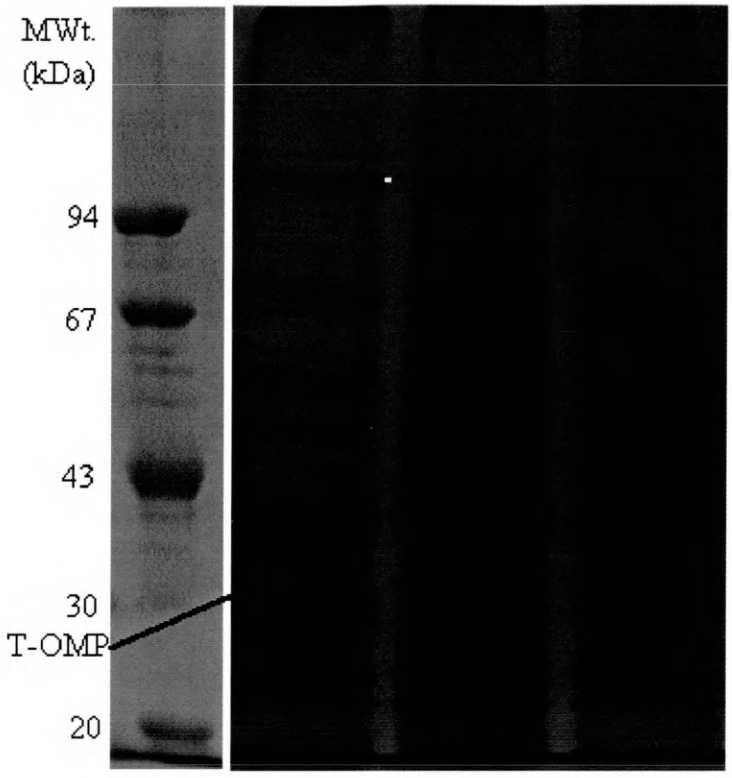
Figure 29 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to BIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 1 cells; Lane 4: passage 11 cells; Lane 5: passage 14 cells; Lane 6: control cells; Lane 7: molecular weight markers. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 60 μg mL^{-1}; Lane 4: 14μg mL^{-1}; Lane 5: 20 μg mL^{-1}.

Table 18: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 Exposed to BIT cultured in R2A media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane 2: N: novel protein)

Lane 1 (molecular weight markers)					Lane 2 (control cells)					Lane 3 (passage 1 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	117	66000	0.37	-	1	99	82543	0.32	-	1	106	78222	0.34	N
2	115	45000	0.49	-	2	139	52368	0.44	-	2	129	59488	0.41	N
3	240	36000	0.76	-	3	145	49700	0.46	-	3	180	40088	0.57	4
4	286	29000	0.91	-	4	181	39290	0.58	-	4	193	37729	0.61	5
					5	192	37421	0.61	-	5	210	35494	0.67	6
					6	206	35604	0.66	-	6	231	33659	0.73	7
					7	216	34605	0.69	-	7	236	33332	0.75	8
					8	229	33591	0.73	-	8	249	32631	0.79	9
					9	240	32930	0.77	-	9	259	32211	0.82	10
					10	255	32249	0.81	-	10	277	31650	0.88	11
					11	275	31625	0.88	-	11	295	31270	0.94	12
					12	297	31188	0.95	-					

Lane 4 (passage 11 cells)					Lane 5 (passage 14 cells)					Lane 6 (control cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	130	57663	0.42	N	1	174	40958	0.55	4	1	99	82543	0.32	1
2	143	50991	0.46	3	2	195	37127	0.62	5	2	145	49700	0.46	3
3	167	42674	0.54	N	3	211	35178	0.67	6	3	163	43493	0.52	N
4	190	37887	0.61	5	4	232	33459	0.73	8	4	178	39882	0.57	4
5	211	35178	0.68	6	5	239	33039	0.76	9	5	191	37573	0.61	5
6	232	33459	0.74	8	6	254	32327	0.80	10	6	199	36444	0.63	6
7	244	32776	0.78	9	7	276	31625	0.87	11	7	215	34695	0.68	7
8	282	31485	0.90	11	8	297	31204	0.94	12	8	230	33524	0.73	8
9	299	31173	0.96	12						9	243	32776	0.77	9
										10	250	32452	0.80	10
										11	265	31903	0.84	11
										12	294	31236	0.94	12

a



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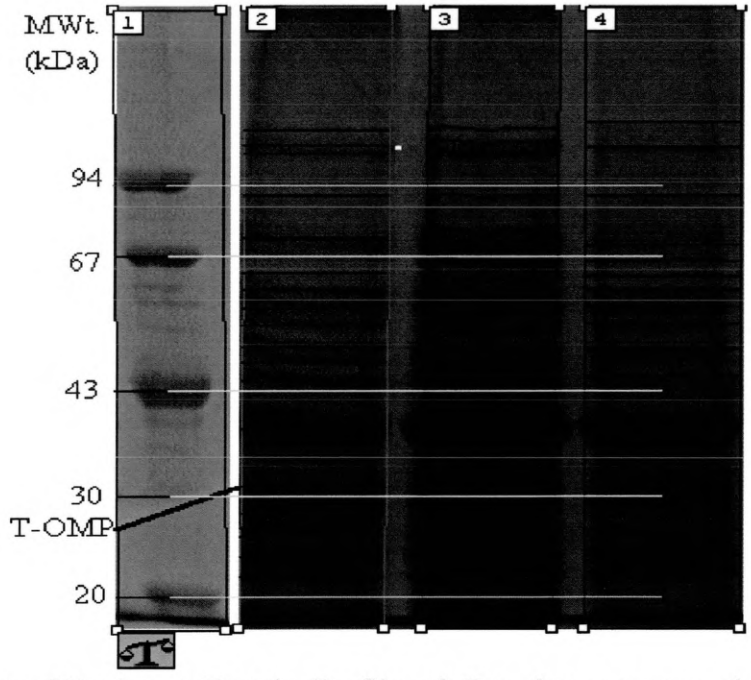

Figure 30 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to MIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 5 cells; Lane 4: passage 6 cells;. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 14 $\mu g\ mL^{-1}$; Lane 4: 14 $\mu g\ mL^{-1}$.

Table 19: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 Exposed to Thiomersal cultured in R2A media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane2; N: novel protein)

Lane 1 (molecular weight markers)					Lane 2 (control cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	105	94000	0.29	-	1	3	224796	0.01	-
2	145	67000	0.40	-	2	72	125349	0.20	-
3	223	43000	0.62	-	3	82	115175	0.22	-
4	283	30000	0.78	-	4	87	110402	0.24	-
5	343	20100	0.95	-	5	123	80801	0.34	-
					6	139	70693	0.38	-
					7	150	65085	0.41	-
					8	171	56879	0.47	-
					9	178	54657	0.49	-
					10	193	50451	0.53	-
					11	204	47690	0.56	-
					12	212	45783	0.58	-
					13	223	43230	0.61	-
					14	253	36438	0.70	-
					15	284	30000	0.78	-
					16	298	27376	0.82	-
					17	312	24944	0.86	-
					18	329	22248	0.91	-
					19	341	20513	0.94	-

Lane 3 (passage 5 cells)					Lane 4 (passage 6 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	72	124293	0.20	2	1	72	124293	0.20	2
2	86	110402	0.24	4	2	84	112287	0.23	3
3	116	85181	0.32	N	3	115	85942	0.32	N
4	140	69593	0.39	6	4	124	79412	0.34	5
5	152	63736	0.42	N	5	142	68529	0.39	6
6	161	60069	0.45	N	6	150	64628	0.42	N
7	172	56224	0.48	8	7	170	56879	0.47	8
8	180	53762	0.50	9	8	179	54057	0.50	9
9	193	50191	0.53	10	9	192	50451	0.53	10
10	204	47448	0.57	11	10	204	47448	0.57	11
11	211	45783	0.58	12	11	220	43692	0.61	13
12	250	36878	0.69	14	12	254	36000	0.70	14
13	296	27557	0.82	16	13	295	27739	0.82	16
14	313	24612	0.87	17	14	312	24777	0.86	17
15	330	21949	0.91	18	15	327	22399	0.91	18
16	342	20237	0.95	19	16	338	20793	0.94	19
17	364	17471	1.00	-	17	364	17471	1.00	-

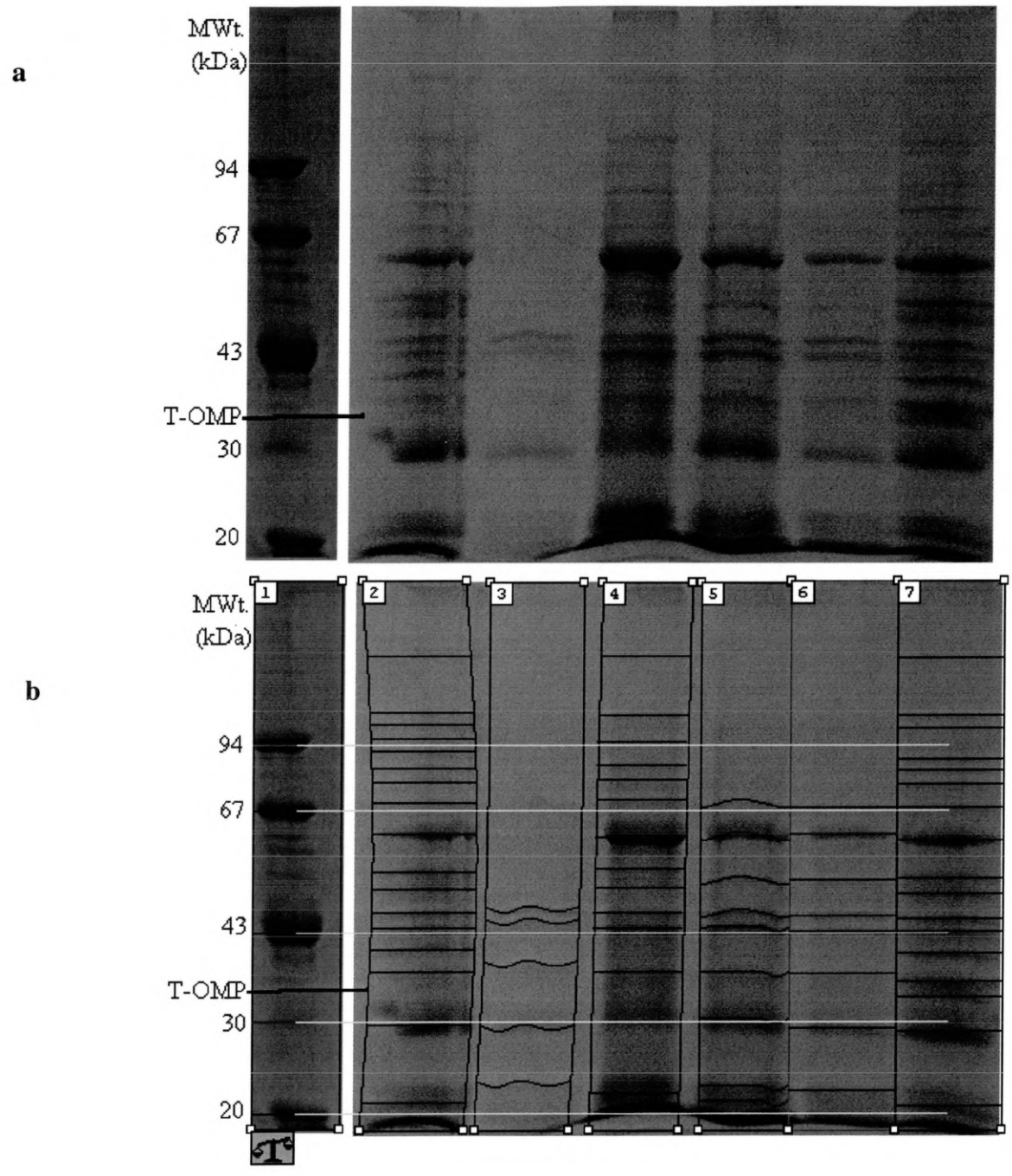

Figure 31 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to CMIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 1 cells; Lane 4: passage 2 cells; Lane 5: passage 3 cells; Lane 6: passage 4 cells; Lane 7: control cells. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 1 μg mL^{-1}; Lane 4: 2μg mL^{-1}; Lane 5: 2 μg mL^{-1}; Lane 6: 2 μg mL^{-1}.

Table 20: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 Exposed to CMIT (Passage 1-4) cultured in CDM media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane 1; N: novel protein).

Lane 1 (control cells)					Lane 2 (passage 1 cells)					Lane 3 (passage 2 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	33	149956	0.09	-	1	208	46948	0.58	13	1	2	192548	0.01	N
2	71	114701	0.20	-	2	248	36635	0.69	15	2	43	143706	0.12	1
3	79	108467	0.22	-	3	280	30333	0.78	17	3	82	109226	0.23	3
4	91	99786	0.25	-	4	324	23790	0.90	4	4	101	95728	0.28	5
5	96	96392	0.27	-	5	346	21240	0.96	5	5	116	86341	0.32	N
6	106	89970	0.30	-						6	122	82870	0.34	N
7	115	84586	0.32	-						7	137	74842	0.38	N
8	130	76377	0.36	-						8	162	63304	0.45	N
9	151	66321	0.42	-						9	183	55140	0.51	N
10	172	57721	0.48	-						10	191	52351	0.53	11
11	186	52691	0.52	-						11	210	46356	0.58	13
12	199	48467	0.55	-						12	219	43799	0.61	14
13	209	45485	0.58	-						13	248	36635	0.69	16
14	220	42451	0.61	-						14	280	30333	0.78	17
15	238	37993	0.66	-						15	326	23541	0.91	N
16	249	35549	0.69	-						16	337	22233	0.94	18
17	272	31041	0.76	-						17	350	20819	0.97	19
18	327	22993	0.91	-										
19	339	21563	0.94	-										

Lane 4 (passage 3 cells)					Lane 5 (passage 4 cells)					Lane 6 (control cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	136	75862	0.38	8	1	144	71874	0.40	N	1	45	141683	0.13	1
2	157	65880	0.44	9	2	161	64150	0.45	9	2	85	106966	0.24	2
3	187	54075	0.52	11	3	189	53377	0.53	11	3	90	103309	0.25	N
4	209	46948	0.58	13	4	213	45773	0.59	13	4	111	89354	0.31	6
5	222	43254	0.62	14	5	224	42717	0.62	14	5	121	83438	0.34	7
6	245	37534	0.68	15	6	251	36196	0.70	15	6	128	79551	0.36	N
7	227	31041	0.77	17	7	285	29646	0.79	N	7	144	71392	0.40	N
8	318	24693	0.89	N	8	325	23790	0.91	18	8	164	62470	0.46	11
9	326	23665	0.91	18	9	345	21455	0.96	19	9	186	54075	0.52	N
10	339	22119	0.94	19						10	199	49723	0.55	12
										11	214	45199	0.60	13
										12	224	42451	0.62	14
										13	236	39413	0.66	15
										14	254	35336	0.71	16
										15	264	33297	0.74	N
										16	286	29310	0.80	N
										17	330	23053	0.92	18
										18	346	21240	0.96	19

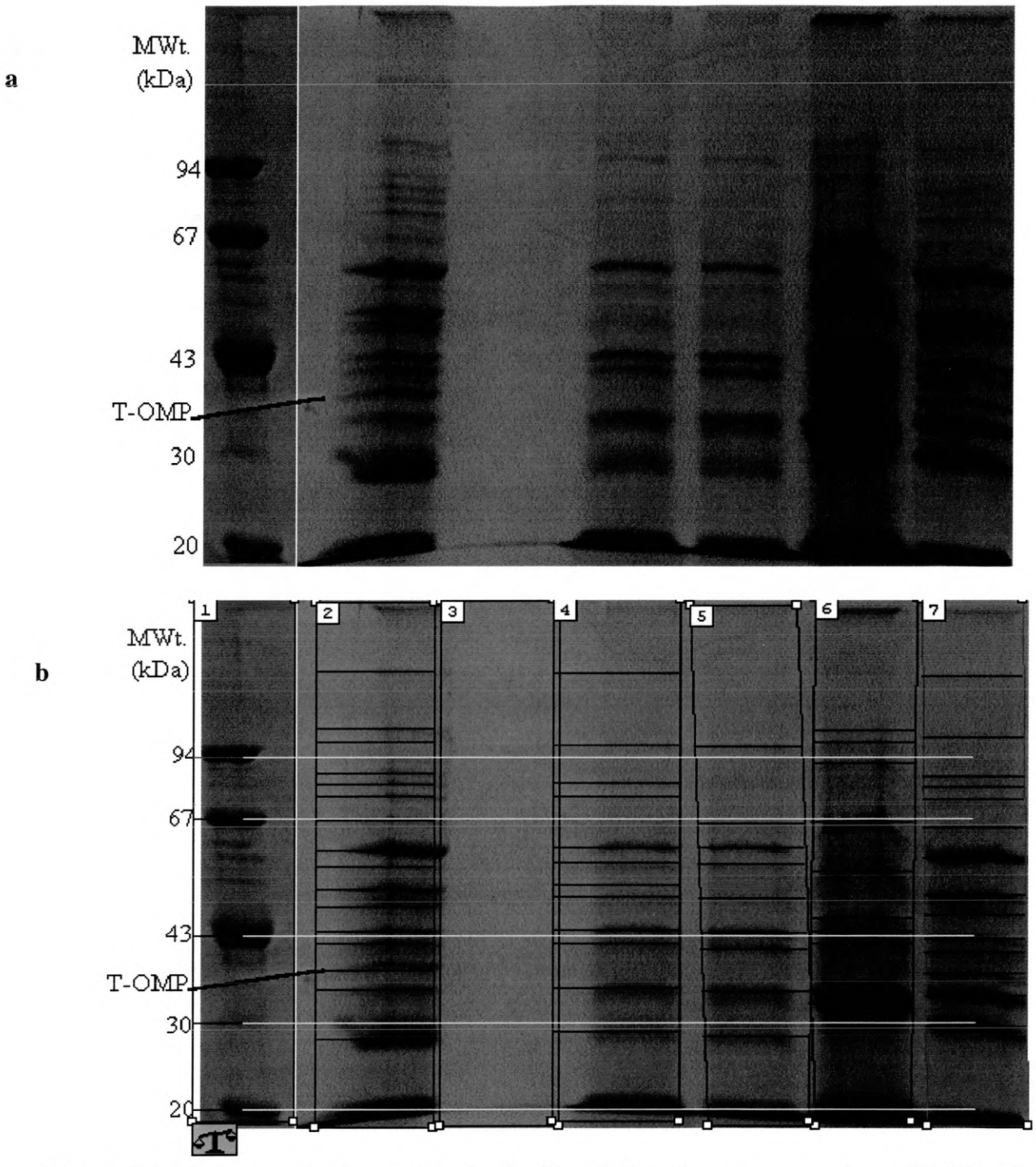

Figure 32 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to CMIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 5 cells; Lane 4: passage 6 cells; Lane 5: passage 7 cells; Lane 6: passage 8 cells; Lane 7: control cells. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 1 μg mL^{-1}; Lane 4: 8μg mL^{-1}; Lane 5: 20 μg mL^{-1}; Lane 6: 40 μg mL^{-1}.

Table 21: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 Exposed to CMIT (Passage 5 to 8) cultured in CDM media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane 1; N: novel protein)

Lane 1 (control cells)					Lane 2 (passage 5 cells)					Lane 3 (passage 6 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	46	130813	0.13	-	1	338	20587	0.96	17	1	3	183219	0.01	N
2	84	98457	0.24	-						2	46	131807	0.13	1
3	104	85051	0.30	-						3	95	91481	0.27	N
4	112	80272	0.32	-						4	119	76886	0.34	5
5	119	76338	0.34	-						5	144	64434	0.41	7
6	128	71599	0.36	-						6	161	57306	0.46	8
7	142	64885	0.40	-						7	176	51786	0.50	9
8	164	55767	0.47	-						8	187	48146	0.53	N
9	177	51100	0.50	-						9	195	45697	0.55	10
10	190	46901	0.54	-						10	217	39729	0.62	12
11	200	43962	0.57	-						11	227	37351	0.64	13
12	218	39238	0.62	-						12	257	31276	0.73	N
13	229	36674	0.65	-						13	285	26801	0.81	16
14	242	33927	0.69	-						14	339	20587	0.96	17
15	258	30921	0.73	-										
16	292	25699	0.83	-										
17	340	20405	0.97	-										

Lane 4 (passage 7 cells)					Lane 5 (passage 8 cells)					Lane 6 (control cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	20	159490	0.06	N	1	4	180407	0.01	N	1	44	132810	0.13	1
2	33	144392	0.09	N	2	84	98457	0.24	2	2	82	99921	0.23	2
3	94	91481	0.27	N	3	93	92153	0.26	N	3	88	95598	0.25	N
4	146	63103	0.41	7	4	105	84436	0.30	3	4	112	80272	0.32	4
5	162	56530	0.46	8	5	125	73140	0.36	N	5	120	75794	0.34	5
6	177	51100	0.50	9	6	146	63103	0.41	7	6	127	72108	0.36	6
7	195	45402	0.55	10	7	177	51100	0.50	9	7	144	63987	0.41	7
8	220	38755	0.63	12	8	216	39729	0.61	12	8	167	54646	0.47	8
9	228	36898	0.65	13	9	265	29725	0.75	15	9	191	46597	0.54	10
10	258	30921	0.73	15	10	340	20405	0.97	17	10	204	42851	0.58	11
11	284	26801	0.81	16						11	219	38995	0.62	12
12	342	20226	0.97	17						12	232	36014	0.66	13
										13	246	33138	0.70	14
										14	260	30573	0.74	15
										15	281	27233	0.80	16
										16	346	19877	0.98	17

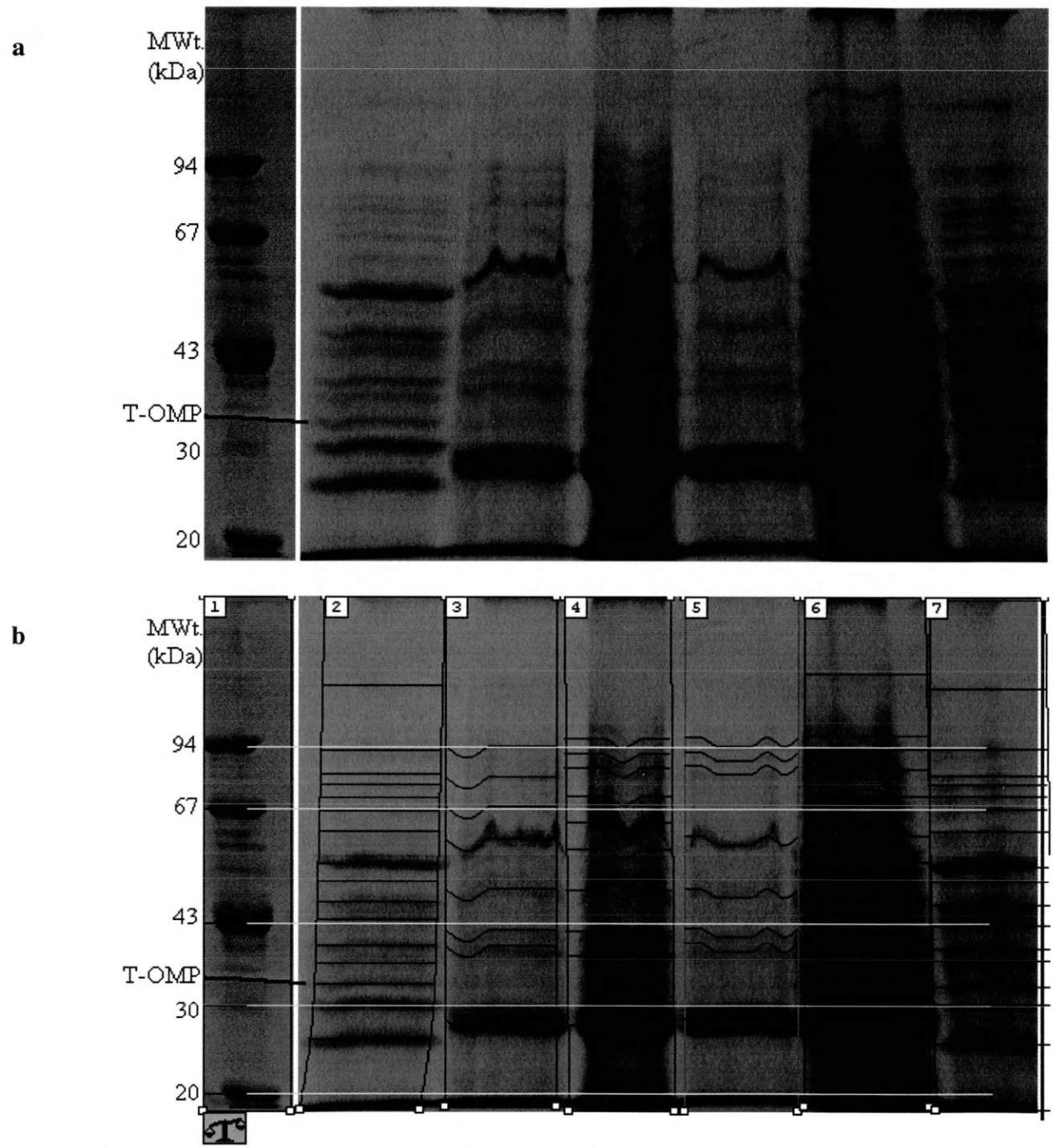

Figure 33 (a) Outer Membrane Protein Profile of *Pseudomonas aeruginosa* PAO1 cells cultured in R2A medium and exposed to CMIT. Lane 1: molecular weight markers; Lane 2: control cells; Lane 3: passage 9 cells; Lane 4: passage 10 cells; Lane 5: passage 11 cells; Lane 6: passage 12 cells; Lane 7: control cells. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 3: 10 μg mL^{-1}; Lane 4: 16μg mL^{-1}; Lane 5: 2 μg mL^{-1}; Lane 6: 48 μg mL^{-1}.

Table 22: Measurement Data indicating Band Position, Molecular Weight and Rf value for Outer Membrane Proteins of *Pseudomonas aeruginosa* PAO1 Exposed to CMIT (Passage 9 to 12) cultured in CDM media. (MW: molecular weight; Rf: refraction factor; Posn: band position on gel; Co: corresponding protein to lane 1; N: novel protein).

Lane 1 (control cells)					Lane 2 (passage 9 cells)					Lane 3 (passage 10 cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	2	177070	0.01	-	1	95	89158	0.28	3	1	87	94445	0.26	N
2	57	117532	0.17	-	2	105	83002	0.31	4	3	108	81248	0.32	4
3	96	88521	0.28	-	3	115	77314	0.34	5	4	130	69581	0.38	6
4	101	85407	0.30	-	4	135	67200	0.40	6	5	144	63145	0.42	N
5	117	76229	0.35	-	5	153	59368	0.45	8	6	184	48234	0.54	10
6	130	68581	0.38	-	6	190	46377	0.56	11	7	214	39768	0.63	N
7	137	66274	0.40	-	7	227	36678	0.67	13	8	229	36230	0.68	14
8	152	59775	0.45	-	8	276	27508	0.81	17	9	274	27816	0.81	17
9	170	52927	0.50	-	9	324	21385	0.96	18	10	323	21490	0.95	18
10	181	49197	0.53	-										
11	194	45187	0.57	-										
12	204	42370	0.60	-										
13	222	37829	0.65	-										
14	233	35355	0.69	-										
15	246	32696	0.73	-										
16	261	29948	0.77	-										
17	283	26464	0.83	-										
18	324	21385	0.96	-										

Lane 4 (passage 11 cells)					Lane 5 (passage 12 cells)					Lane 6 (control cells)				
Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co	Band	Posn	MW	Rf	Co
1	90	92423	0.27	N	1	3	177070	0.01	1	1	54	118400	0.16	2
2	101	85407	0.30	4	2	51	123753	0.15	N	2	94	88521	0.28	3
3	113	78416	0.33	5	3	87	95130	0.26	N	3	112	77863	0.33	5
4	136	66736	0.40	7	4	98	87888	0.29	3	4	118	74634	0.35	N
5	156	58168	0.46	8	5	109	81248	0.32	N	5	126	70560	0.37	6
6	169	53282	0.50	9	6	128	71055	0.38	N	6	134	66736	0.40	7
7	186	47605	0.55	10	7	154	59368	0.45	8	7	148	60598	0.44	8
8	214	39768	0.63	N	8	168	54002	0.50	9	8	170	52224	0.50	9
9	223	37595	0.66	13	9	188	47295	0.55	10	9	178	49523	0.53	10
10	275	27661	0.81	17	10	222	38065	0.65	13	10	194	44605	0.57	11
11	321	21703	0.95	18	11	250	32120	0.74	15	11	205	41568	0.60	12
					12	281	26904	0.83	17	12	222	37363	0.65	13
					13	323	21596	0.95	18	13	231	35355	0.68	14
										14	246	32310	0.73	15
										15	260	29776	0.77	16
										16	282	26319	0.83	17
										17	324	21177	0.96	18

The Use of Two-Dimensional Polyacrylamide Gel Electrophoresis to investigate T-OMP shifts in *Pseudomonas aeruginosa* PAO1

Two-dimensional gel electrophoresis (2-D PAGE) was introduced by O'Farrell (1975). It is a powerful tool in the separation and quantification of proteins from complex mixtures due to its resolution and sensitivity. Proteins are separated in the first dimension by isoelectric focussing according to their isoelectric point and in the second dimension according to molecular weight by SDS polyacrylamide gel electrophoresis. The system separates individual proteins as discrete spots on gels and is sufficiently sensitive as to resolve proteins differing in a single charge. The first-dimensional stage (isoelectric focussing; IEF) of the technique can be performed in two different ways. The first-dimensional gel (tube-gel) can be pre-run (without sample) to establish the pH gradient, this is known as the pre-formed stage. Alternatively, the sample can be immediately applied to the tube-gel and the pH gradient established during the isoelectric focussing stage (the non-equilibrium pH gradient electrophoresis technique; NEPHGE).

Preparation of Glass Tubes for the First-Dimensional stage

The glass tubes (1 mm diameter) were soaked in 5% Decon (BDH) overnight, rinsed thoroughly in deionised water and placed in fresh potassium hydroxide solution (KOH, 0.4 g; ethanol, 20 mL). The tubes were rinsed in deionised water, placed in 100% ethanol and then allowed to air dry. Once dried the tubes were sealed with three layers of parafilm and placed in the pouring stand. The tubes were marked 5 mm from the top to ensure all tubes gels were the same length in order to facilitate reproducibility between runs.

Preparation of Tube-Gel (First Dimension Protein Separation)

An aliquot (10 mL) of the gel mixture (urea, 5.5 g; acrylamide stock, 1.33 mL; acrylamide, 14.19 g; bis-acrylamide, 0.8 g; deionised water, 50 mL), Igepal CA-630 stock, 2 ml, 10 v/v; Resolyte (BDH, Poole, pH 3.5 to 10, 0.3 mL), Resolyte (BDH, Poole, pH 4 to

8, 0.2 mL); deionised water, 1.97 mL) was prepared. The flask was mixed constantly at 37°C in a water bath (Gallenkamp, England) until the urea completely dissolved. The gel mixture was then degassed. For the NEPHGE method ammonium persulphate (20 μL; 10% w/v) and TEMED (14 μL) were added. The solution was loaded into gel tubes using a syringe with a narrow gauge hypodermic needle. The tubes were filled approximately 5 mm from the top. The gels were overlaid with gel overlay solution (20 μL; urea, 4.81 g; deionised water, 10 mL) and allowed to polymerise for one hour. The tubes were removed from the pouring stand, parafilm carefully removed and the gel overlay solution was removed. The surface of the gel was rinsed three times with deionised water.

Isoelectric Focusing of Protein Samples

The tube gels were placed in the 2-D gel rig and the bottom reservoir chamber was filled with freshly prepared 0.2M NaOH. An aliquot (20 μL) of lysis buffer (urea, 9.5M; Igepal CA-630, 10% v/v; dithiothreitol, 1M; Resolyte (BDH, pH 3.5 to 10, 0.3 mL); Resolyte (BDH, pH 4 to 8, 0.2 mL); distilled water, 3 mL) was added to the surface of the tube gels. An aliquot (20 μL) of the test samples containing 9M urea was added to the tube gels. The samples were overlaid with sample overlay (10 μL; urea, 8M; Resolyte pH 3.5 to 10, 250 μL; distilled water, 5 mL). The top reservoir chamber was filled with freshly prepared 0.1M H_3PO_4 ensuring the remainder of the tubes was filled and no air bubbles were present. The system was run at 500V for five hours (2197 power supply, LKB). The tube gels were then removed from the glass tubes, rolled in parafilm and placed in SDS sample buffer (5 mL; glycerol, 10% w/v; DTT, 15 w/v; SDS, 2.3% w/v; Tris, 0.0625M; pH adjusted to pH 6.8) for 30 minutes. The tubes were then stored at -20°C for later analysis.

Preparation of Slab Gel (Second Dimension Protein Separation)

Stock acrylamide solution (10 mL; acrylamide, 73 g; bis-acrylamide, 2 g; distilled water, 250 mL), distilled water (12 mL), and stock separating buffer (7.5 mL; SDS, 1 g; Tris buffer, 45.5 g; made up to 250 mL with deionised water, pH 8.0) were added together and degassed. Stock ammonium persulphate solution (45 μL; 10% w/v), and N,N,N',N'-

tetramethyl-ethylenediamine (TEMED; 15 μL), were added to begin the polymerisation process. The mixture was mixed gently to avoid re-gassing, and poured into a prepared gel cast (1.0 mm thick: mini-PROTEAN II, Biorad). The gel was overlayed with water-saturated butan-2-ol, and left to set for approximately 1h. The butan-2-ol was removed and the surface of the gel was rinsed with deionised water. The tube gel which had previously been defrosted and placed in fresh SDS-sample buffer for 30 minutes was placed on the surface of the separating slab gel, ensuring no air bubbles were present between the tube and slab gels. A thin layer of 0.1% bromophenol blue was placed along the surface of the tube gel. The gel was transferred to a mini-PROTEAN II system, (Biorad). The upper and lower buffer chambers were filled with reservoir buffer (glycine, 0.192M; Tris, 0.025M; SDS, 0.1% w/w). A constant current (35 mA) was applied to the gels by a powerpack (LKB) for approximately 3 h, or until the bromophenol blue front reached the bottom of the gel. The gels were removed from the mini-PROTEAN II system and silver stained (Chapter 2).

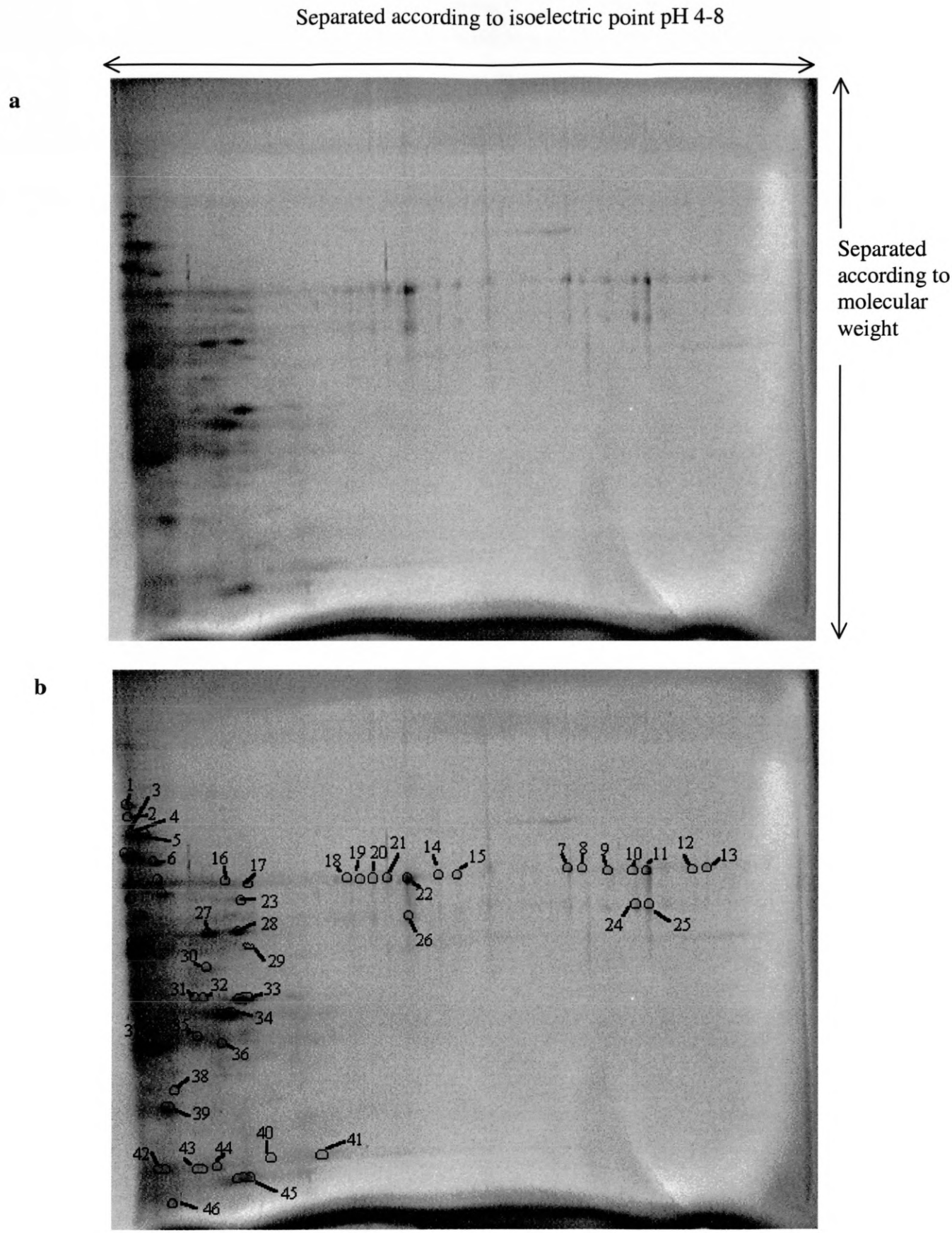

Figure 34: **(a)** Two-Dimensional Gel Electrophoresis of outer membrane protein (OMP) profiles of sensitive cells of *Pseudomonas aeruginosa* PAO1 cultured in R2A medium. The gel exhibits OMPs separated by the isoelectric point (reading from left to right) and molecular weight (reading top to bottom). **(b)** The same gel showing the position of the identified proteins.

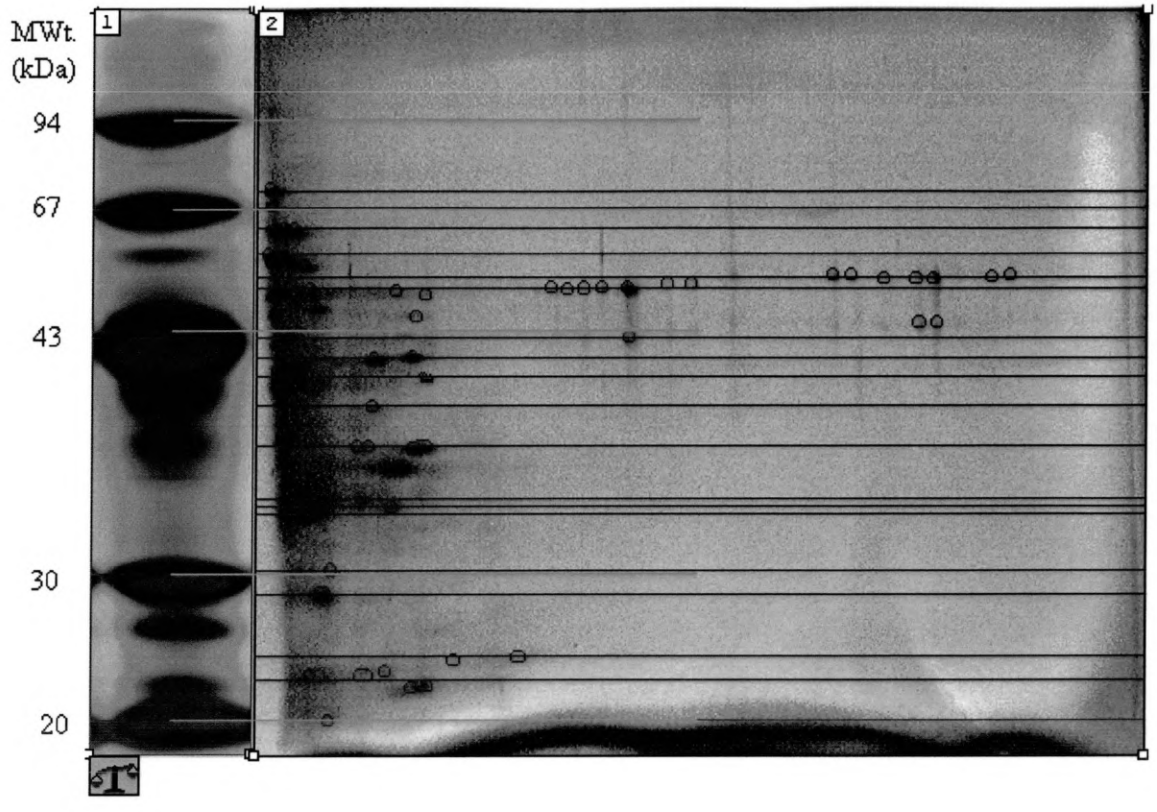

Figure 34 (c):Two-dimensional gel electrophoresis of outer membrane protein (OMP) profile of sensitive cells of *Pseudomonas aeruginosa* PAO1 cultured in R2A medium. The same gel image as previously shown in figures 34 a and b after Phoretix treatment. Lane 1 is a molecular weight marker, where the protein origin and end point have been prepared to exactly correspond to the origin and end point of the two-dimensional gel. Black lines on the image indicate the molecular weight of specific marker proteins chosen from the two-dimensional gel. White lines on the image indicate computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Lane 2 illustrates the separation of the protein sample, the total protein concentration of this lane is 0.08 μg ml^{-1}.

Table 23: Measurement data indicating Protein position, Molecular weight and Isoelectric points of the proteins identified from the Two-Dimensional gel analysis of the OMPs from the sensitive cells of *Pseudomonas aeruginosa* PAO1 and the appropriate corresponding protein numbers for the resistant cells.

Sensitive cells		Corresponding protein number of resistant cells				
Protein Number	MWt (Da).	IEF	BIT	MIT	CMIT	Thio
1	71440	4	nd	-	nd	-
2	66946	4	nd	-	nd	-
3	62276	4	nd	-	nd	-
4	62276	4.1	nd	-	nd	-
5	62276	4.2	nd	-	nd	-
6	56983	4	nd	-	nd	-
7	52650	6.6	nd	-	nd	-
8	52650	6.7	nd	-	nd	-
9	52650	6.9	nd	-	nd	-
10	52650	7	nd	-	nd	-
11	52650	7.1	nd	8	nd	-
12	52650	7.4	nd	-	nd	-
13	52650	7.5	nd	-	nd	-
14	51735	4.75	nd	-	nd	-
15	51735	5.9	nd	-	nd	-
16	50820	4.65	nd	10	nd	-
17	50096	5.4	nd	11	nd	-
18	50820	5.5	nd	-	nd	-
19	50820	5.6	nd	-	nd	-
20	50820	5.65	nd	-	nd	-
21	50820	5.7	nd	-	nd	-
22	50820	5.8	nd	-	nd	-
23	46158	7	nd	-	nd	-
24	46158	7	nd	-	nd	-
25	46158	7.1	nd	22	nd	-
26	43741	4.55	nd	-	nd	-
27	41330	4.7	nd	-	nd	-
28	41330	4.8	nd	-	nd	-
29	39342	4.5	nd	27	nd	-
30	36523	4.8	nd	-	nd	-
31	**33272**	**4.65**	**nd**	**30**	**nd**	**-**
32	**33272**	**4.7**	**nd**	**30**	**nd**	**-**
33	**33272**	**4.8**	**nd**	**31**	**nd**	**-**
34	**31642**	**4.7**	**nd**	**33**	**nd**	**22**
35	29889	4.65	nd	34	nd	27
36	29489	4.8	nd	35	nd	-
37	29105	4.1	nd	-	nd	28
38	26565	4.5	nd	-	nd	-
39	25700	4.45	nd	-	nd	-
40	23784	5.2	nd	-	nd	32
41	23784	5.5	nd	-	nd	-
42	23316	4.2	nd	-	nd	33
43	23316	4.5	nd	-	nd	-
44	23595	4.7	nd	-	nd	-
45	23236	4.8	nd	-	nd	-
46	22512	4.4	nd	-	nd	34

nd = not possible to triangulate proteins; - = no appropriate protein; embolden: T-OMP range

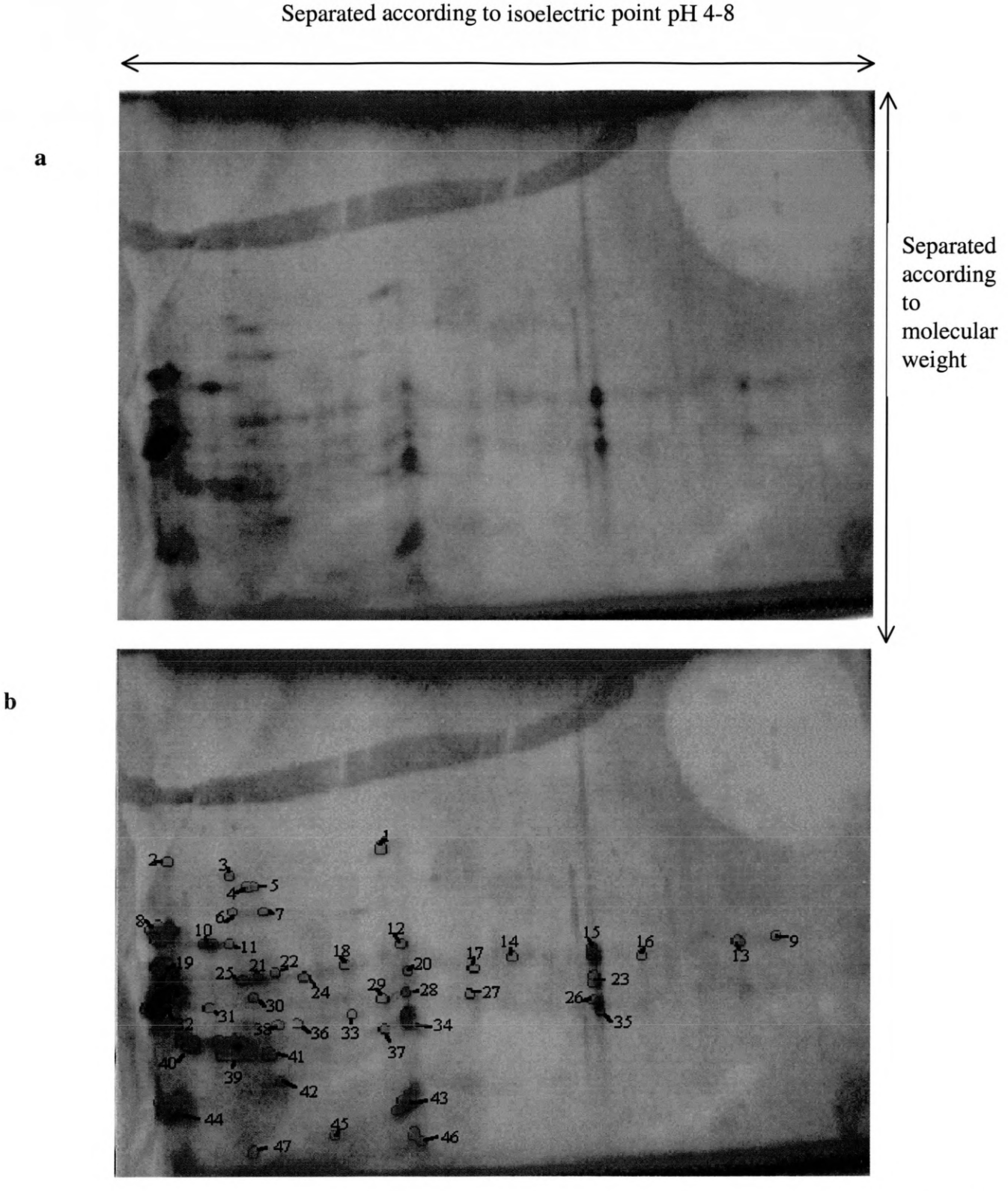

Figure 35: **(a)** Two-Dimensional Gel Electrophoresis of outer membrane protein (OMP) profiles of cells of *Pseudomonas aeruginosa* PAO1 resistant to BIT cultured in R2A medium. The gel exhibits OMPs separated by the isoelectric point (reading from left to right) and molecular weight (reading top to bottom). **(b)** The same gel image indicating the presence of identifiable separated proteins, which were then plotted for gel position using their molecular weight and isoelectric focussing conditions. The total protein concentration is 0.08 μg mL^{-1}.

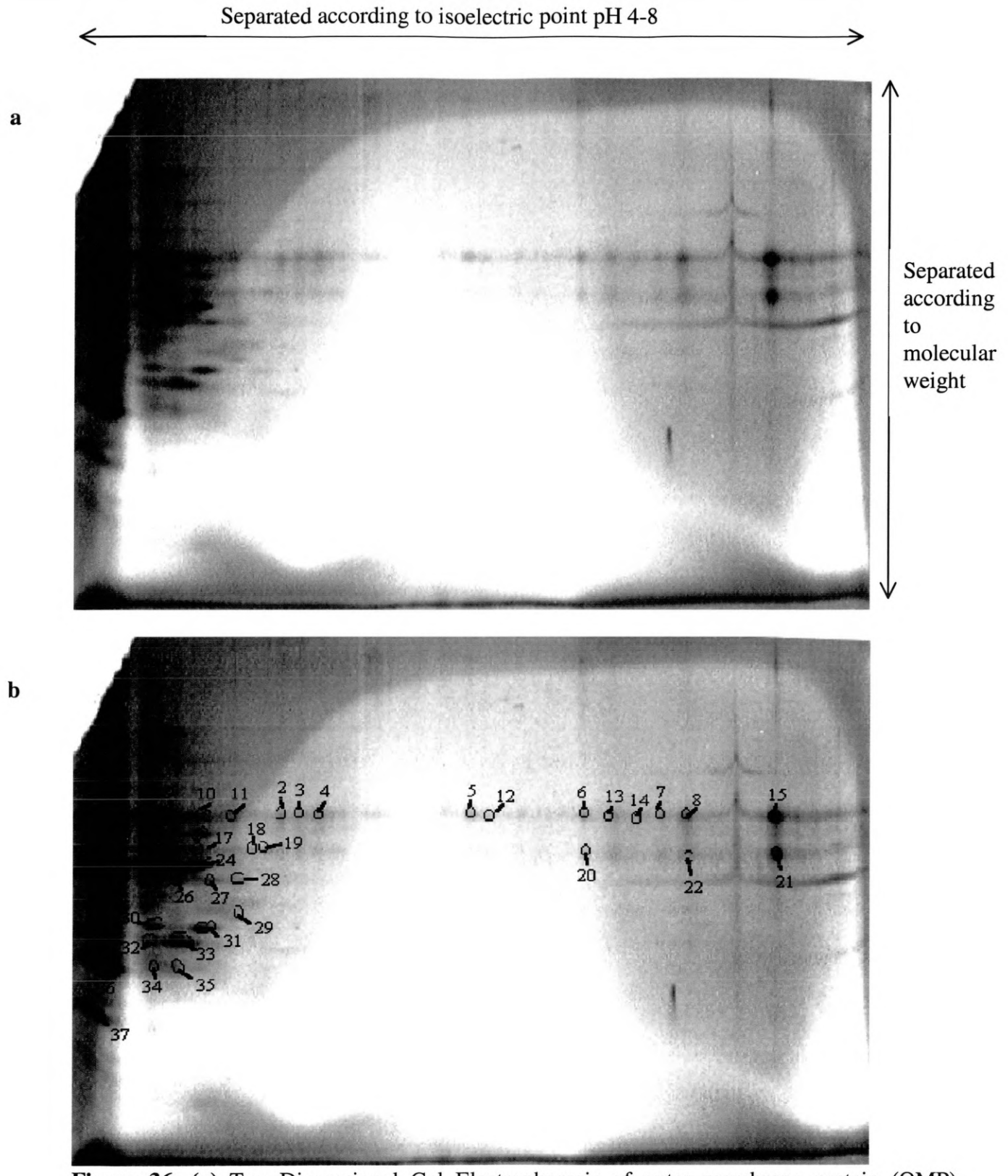

Figure 36: **(a)** Two-Dimensional Gel Electrophoresis of outer membrane protein (OMP) profiles of cells of *Pseudomonas aeruginosa* PAO1 resistant to MIT cultured in R2A medium. The gel exhibits OMPs separated by the isoelectric point (reading from left to right) and molecular weight (reading top to bottom). **(b)** The same gel image indicating the presence of identifiable separated proteins, which were then plotted for gel position using their molecular weight and isoelectric focussing conditions. The total protein concentration is 0.23 μg mL^{-1}.

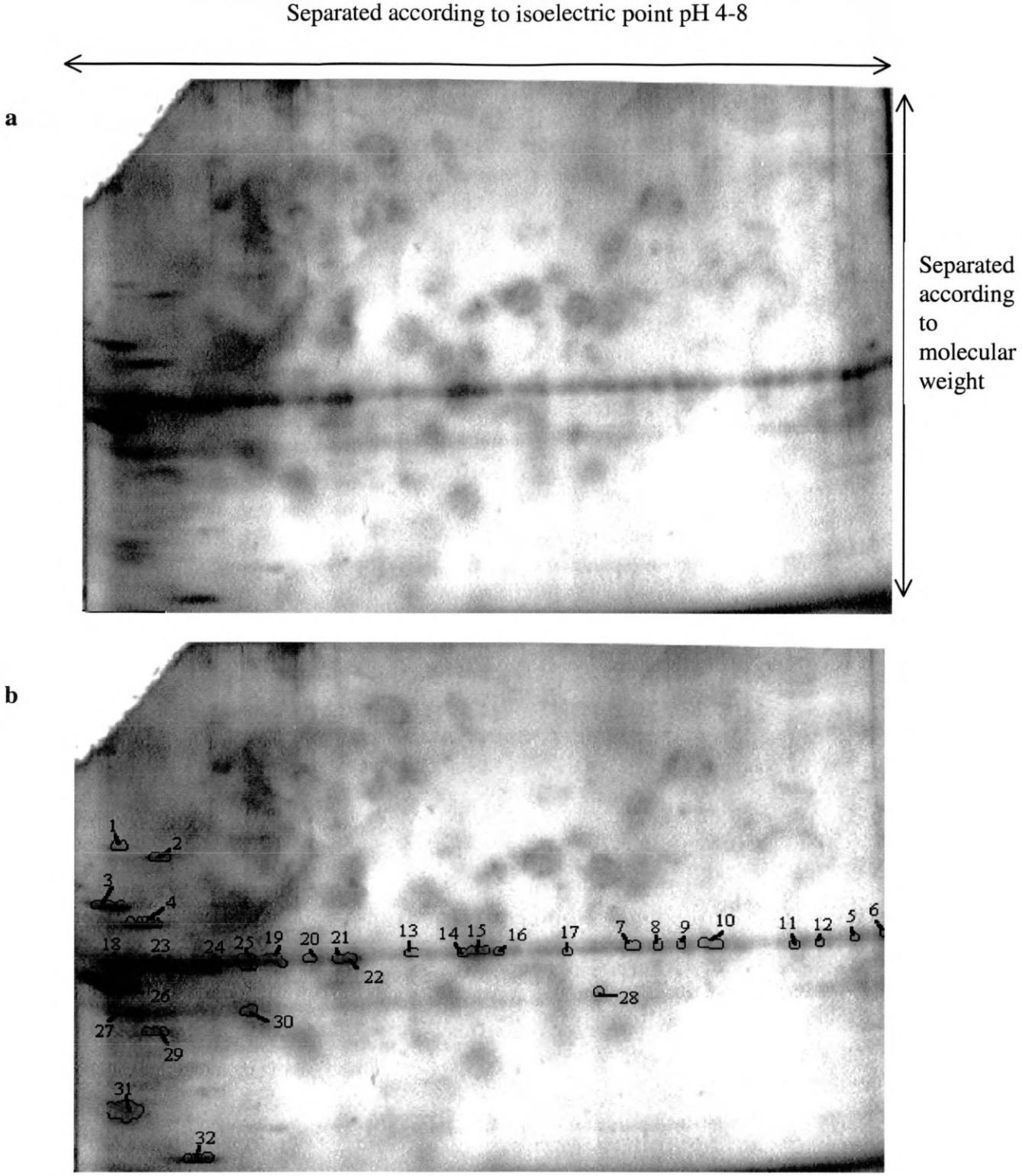

Figure 37: **(a)** Two-Dimensional Gel Electrophoresis of outer membrane protein (OMP) profiles of cells of *Pseudomonas aeruginosa* PAO1 resistant to CMIT cultured in R2A medium. The gel exhibits OMPs separated by the isoelectric point (reading from left to right) and molecular weight (reading top to bottom). **(b)** The same gel image indicating the presence of identifiable separated proteins, which were then plotted for gel position using their molecular weight and isoelectric focussing conditions. The total protein concentration is 0.12 μg mL^{-1}.

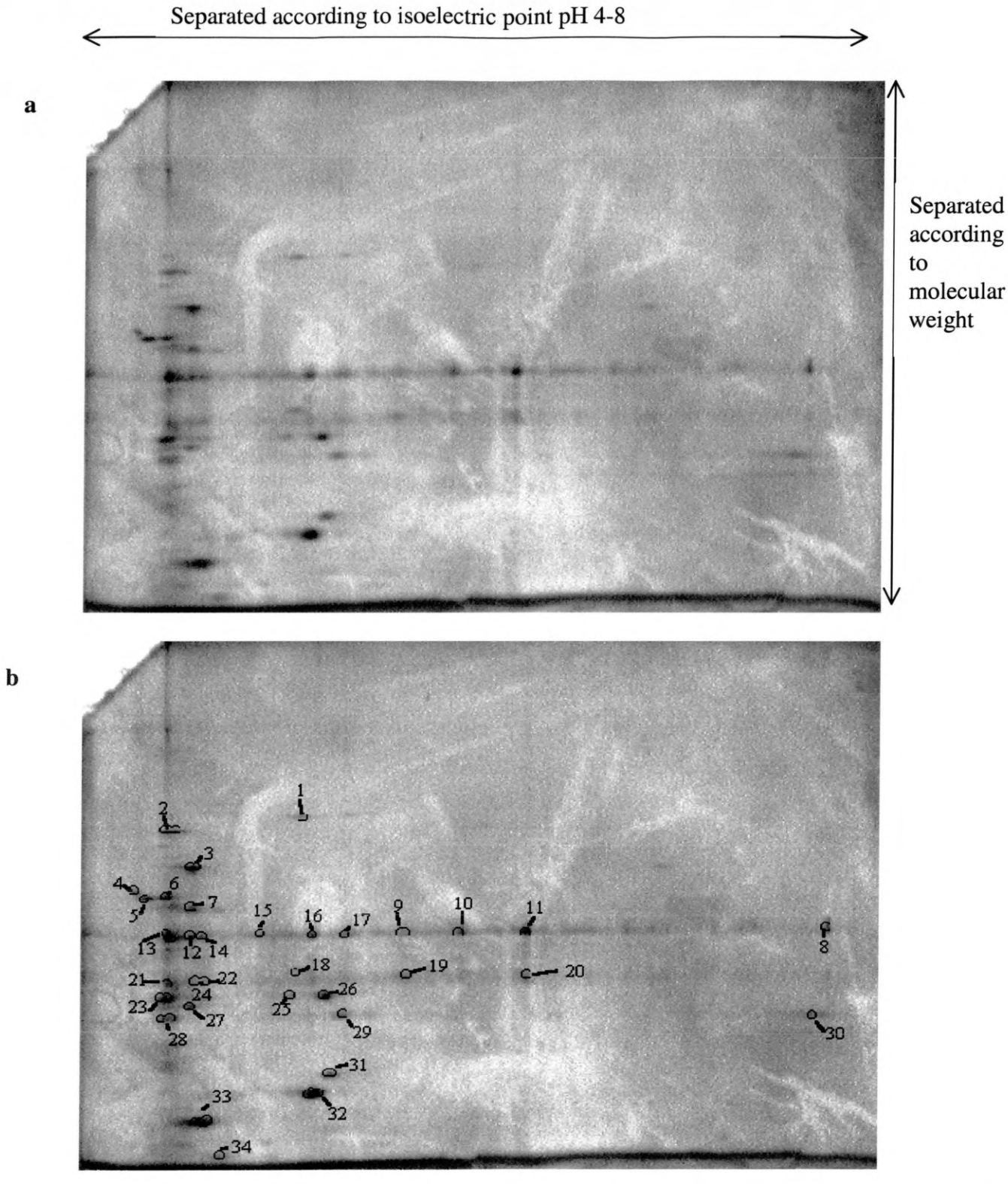

Figure 38: **(a)** Two-Dimensional Gel Electrophoresis of outer membrane protein (OMP) profiles of cells of *Pseudomonas aeruginosa* PAO1 resistant to Thiomersal cultured in R2A medium. The gel exhibits OMPs separated by the isoelectric point (reading from left to right) and molecular weight (reading top to bottom). **(b)** The same gel image indicating the presence of identifiable separated proteins, which were then plotted for gel position using their molecular weight and isoelectric focussing conditions. The total protein concentration is 0.08 $\mu g\ mL^{-1}$.

Discussion

Figure 34 illustrates sensitive cells of *P. aeruginosa* PAO1 separated by two-dimensional gel electrophoresis according to molecular weight and isoelectric point. The individual numbered proteins (Figure 34b) and their corresponding estimated molecular weights (analysed by the Phoretix software; Figure 34c) and isoelectric points are shown in Table 23. It has already been suggested by the one-dimensional SDS-PAGE analysis that the molecular weight of T-OMP is between the range of 31.8 - 34.1 kDa and therefore, the protein T-OMP may be any of the proteins between numbers 31-34 (Figure 34b). The OMP profiles of the resistant cultures were analysed by a triangulation method against proteins from the sensitive cells. Key proteins that were visible in the sensitive and resistant cells were identified and compared, such that the molecular weight and isoelectric points of the resistant cells could be estimated against that of the sensitive cells. This method of analysis was relatively successful for the cells resistant to MIT (Figure 36) and thiomersal (Figure 38). It was possible to compare the proteins in order to isolate the region with the appropriate molecular weight. The sensitive cell proteins 33 and 34 appear to be present in the cells resistant to MIT with the corresponding numbers of 31 and 33 respectively (Figures 36). It is not clear as to whether protein 30 of the cells resistant to MIT is representative of one or both of the proteins 31 and 32 from the sensitive cells. The cells resistant to thiomersal appear to be have three proteins missing from the T-OMP range in comparison to the sensitive cells (numbers 31, 32 and 33). The triangulation method of analysis in comparison to the sensitive gel was not successful for cells resistant to BIT and CMIT. Therefore, it was not possible to conclude whether T-OMP had disappeared in the resistant cells in these cases. Therefore, it is not possible to definitely conclude whether or not T-OMP is present in the resistant cells when using the two-dimensional gel electrophoresis technique. However, the gels do illustrate differences between the sensitive and resistant cells. It is possible to identify a greater number of proteins in the sensitive cells than those resistant to MIT, CMIT and thiomersal. Hence, this may be an indication that significant changes are occurring in the protein profiles, apart from the previously noted loss of T-OMP.

Changes in the Outer Membrane Protein Profiles of *Pseudomonas aeruginosa* PAO1 dependent upon specific Nutrient Limitation.

The outer membrane proteins of *P. aeruginosa* PAO1 were analysed following periods of nutrient limitation. This was performed in order to eliminate the possibility that the alterations in the outer membrane were a result of nutrient limitation and not biocide exposure. In conditions of nutrient limitation the cell becomes economical with its metabolic processes, in order to, redirect the energy towards more essential services. This may result in T-OMP shifts. *P. aeruginosa* was cultured in chemically defined medium (Dinning, 1995) which was limited for sources of carbon, nitrogen, potassium and phosphorus at separate time intervals. The growth period was observed for 24 hours, at the end of this period the cells were centrifuged and stored for later analysis of their OMP profiles using SDS-PAGE.

Preparation of overnight culture

P. aeruginosa was inoculated into a deionised flask (Chapter 2) containing an aliquot (25 mL) of non-limited chemically defined medium (Chapter 2). The culture was incubated for 19 hours at 37°C, 200 osc min^{-1}. This culture was used to inoculate the nutrient limited flasks.

Preparation of nutrient limited media

The nutrient limited media were prepared in deionised glassware. The glassware was soaked overnight in 5% Decon 90 (BDH) then rinsed in deionised water. The glassware was soaked in 1% HCl for at least one hour and then rinsed six times in deionised water (Lee *et al,* 1982).

Limitation of Potassium and Phosphorus

When potassium and phosphorus were limited an alternative buffering system was prepared in order to maintain the pH of the medium whilst removing K_2HPO_4 and

KH_2PO_4. The alternative buffering system was prepared by mixing solution A (75 mL; trizma hydrochloride, 1.815 g; maleic acid, 1.74 g; dissolved in 75 mL of deionised water), solution B (0.2N NaOH; 65.5 mL) and 157.5 mL of deionised water. The NH_4Cl (0.3 g) normally present in solution A of the chemically defined buffer was added to the alternative buffering system. Varying dilutions of potassium chloride (KCl) in concentrations ranging from 1.2×10^{-4} to 3.0×10^{-2} M were added during the limitation of the potassium. The normal concentration of phosphorus was added *via* phosphoric acid (H_3PO_4). In order to limit the phosphorus available to the cells, varying concentrations of H_3PO_4 were prepared in the range of 1.9×10^{-5} to 3.8×10^{-2} M. The normal concentration of potassium was added to the cells *via* KCl.

Limitation of carbon

The limitation of carbon was achieved by limiting the concentration of glycerol. Ten flasks were prepared ranging from a glycerol concentration of 1×10^{-4} to 1M.

Limitation of nitrogen

The limitation of nitrogen was achieved by limiting the concentration of ferric ammonium citrate to the chemically defined medium. Nine flasks were prepared with concentrations ranging from 1.908×10^{-5} to 3.818×10^{-2}.

Growth of P. aeruginosa under conditions of nutrient limitation

The flasks containing the nutrient limited medium were inoculated from an overnight culture of *P. aeruginosa*. The cultures were grown at 37°C, 200 osc min^{-1} for 24 hours. Optical density readings were taken at hourly intervals 0 to 8 hours and at 24 hours. The culture was then centrifuged at 5000 g for 15 minutes in order to remove the cells. The cells were washed twice in tris buffer (0.5M, pH 6.8). The cells were stored for later analysis.

Figures 39 to 42 illustrate the growth curves of *Pseudomonas aeruginosa* under varying concentrations of carbon, nitrogen, phosphorus and potassium limitation. The cells

from the cultures were analysed for any alterations in their OMP profiles following conditions of nutrient limitation.

Analysis of outer membrane protein profiles

The outer membranes were extracted using the modified technique of Pugsley *et al,* 1986 (Chapter 5). The protein profiles were prepared using SDS-PAGE using a 12% separator gel. The gels were stained using Coomassie Brilliant Blue R250 (Chapter 2).

Upon analysis of the gels using the Phoretix software package it was observed that the T-OMP protein did not disappear following the nutrient limitation studies (Figure 43).

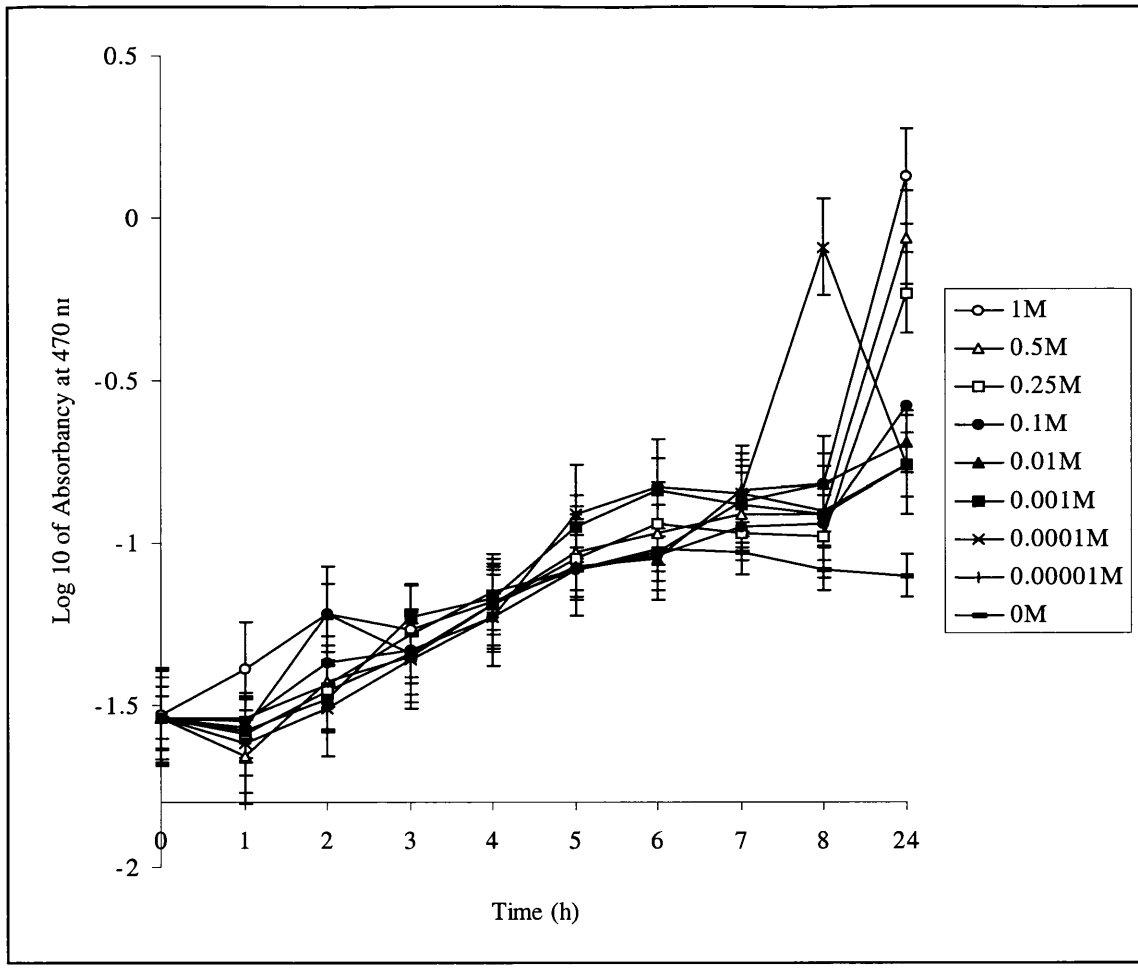

Figure 39: The effect of varying concentrations of Carbon upon the rate of growth of *Pseudomonas aeruginosa* PAO1 when added to early logarithmic phase cultures in CDM.

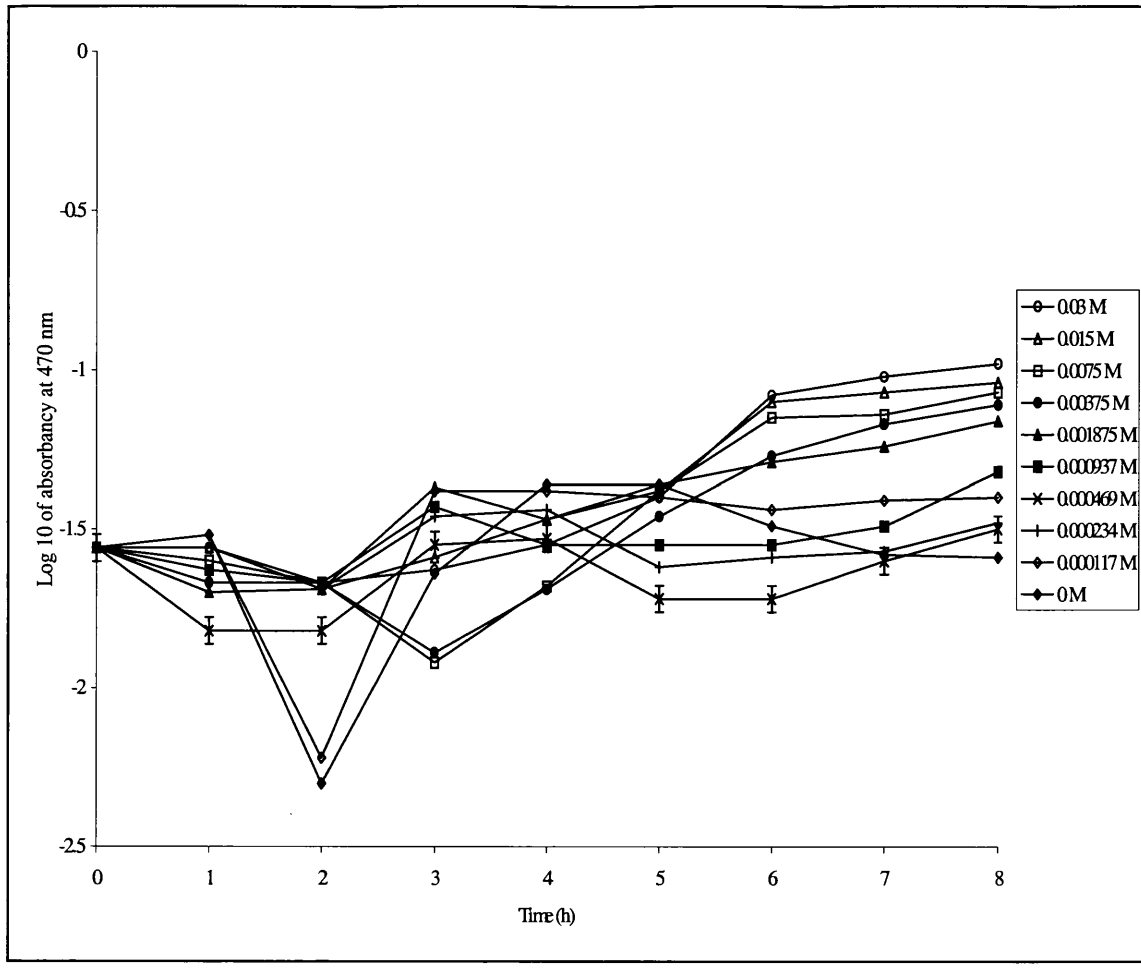

Figure 40: The effect of varying concentrations of Nitrogen upon the rate of growth of *Pseudomonas aeruginosa* PAO1 when added to early logarithmic phase cultures in CDM

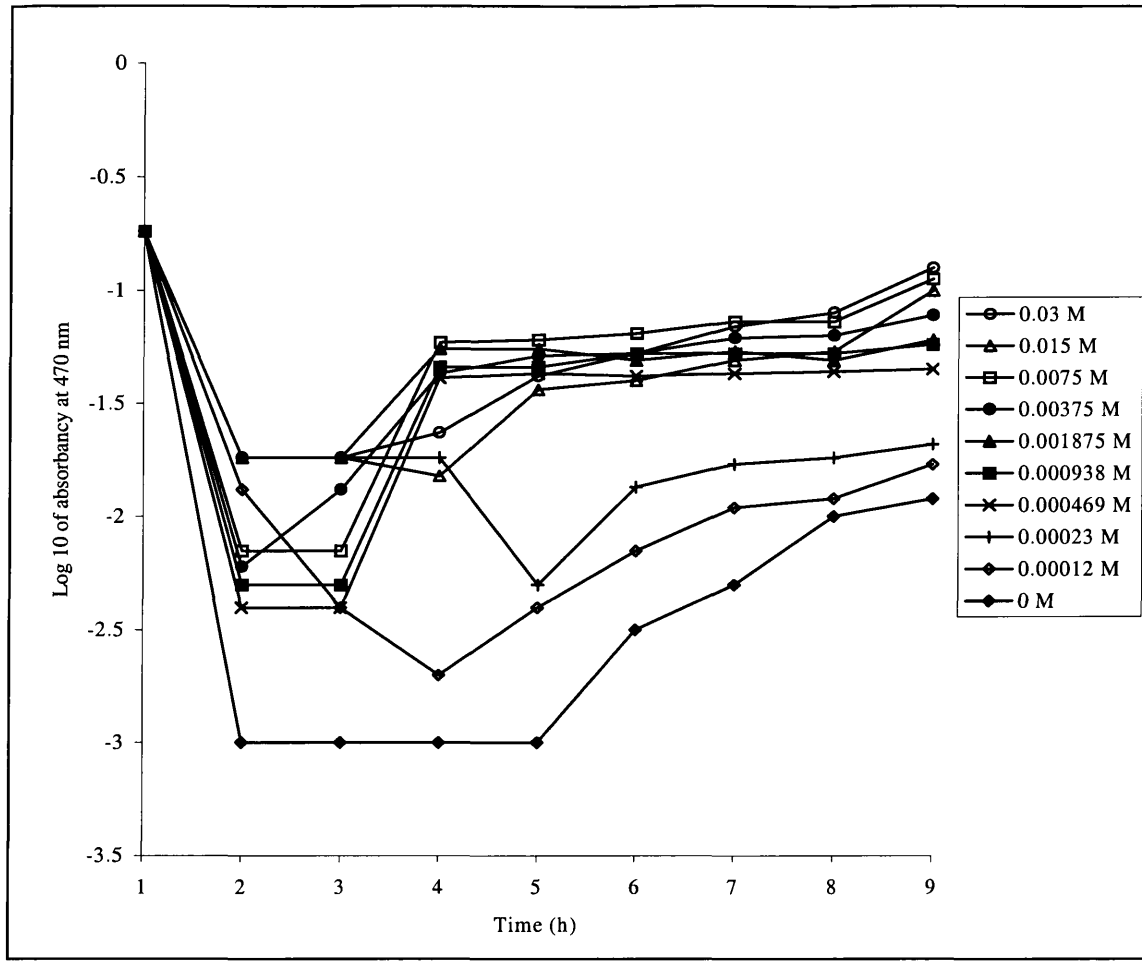

Figure 41: The effect of varying concentrations of Potassium upon the rate of growth of *Pseudomonas aeruginosa* PAO1 when added to early logarithmic phase cultures in CDM

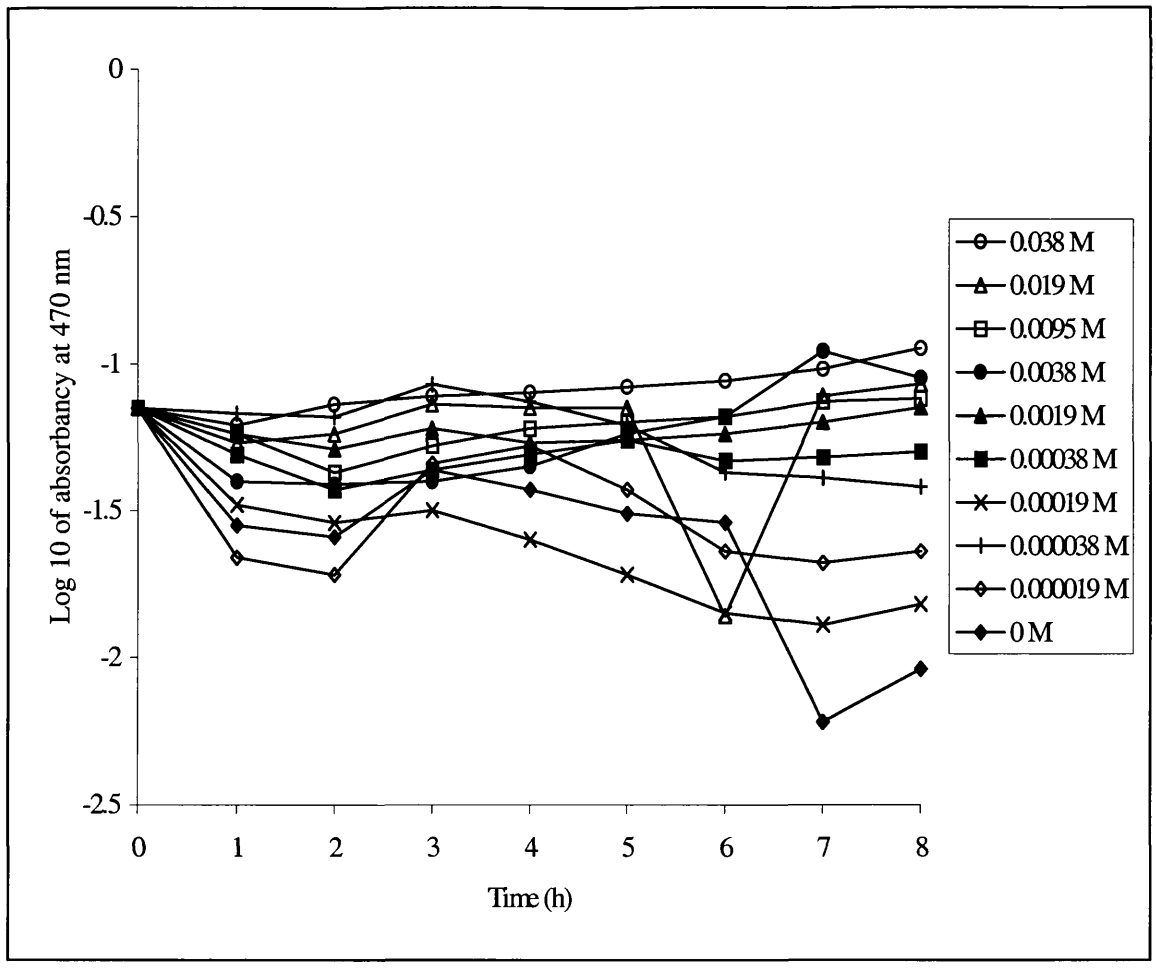

Figure 42: The effect of varying concentrations of Phosphorus upon the rate of growth of *Pseudomonas aeruginosa* PAO1 when added to early logarithmic phase cultures in CDM

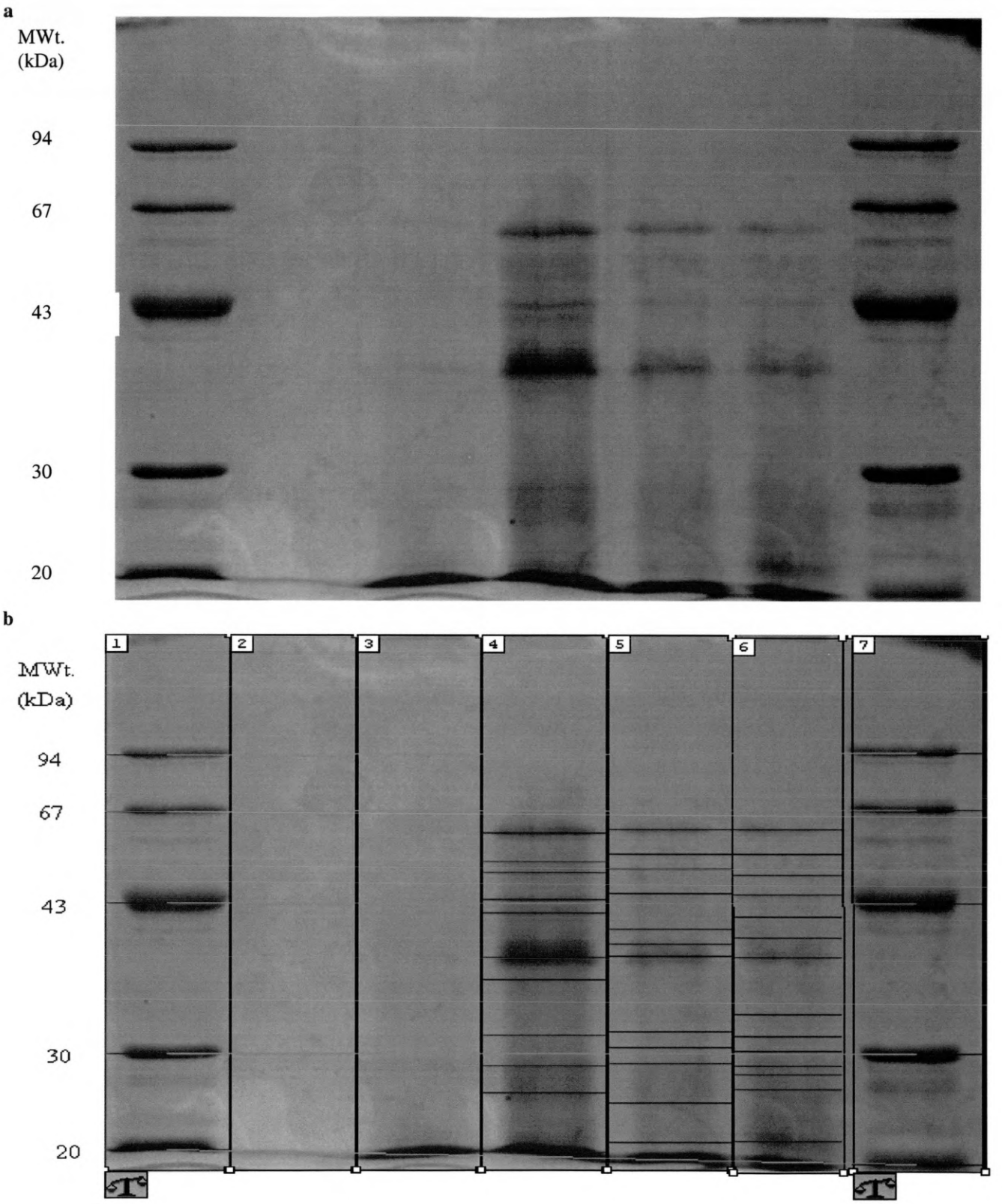

Figure 43 (a) Outer Membrane Protein Profile of sensitive cells of *Pseudomonas aeruginosa* PAO1 cells cultured in varying conditions of carbon limitation. **(b)** The same gel image after Phoretix analysis. Black lines on the image indictae the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes.

Conclusions

Sub-inhibitory concentrations of the isothiazolone biocides and thiomersal appear to inhibit the presence of the protein T-OMP. Therefore, this implies that T-OMP may be a possible route for the entry of the biocides into the cell. However, once the presence of the biocides is removed T-OMP re-appears. Therefore, this suggests that this is a phenotypic adaptation brought about as a direct result of the presence of the biocide. The 2-D gel analysis illustrates that alterations in protein movement are occurring in the cells. However, it is not possible to conclude from the two-dimensional gels that alterations are definitely the cause of the loss of T-OMP. It is also evident that other proteins may be lost from the cells following the induction of resistance. Therefore, this suggests that some other mechanism of resistance may be occurring in the cells and hence, contributes to the resistance mechanism. Upon exposure of *P. aeruginosa* to conditions of nutrient limitation, there were no observable alterations in the OMP profiles. This suggests that the observed alterations in the OMPs are a result of direct biocide challenges and not nutrient limitation.

Chapter Six

Investigations into the Morphological and cell surface ultrastructural Differences observed in resistant Isolates of *Pseudomonas aeruginosa* PAO1

The cultures of *Pseudomonas aeruginosa* PAO1 resistant to the isothiazolone biocides and thiomersal were maintained on biocide gradient plates (Chapter 4). During this procedure two different morphological types were noted. They were designated as non-mucoid and mucoid forms of PAO1. The non-mucoid and mucoid forms were sub-cultured onto biocide-free media and then cultured back onto biocide gradient plates. The mucoid form reverted back to a non-mucoid phenotype when placed on biocide-free media. However, when placed back on the biocide gradient plates this strain displayed the mucoid phenotype. The non-mucoid form displayed the same phenotype on biocide-free media and biocide containing media. The two forms were identified using the API 20 NE system (Biomerieux, France) to establish that they were *P. aeruginosa* and not some contaminating species. The non-mucoid and mucoid forms were investigated to determine whether the two forms displayed any differences in the lipopolysaccharide structure in the outer membrane.

The penetration of antimicrobial agents into the Gram-negative bacterial cell is dependent upon the hydrophobic or hydrophilic properties of the antimicrobial and the nature of the outer membrane (Russell & Furr, 1986). The lipopolysaccharide (Figure 51; LPS) component constitutes the outer half (outer leaflet) of the bacterial outer membrane. Many Gram-negative bacteria produce an extracellular polysaccharide, which forms either a discrete capsule or a slime layer attached to the cell surface (Hammond *et al,* 1984). The exopolysaccharides (EPS) are immunogenic, corresponding to the K (Kapsel)-antigen and tend to dissolve when cultured in liquid medium (Hammond *et al*, 1984). The EPS are built from a range of monosaccharides with the addition of amino sugars and KDO in a limited number of *E. coli* strains (Hammond *et al,* 1984). The LPS is the major antigenic determinant in Gram-negative bacteria and is used in serological typing (Jann & Jann,

1999). The LPS is composed of three parts, lipid A, the core oligosaccharide and the O-specific polysaccharide (Jann & Jann, 1999). Formation of the O-specific polysaccharide may be affected by mutations. Mutants which lack the O-specific polysaccharide have a rough appearance when grown on agar and growth occurs in brittle colonies. These mutants are called "R-mutants" (Jann & Jann, 1999). Whereas, the wild-type bacteria possess intact O-specific polysaccharide, and their appearance on agar is smooth and glossy. These strains are therefore called S-forms (smooth forms; Jann & Jann, 1999). The LPS molecules are restricted to the outer leaflet of the outer membrane and tightly associated with the OMPs (Jann & Jann, 1999). The OMPs are often found to constitute a lower proportion in the R-mutants than in S-forms, the corresponding space being filled with phospholipids (Jann & Jann, 1999). Therefore, the R-mutants contain phospholipid bilayer areas in the outer membrane, and hence, demonstrate greater sensitivity towards certain hydrophobic agents (Jann & Jann, 1999). In a study which reviewed the sensitivity of wild-type, LPS-deficient and porin-deficient strains of *E. coli* towards parabens (a homologous series of esters of para-hydroxybenzoic acid), the LPS-deficient mutants were found to be deep-rough heptoseless strains (Russell *et al*, 1987). They demonstrated sensitivity towards methyl and ethyl esters and especially propyl and butyl esters. This alteration in cell sensitivity is thought to be related to LPS and OMP deficiency and especially towards surface exposure of the phospholipids and paraben hydrophobicity (Russell *et al,* 1987). Hydrophobic antimicrobial agents can cross the outer membrane *via* the phospholipid component, which is normally protected from the environment by intact LPS and OMPs. Therefore, removal of the LPS and OMPs may be expected to result in modifications in the cellular response to hydrophobic antimicrobials (Russell & Furr, 1986). An increase in the roughness of the LPS (a decrease in the amount of R-core) enables hydrophobic antimicrobials to enter the Gram-negative bacteria (Russell & Furr, 1987). The metal chelator and bacterial outer membrane permeabiliser, ethylenediamine tetraacetate (EDTA) increases the sensitivity of Gram-negative bacteria towards many antimicrobial agents (Sawer *et al,* 1997). It has been demonstrated that EDTA removes Mg^{2+} ions and a considerable amount of LPS from the membrane structure (Russell *et al*,

1987). This enables the subsequent passage of antimicrobial agents across the outer membrane. For example, LPS-deficient strains of *E. coli* are considerably more sensitive to benzalkonium chloride and cetylpyridinium chloride than wild-type organisms (Russell & Furr, 1987).

The aims of the investigation were to determine whether the non-mucoid and mucoid cell morphology types identified from the gradient plates displayed any differences in lipopolysaccharide structure, or cell surface morphology.

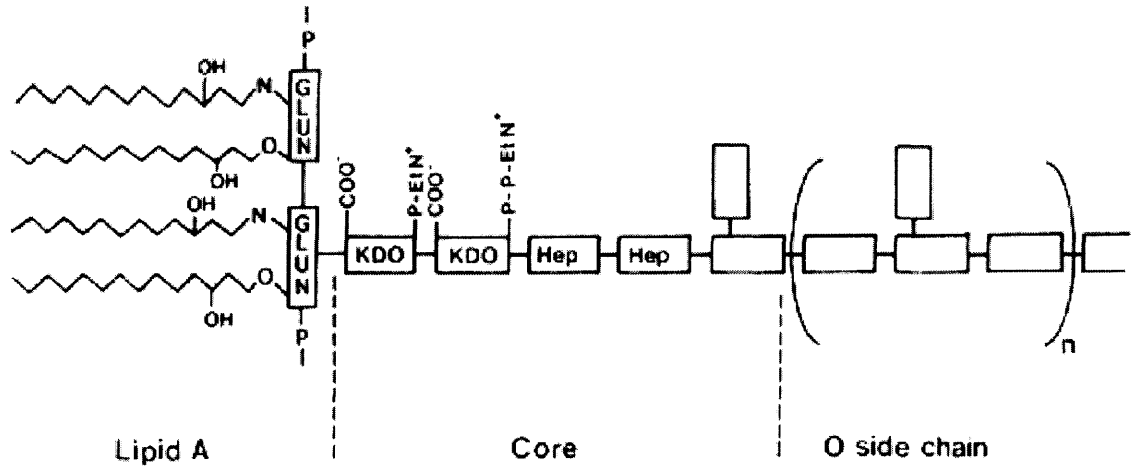

Figure 44: Generalised Structure of Bacterial Lipopolysaccharide. The structure of the lipid A region is highly conserved amongst a wide range of Gram-negative bacteria. Similarly little variation is found in the inner core (KDO-heptose) region. Considerable variation is present in the monosaccharide that constitute the outer core and O-side chain regions and consequently they have been left as empty blocks. (Taken from Hammond *et al,* 1984).

Determination of Cell-surface Hydrophobicity

Non-mucoid and mucoid colonies were identified during routine passaging of the cultures in the presence of biocide, in order, to maintain the presence of resistance. It has been reported that alterations in colonial morphology are associated with changes in hydrophobicity of the cell surface (Sunairi, 1997). The cultures were maintained on gradient plates in which a biocide gradient of varying concentrations of agar extended along the length of the plate. The non-mucoid and mucoid colonies (Figure 45) were observed growing at approximately the same point along the gradient. The colonies were sub-cultured separately onto biocide-containing agar in order to obtain pure cultures. The pure cultures were identified using API 20NE tests (Biomerieux, France) to confirm they were *P. aeruginosa* and not contaminants. The non-mucoid and mucoid forms were tested for cell-surface hydrophobicity according to the method of Rosenberg *et al,* (1980). In order to determine if the alterations, in the cell surface phenotype, could possibly be related to the observed resistance towards the isothiazolone biocides and thiomersal.

The conversion from a non-mucoid to a mucoid phenotype can be related to the copious production of exopolysaccharide alginate (Rocchetta *et al,* 1999). This cell-surface alteration is commonly observed within the lungs of patients with cystic fibrosis and is correlated with poor lung function, due to the bacteria embedded with an alginate matrix behaving as a bacterial biofilm (Rocchetta *et al,* 1999). The emergence of mucoid strains is often accompanied by alterations in the lipopolysaccharide and is commonly observed in biofilm cells. Mucoid strains are observed to lack or to express smaller quantities of B-band O-polysaccharide, whilst maintaining the level of A-band O-polysaccharide (Rocchetta *et al,* 1999). Whereas, in non-mucoid cells devoid of alginate, the predominant surface O-polysaccharide is B-band (Rocchetta *et al,* 1999). The B-band O-polysaccharides are highly anionic and they extend through the layers of the A-band polymers and OMPs (Rocchetta *et al,* 1999). Cells which posses the B-band polymers demonstrate low surface hydrophobicity and surface charge and are observed to adhere to glass and polystyrene more effectively than those with an absence of the B-band polymers

(Rocchetta *et al,* 1999). An absence in the production of the A-band polymer has been related to a deficiency in the core LPS. An increase in hydrophobicity in rough mutants of *E. coli* is thought to be due to the exposure of the inner core of the LPS (Rosenberg *et al,* 1980). The loss of the oligosaccharide core components on the outer surface of *E. coli* rough mutants was observed to be accompanied by a significant increase in affinity towards the hydrocarbons hexadecane, octane and xylene (Rosenberg *et al,* 1980).

Preparation of Bacteria

An aliquot (24 mL) of nutrient broth was inoculated with mucoid or non-mucoid forms of *P. aeruginosa* PAO1 which were deemed resistant to the three isothiazolone biocides and thiomersal. An aliquot (1 mL) of appropriate biocide stock (in order to obtain a quarter strength of the MIC determined for passage 11 during the inducement of resistance in the nutrient broth) was added to the nutrient broth. The cultures were incubated at 37°C, 200 osc min^{-1} for 19 hours. The bacteria were harvested, centrifuged at 3000 g (IEC, Centra-4B) for ten minutes and 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 deionised water, the pH was adjusted to pH 7.1, and the volume was made up to 1 L). The cells were resuspended in 5 mL PUM buffer.

Assay Procedure

An aliquot (1.2 mL) of washed cells suspended in PUM buffer was placed in round bottom test-tubes (10 mm diameter). Various volumes of the test hydrocarbon, p-xylene, was added to the suspensions in the range of 0 to 0.2 mL. The tubes were incubated for 10 minutes at 30°C, and the mixtures were agitated uniformly for 120 seconds. The solutions were allowed to settle for 15 minutes in order to allow the hydrocarbon phase to completely separate from the aqueous phase. The aqueous phase was carefully removed using a Pasteur pipette and transferred to a 1 mL cuvette. The optical density was determined at 400nm using a spectrophotometer (Novaspec II, LKB).

Following separation of the two layers, the upper layer is composed of an oil in water emulsion, with xylene droplets covered with patches of bacteria, whilst the lower layer contains the remaining bacteria suspended in PUM buffer. A decrease in the optical density of the lower aqueous phase between that of the non-mucoid and that of the mucoid cells was used to measure the relative cell-surface hydrophobicity of the isolates. If the optical density decreased between the aqueous layer of the non-mucoid cells and that of the mucoid cells resistant to a particular biocide, then a greater proportion of the cells adhered to the xylene droplets and hence, the cell-surface of the bacteria was more hydrophobic. However, if the optical density increased between the lower aqueous layer of the non-mucoid cells and that of the mucoid cells resistant to a particular biocide, then a greater proportion of the cells have remained in the aqueous layer and hence, the cell-surface of the bacteria was more hydrophilic. Due to the discontinuous nature of the test samples used in this experiment, the data is presented as absolute absorbance values rather than absorbance changes, as is normal with this procedure.

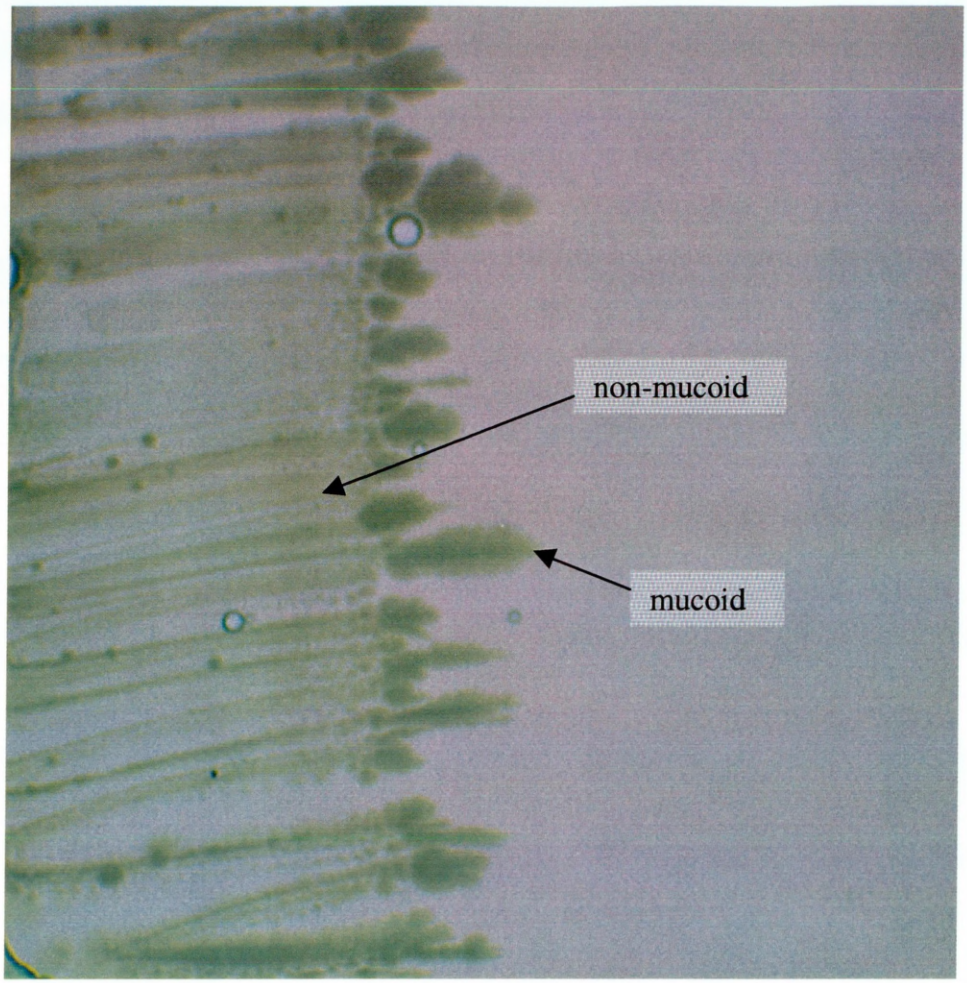

Figure 45: A Gradient Plate Illustrating the Mucoid and Non-Mucoid forms of *Pseudomonas aeruginosa*PAO1 Resistant to BIT (147.5 μg mL^{-1}).

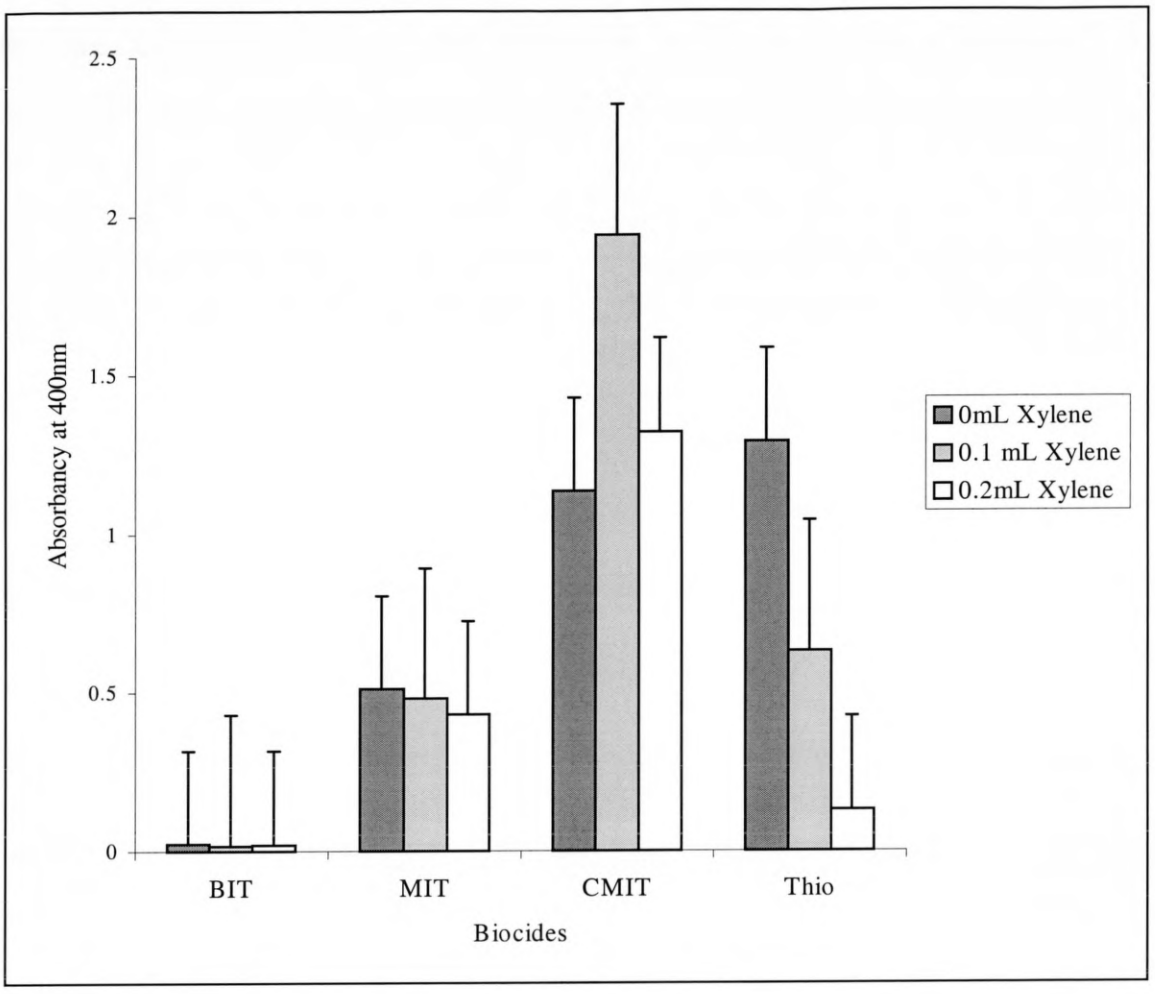

Figure 46: The absorbancy of the non-mucoid cells of *Pseudomonas aeruginosa* PAO1 cultured in R2A medium in the presence of sub-inhibitory levels of the three isothiazolone biocides and thiomersal in relation to the volume of xylene. The error bars are calculated as the standard error of the data set.

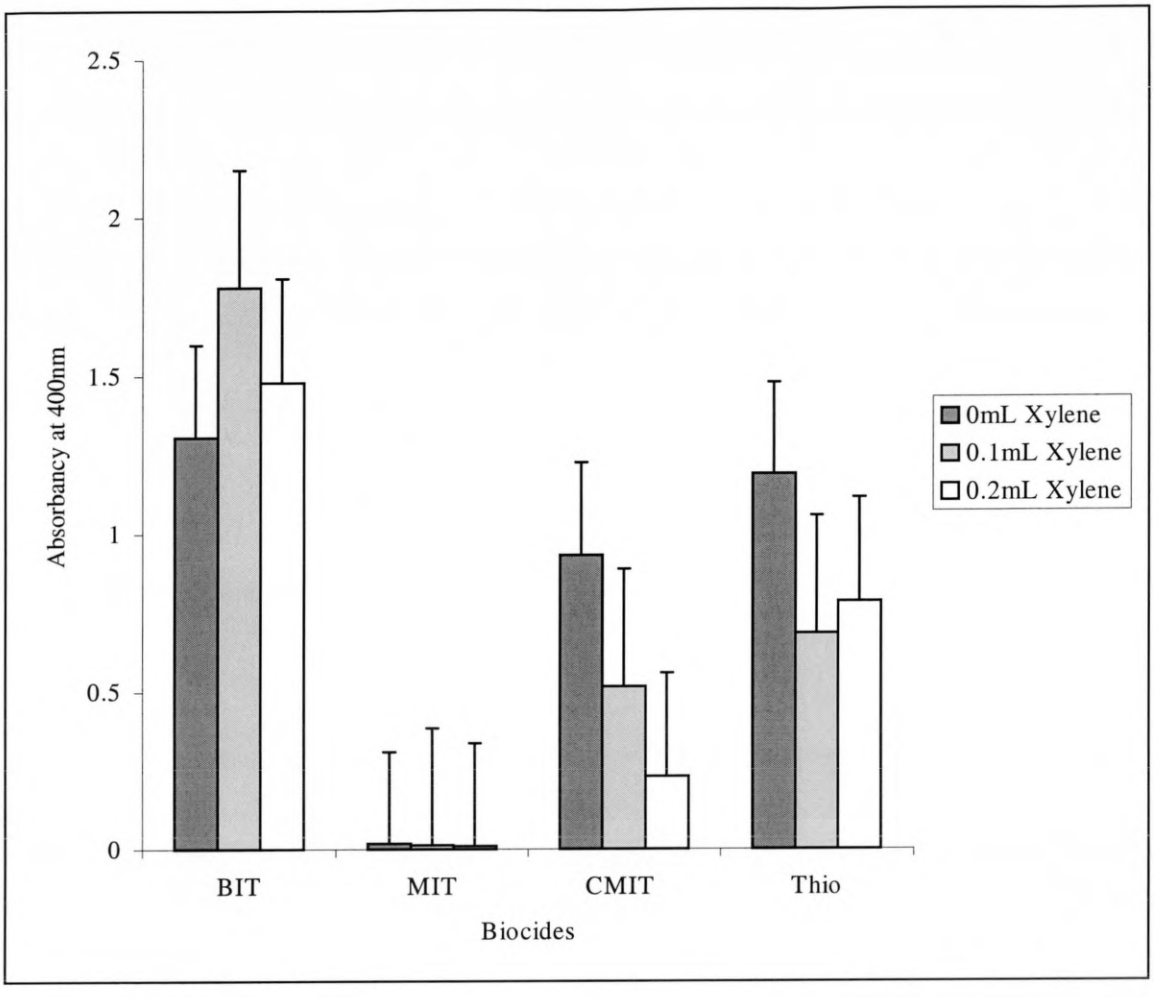

Figure 47: The absorbancy of the mucoid cells of *Pseudomonas aeruginosa* PAO1 cultured in R2A medium in the presence of sub-inhibitory levels of the three isothiazolone biocides and thiomersal in relation to the volume of xylene. The error bars are calculated as the standard error of the data set.

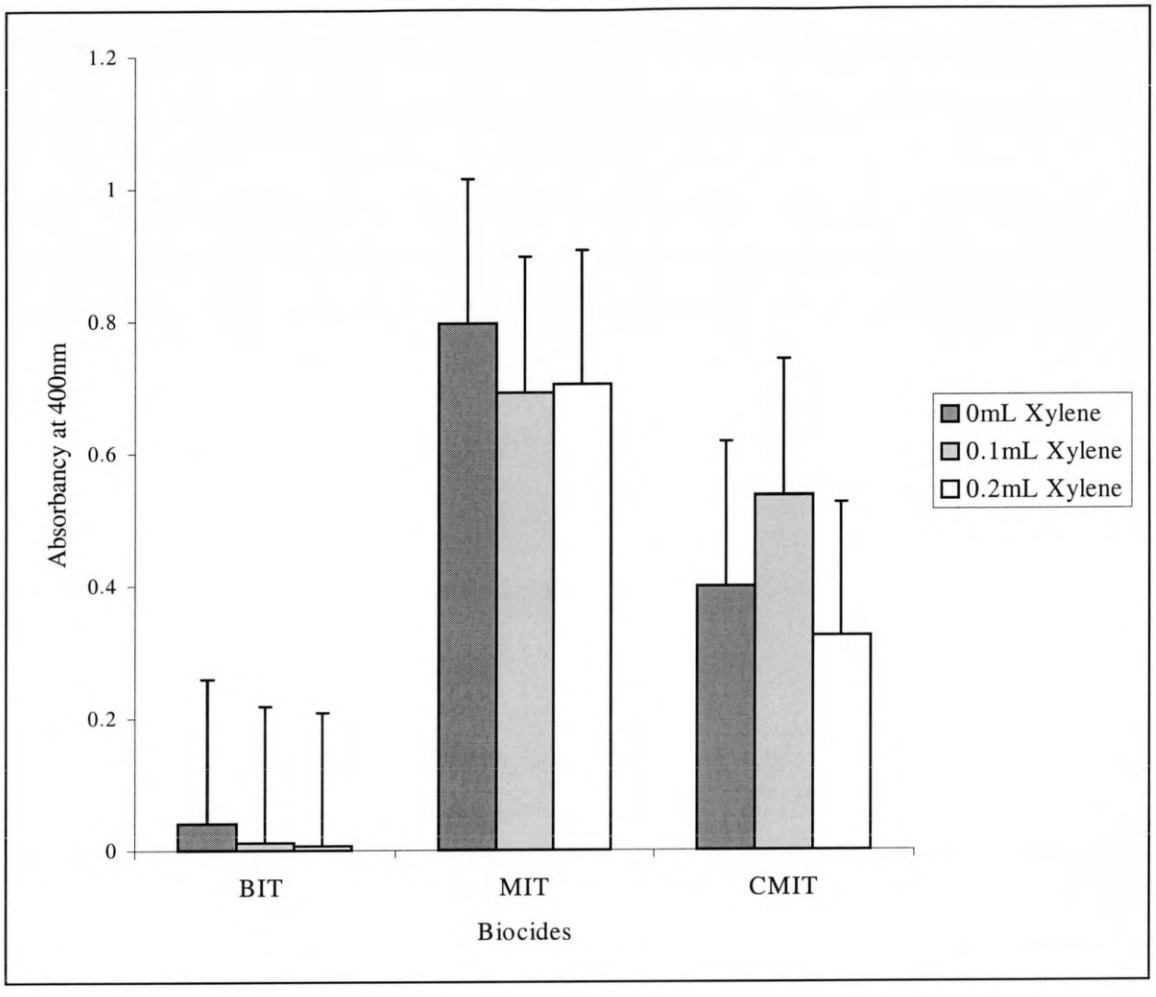

Figure 48: The absorbancy of the non-mucoid cells of *Pseudomonas aeruginosa* PAO1 cultured in CDM medium in the presence of sub-inhibitory levels of the three isothiazolone biocides and thiomersal in relation to the volume of xylene. The error bars are calculated as the standard error of the data set.

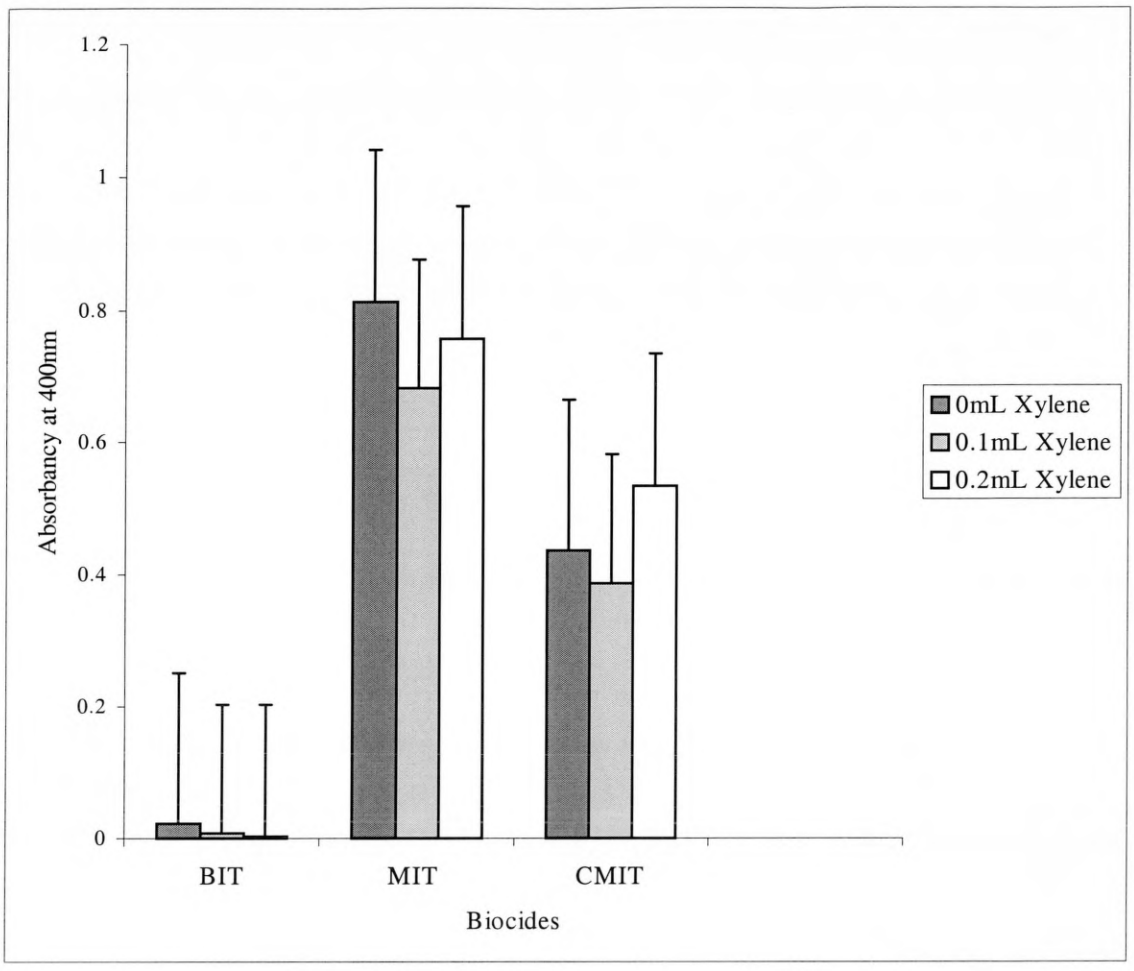

Figure 49: The absorbancy of the mucoid cells of *Pseudomonas aeruginosa* PAO1 cultured in CDM medium in the presence of sub-inhibitory levels of the three isothiazolone biocides and thiomersal in relation to the volume of xylene. The error bars are calculated as the standard error of the data set.

The following discussion relates to the results given in figures 39 to 41.

BIT resistant Pseudomonas aeruginosa.

The absorbancy increased between the non-mucoid and mucoid cells resistant to BIT grown in R2A. The cells mixed with 0.1 mL of xylene increased from an absorbancy (400 nm) of 0.017 to 1.778, whereas the absorbancy increased from 0.020 to 1.478 when the cells were mixed with 0.2 mL of xylene. This indicated less relative affinity towards the test hydrocarbon xylene by the mucoid cells, particularly at the lower xylene level. The cell surface of the mucoid cells is, therefore, relatively more hydrophilic than the non-mucoid cells. There was no observable difference in the absorbancy between the non-mucoid and mucoid cells resistant to BIT when grown in CDM. It is, therefore, not possible to conclude if any significant alteration has occurred in the cell surface hydrophobicity. This may be due to the conditions of nutrient limitation of the cells cultured in CDM.

MIT resistant Pseudomonas aeruginosa.

The absorbancy decreased between the non-mucoid and mucoid cells resistant to MIT grown in R2A. The absorbancy decreased from 0.418 to 0.013 when the cells were mixed with 0.1 mL of xylene and 0.413 to 0.009 when mixed with 0.2 mL of xylene. There was, therefore, no significant difference between the readings for the two levels of xylene. This relative decrease in absorbancy between the non-mucoid and mucoid cells indicated less affinity towards the test hydrocarbon xylene and, therefore, an increase in the cell surface hydrophobicity in mucoid cells. This indicated a possible loss or reduction of B-band O-polysaccharide in the LPS core on the outer membrane of mucoid cells. There was no observable difference in the absorbancy between the non-mucoid and mucoid cells grown in CDM.

CMIT resistant Pseudomonas aeruginosa.

The absorbancy of the cells resistant to CMIT grown in R2A when mixed with 0.1 mL of xylene decreased from 1.940 to 0.516, whereas the cells mixed with 0.2 mL of xylene decreased from 1.320 to 0.232. This relative decrease in absorbancy between non-mucoid and mucoid cells indicated an increase in cell surface hydrophobicity, and a possible loss or reduction in the B-band O-polysaccharide in the LPS core in mucoid cells. A greater affinity towards the test hydrocarbon at the lower volume may possibly be due to CMIT being the most reactive of the isothiazolone biocides. The absorbancy of the cells resistant to CMIT grown in CDM decreased from 0.536 to 0.386 between non-mucoid and mucoid cells when mixed with 0.1 mL of xylene, indicating a slight increase in cell surface hydrophobicity in mucoid cells. However, when the cells were mixed with 0.2 mL of xylene the absorbancy increased from 0.324 to 0.534 between the non-mucoid and mucoid cells. This indicated an increase in cell surface hydrophilicity in mucoid cells under these conditions.

Thiomersal resistant Pseudomonas aeruginosa.

The absorbancy of the cells did not significantly alter between the non-mucoid and mucoid cells resistant to thiomersal grown in R2A when mixed with 0.1 mL of xylene. However, the absorbancy increased from 0.129 to 0.786 between the non-mucoid and mucoid cells when mixed with 0.2 mL of xylene. This indicated a relative increase in cell surface hydrophilicity by the mucoid cells.

Summary

The observed increase in cell hydrophobicity between the non-mucoid and mucoid cells for MIT, CMIT and thiomersal in R2A indicates a loss or reduction in the B-band O-polysaccharide present. The amount of the A-band O-polysaccharide remains the same. The non-mucoid cells are devoid of alginate and have the predominant B-band O-polysaccharide. The B-band polysaccharides are highly anionic and extend through the layers of the A-band polysaccharides and OMPs. The non-mucoid cells demonstrate low

surface hydrophobicity and posses surface charges that adhere to glass and polystyrene more effectively than those with a B-band absence. The mucoid cells have cell-surface alterations commonly observed with patients with cystic fibrosis, which are connected with the formation of biofilms and alginate matrix. Therefore, the mucoid resistant forms may indicate cell-surface alterations that would aid the formation of a biofilm.

Determination of Lipopolysaccharide Profiles

Alteration in the lipopolysaccharide (LPS) layer of the outer membrane can be related to observed increases in resistance to antimicrobial agents. Hydrophobic antibiotics appear unable to readily enter Gram-negative cells until the cells demonstrate a degree of roughness (Russell & Furr, 1987). The R-mutants are often found to contain a lower proportion of proteins in the outer membrane than S-forms. The protein is replaced by a larger quantity of phospholipids (Jann & Jann, 1999). These mutants demonstrate greater sensitivity towards hydrophobic antimicrobial agents. It is thought that the observed loss of the outer membrane protein T-OMP in cells of *P. aeruginosa* resistant to the isothiazolone biocides, occurs because T-OMP is the route of entry for these biocides into the cell (Brözel & Cloete, 1994). The isolation of lipopolysaccharide was performed according to the method of Preston and Penner (1987), in order to establish whether any alterations occurred in the lipopolysaccharide structure between the mucoid and non-mucoid cells. This may be related to the observed resistance towards the isothiazolone biocides and thiomersal.

Extraction of Lipopolysaccharide

An aliquot (24 mL) of appropriate medium (R2A or CDM) was inoculated with *P. aeruginosa* PAO1 deemed resistant to the appropriate biocide (Passage 11). An aliquot (1 mL) of appropriate biocide stock (quarter the highest established MIC) was added to the culture. The flasks were incubated at 37°C, 200 osc min^{-1} for 19 hours. An aliquot (10 mL) of the culture was centrifuged for 10 minutes at 4000 g (IEC Centra-4B). The pellet was

resuspended in 1.0 mL of Tris buffer (0.5 M, pH 6.8) and centrifuged for 1.5 minutes at 11,500 g (Microcentaur, MSE). The pellet was solubilised in 0.2 mL of lysis buffer (Glycerol, 20%; ß-mercaptoethanol, 5%; SDS, 4.6%; Tris, 2%; 0.1%; pH 6.8) and heated at 100°C for 10 minutes. The samples were cooled to room temperature and 0.04 mL of lysis buffer was added containing 2.5 mg mL^{-1} proteinase K. The samples were incubated at 60 °C for one hour. The samples were centrifuged at 5000 g for 1.5 minutes (Microcentaur, MSE), retaining the supernatant. An aliquot of bromophenol blue stock (0.1%)(15 μL) was added to each sample.

Determination of Lipopolysaccharide Profile

The LPS profiles were prepared using a 12% separator and 5% stacker gel as previously described (Chapter 2) without the addition of SDS. An aliquot (40 μL) of the samples were added to the wells and the gels were run at 35 mA for approximately 1.5 hours or until the leading samples had reached the end of the gel. The gels were fixed and stained according to the method of Preston & Penner, (1987) (Chapter 2).

Observation of the Lipopolysaccharide Profile

There were no significant differences (Figure 50) in the banding patterns between the mucoid and non-mucoid isolates for all four biocides when grown in R2A or CDM medium (Figure 50). This indicates the composition of the O-specific polysaccharide did not alter between the mucoid and non-mucoid forms of the resistant cells. It is, therefore, unlikely that the outer membrane contains larger patches of phospholipids which are more sensitive to the penetration of hydrophobic antimicrobials.

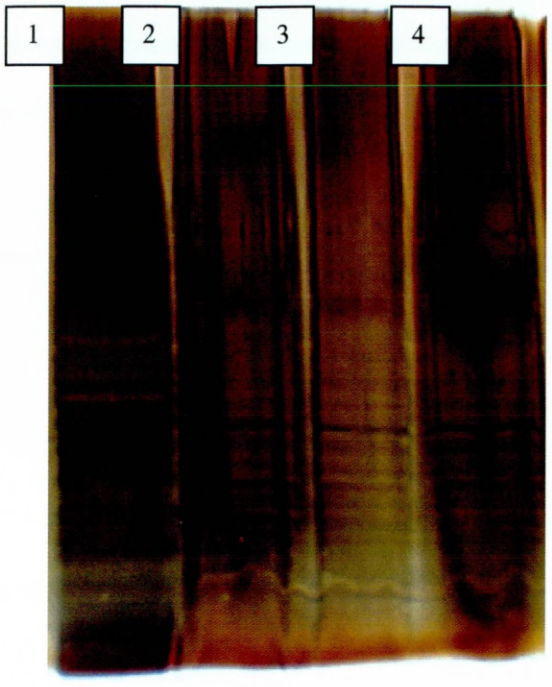

Figure 50: The Lipopolysaccharide Banding Pattern of Non-Mucoid and Mucoid Cells of *Pseudomonas aeruginosa* PAO1 resistant to MIT and CMIT. Lane 1: non-mucoid cells resistant to MIT; Lane 2: mucoid cells resistant to MIT; Lane 3: non-mucoid cells resistant to CMIT; Lane 4: mucoid cells resistant to CMIT.

The Leakage of Intracellular Potassium Ions

A membrane active agent can induce damage to the membrane by acting upon either the membrane potentials, bound enzymes or permeability (AL-Adham *et al,* 1998) An example of such is an ionophore. An ionophore is a compound which promotes the specific movement of ions across membranes (Kroll & Patchett, 1991). This is suggested as the mode of action for many antimicrobial agents. For example, chlorohexidine causes leakage of intracellular ions from *E. coli* and *Staphylococcus aureus* (AL-Adham *et al,* 1998). Initially, this intracellular leakage increases with increasing concentrations of the antimicrobial. However, at high concentrations of antimicrobials, coagulation is observed between the protoplasmic contents and / or cytoplasmic membrane (AL-Adham *et al,* 1998), thereby resulting in a decrease in leakage. This results in subsequent changes in membrane permeability. The observed increase in antimicrobial resistance to the isothiazolone biocides and thiomersal may be related to the permeability of the outer membrane and OMPs. It is therefore, necessary to eliminate any cytoplasmic membrane permeability alterations as a direct result of the biocides activity on the bacterial membrane. Such changes can be monitored by observing the concentration of potassium ions in the bathing solution of the bacterium exposed to a particular antimicrobial agent.

In order to establish whether the biocides had a direct effect on the membrane, the cell was observed to determine any leakage from the cell interior. The leakage of potassium ions was monitored using a potassium sensing electrode in order to determine if the action of the isothiazolone biocides and thiomersal caused direct damage to the membrane of *P. aeruginosa*, indicated by an efflux of potassium ions. A control agent, cetrimide was used in the experiment due to its known cell leakage activity.

The calibration of the potassium electrode

The sensing electrode (Qualiprobe QSE 314, EDT Instruments, Dover) was placed in a solution of 10^{-1} M KCl. This was performed two hours prior to monitoring the potassium ion content of the bacterial cell bathing solutions. The reference electrode

(Qualiprobe double junction reference electrode E8092, EDT Instruments, Dover) was topped up with electrolyte (0.1 M $NaNO_3$) and the tip was replaced until the probes were required. The reference and sensing electrodes were taped together in order to ensure both electrodes would be at the same level in the test solutions.

Solutions of potassium chloride were volumetrically prepared in the range of 10^{-1} to 10^{-6} M using deionised glassware. An aliquot (50 mL) of the standard solutions was added to 5 mL of ionic strength adjustment buffer (ISAB; tetraethylammonium chloride; 18.37 g in 100 mL^{-1}). The adjustment buffer ensured the background ionic strength of all the test solutions was kept constant by blocking the potassium channels. The electrodes were placed in the solutions and were constantly stirred during the observation period by a magnetic stirrer (Rotamixer, Hook & Tucker). The potential derived by the electrodes was measured for a period of five minutes using a Whatman PHA 220 pH / mV meter (Whatman, Maidstone, Kent) set on mV and chart recorder (Belmont Instruments, Glasgow). A calibration graph was constructed of potassium ion concentration against mV (Figure 58).

Observation of Leakage of Potassium Ions from Challenged and non-Challenged cultures of P. aeruginosa

Cultures of sensitive cells of *P. aeruginosa* grown in R2A or CDM medium were tested against the three isothiazolone biocides, thiomersal and cetrimide (positive control). Cells resistant to the isothiazolone biocides and thiomersal were tested against all five biocides. An aliquot (49 mL) of appropriate media (R2A or CDM) was inoculated with *P. aeruginosa* and incubated at 37°C, 200 osc min^{-1} for 19 hours. The culture was centrifuged for 20 minutes at 3000 g (IEC, Centra-4B), and washed twice in Tris-HCl buffer (pH 7.0, 0.2 M). The cells were resuspended in an aliquot (49 mL) of Tris-HCl buffer (pH 7.0, 0.2 M) in a deionised beaker (100 mL) and 5 mL of ISAB was added. The cell suspension was stirred at a constant speed on a magnetic stirrer and the pre-calibrated reference and sensing electrodes connected to the pH / mV meter were placed in the suspension. The

background potassium ion content of the suspension was monitored for five minutes. An aliquot (1 mL) of appropriate biocide stock was added to the suspension in order to give a final biocide concentration equivalent to the highest determined MIC obtained during the inducement of resistance. The fluctuation in potassium ion content was monitored over a period of 20 minutes by a chart recorder (Belmont Instruments, Glasgow).

Figures 58 and 59 illustrate the calibration of potassium concentration and voltage and the observation of potassium leakage from *P. aeruginosa* upon exposure to cetrimide and BIT.

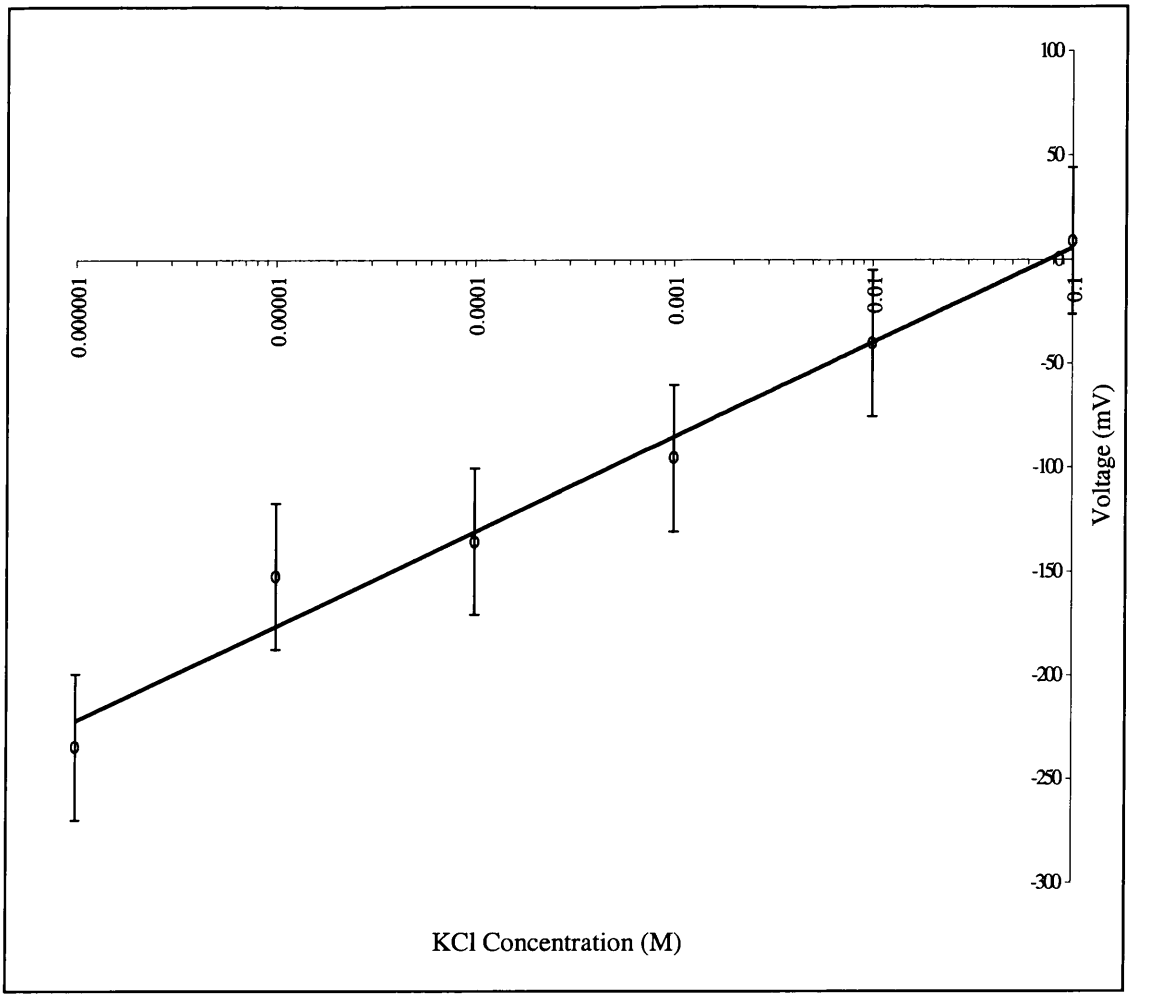

Figure 51: A logarithmic standard curve illustrating the relationship between potassium ion concentration and voltage. The error bars are calculated as the standard deviation of the individual data points.

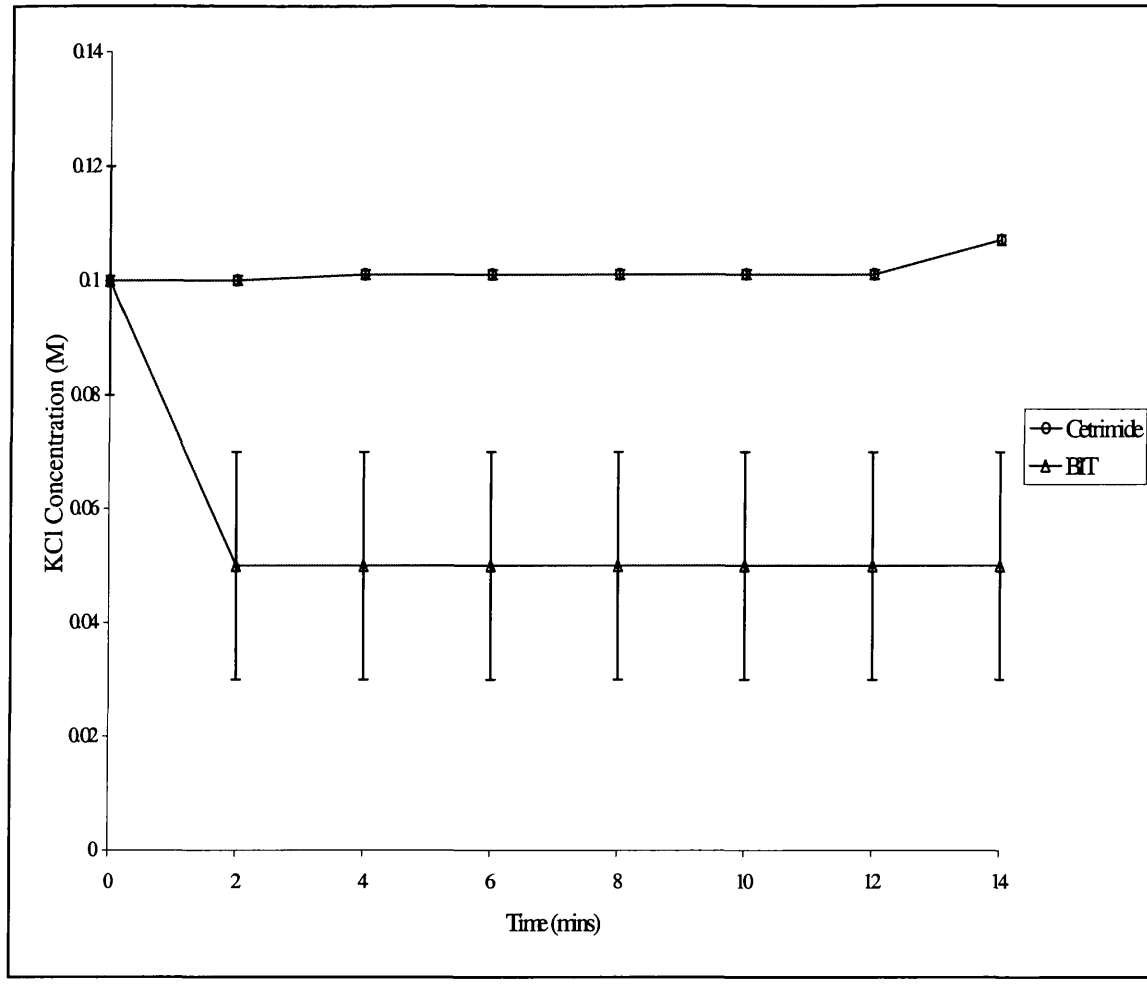

Figure 52: Potassium ion leakage from wild-type cells of *Pseudomonas aeruginosa* PAO1 upon exposure to cetrimide (140 μg mL^{-1}) and BIT (56 μg mL^{-1}) after 5 minutes. The error bars are calculated as the standard error of the data set.

Discussion

Cetrimide was used as a positive control because it is characterised as having a membrane active mode of action. The potassium ion concentration remained stable at 0.1005M in the bathing solution between 4 and 14 minutes (Figure 52). At five minutes the cetrimide was added. At fourteen minutes, after the addition of the biocide there was an increase of potassium ion concentration in the bathing solution to 0.107M indicating disruption of the outer membrane and cell leakage. The cells exposed to BIT did not demonstrate a membrane active mode of action. The study conducted by AL-Adham *et al,* (1998) observed a low rate of potassium ion leakage in comparison to the leakage observed following exposure to cetrimide, which only became apparent over the 15 minute period of the experiment. The potassium ion concentration was monitored for five minutes prior to the addition of the biocide. The potassium ion concentration stabilised at the two minute interval point to 0.05M. The potassium ion concentration was monitored for a further nine minutes after the addition of biocide, no observable alteration in the potassium ion concentration was observed. Therefore, these results indicate that the isothiazolone biocide BIT does not induce cell leakage or membrane disruption.

The Effect of Permeabilisers on the Antimicrobial Sensitivity of *P. aeruginosa*

Pseudomonas aeruginosa demonstrates intrinsic resistance to many antimicrobial agents. This is due to the impermeability of the outer membrane to hydrophobic antimicrobials and high molecular weight hydrophilic drugs (Ayres *et al,* 1999). A variety of polycationic substances can increase the susceptibility of *P. aeruginosa* to biocides by acting as outer membrane permeabilisers (Mann *et al,* 2000). Permeabilisers sensitise the bacteria towards antimicrobial agents without being directly toxic to the bacteria (Helander & Mattila-Sandholm, 2000). The permeabilisers disrupt the integrity of the outer membrane by weakening the stabilising interactions of the outer membrane components. This occurs by either releasing the outer membrane components or intercalating within the

outer membrane (Helander & Mattila-Sandholm, 2000). Permeabilisers may play an essential role, in combination with antimicrobial agents, to prevent the growth of Gram-negative microorganisms (Helander & Mattila-Sandholm, 2000). Impermeability is a major mechanism of intrinsic resistance. The characteristic of impermeability is enhanced when bacteria develop resistance to antimicrobial agents. This is because the development of resistance is associated with the loss of permeability to a particular antimicrobial agent. Recent studies have investigated the exploitation of permeabilising agents in order to overcome resistance. This approach may be particularly useful in biofilms where greater resistance is observed than with planktonic cells (Ayres *et al,* 1998).

Permeabiliser Agents

Three permeabiliser agents were used in the study: EDTA (0.001 M and 0.002 M), sodium polyphosphate (SPP; 0.25%, 0.5%, 1% and 1.25% w/v) and trisodium citrate dihydrate (TCD; 0.25%, 0.5% and 1.0% w/v).

Preparation of Cultures

An aliquot (24 mL) of appropriate media (R2A or CDM) was inoculated with *P. aeruginosa* cells deemed resistant to the appropriate biocides (Passage 11 of the resistance study). An aliquot (1 mL) of biocide stock was added to the flasks to establish a quarter strength of the previously established MIC. The flasks were incubated at 37°C, 200 osc min^{-1} for 19 hours.

Preparation of Tube Dilutions

An aliquot (4.5 mL) of appropriate double strength medium (R2A or CDM) was added to 4.5 mL of the various concentrations of the three permeabilisers in test-tubes. The tubes were autoclaved at 121°C for 15 minutes. Various concentrations of the appropriate biocides were aseptically added to the tubes in duplicate. They were added in the range of the highest previously established MIC for the resistant cultures. The biocide ranges were for R2A BIT, 86 to 100 $\mu g\ mL^{-1}$; MIT, 34 to 48 $\mu g\ mL^{-1}$; CMIT, 1.8 to 2.5 $\mu g\ mL^{-1}$ and

thiomersal, 16.8 to 18.2 $\mu g\ mL^{-1}$ and for CDM; BIT, 7.8 to 9.2 $\mu g\ mL^{-1}$; MIT, 8.2 to 9.6 $\mu g\ mL^{-1}$ and CMIT, 0.1 to 0.6 $\mu g\ mL^{-1}$. The tubes were inoculated with 100 μL of the overnight culture. The tubes were incubated at 37°C for 48 hours and were observed for the presence of growth at 19 and 48 hours.

Table 24: MICs observed for the resistant cultures following exposure to the isothiazolone biocides and thiomersal during the presence of permeabilisers in R2A medium.

Permeabiliser	Biocide ($\mu g\ mL^{-1}$)			
	BIT	MIT	CMIT	Thiomersal
Control	>100	>48	2.5	18.2
EDTA (0.001M)	<86	<34	<1.8	<16.8
EDTA (0.002M)	<86	<34	<1.8	<16.8
TCD (0.25%)	88	44	2.3	<16.8
TCD (0.5%)	88	44	2.3	<16.8
TCD (1%)	88	36	2.3	<16.8
SPP (0.25%)	<86	44	2.5	<16.8
SPP (0.5%)	<86	44	2.5	<16.8
SPP (1%)	<86	36	1.9	<16.8
SPP (1.5%)	<86	36	1.9	<16.8

The presence of the permeabilisers sodium polyphosphate (SPP) and trisodium citrate dihydrate (TCD) resulted in a decrease in the MICs for all four biocides grown in R2A medium. The increasing concentrations of TCD did not significantly enhance the decrease in MIC. However, when the concentration of the permeabiliser sodium polyphosphate was increased, the reduction in the MIC was more pronounced when observing the biocides MIT and CMIT. The MIC for MIT, when using 0.25% sodium polyphosphate was 46 $\mu g\ mL^{-1}$. However, in the presence of 1.5% sodium polyphosphate the MIC decreased to 36 $\mu g\ mL^{-1}$. The permeabilisers render the outer membrane more susceptible to antimicrobial agents because they disrupt the integrity of the outer membrane. In the presence of the permeabilisers the MICs of the resistant cells was reduced towards the three isothiazolone biocides and thiomersal. Therefore, this indicated the observed resistance towards the three isothiazolone biocides and thiomersal was possibly related to the outer membrane and the loss of the outer membrane T-OMP.

Table 25: MICs observed for the resistant cultures following exposure to the isothiazolone biocides and thiomersal during the presence of permeabilisers in CDM medium.

Permeabiliser	Biocide (μg mL^{-1})			
	BIT	MIT	CMIT	Thiomersal
Control	9.2	9.4	0.5	nd
EDTA (0.001M)	<7.8	<8.2	<0.1	nd
EDTA (0.002M)	<7.8	<8.2	<0.1	nd
TCD (0.25%)	9.2	9.4	0.5	nd
TCD (0.5%)	9.2	9.4	0.5	nd
TCD (1%)	9.0	9.4	0.5	nd
SPP (0.25%)	8.0	8.4	0.5	nd
SPP (0.5%)	8.0	<8.2	0.5	nd
SPP (1%)	<7.8	<8.2	0.4	nd
SPP (1.5%)	<7.8	<8.2	0.4	nd

nd = not done

The MICs decreased for all three isothiazolone biocides grown in CDM medium, in the presence of the permeabiliser sodium polyphosphate. With increasing concentrations of the permeabiliser sodium polyphosphate, a decrease in the MIC was observed. The permeabiliser trisodium citrate does not appear to have any effect on the MICs for the biocides MIT and CMIT. However, at 1% w/v the MIC for BIT decreased for 9.2 to 9.0 μg mL^{-1}. There was no observable growth in the presence of EDTA, this was due to the antimicrobial properties demonstrated by EDTA towards *P. aeruginosa*. The results indicated that in the presence of an agent that disrupted the outer membrane the resistant cells became sensitive to their corresponding biocides. Therefore, the observed resistance was a result of some alteration in the outer membrane which prevented the biocides gaining entry into the cell interior.

Summary.

The observed increase in the relative cell surface hydrophobicity between the mucoid and non-mucoid cells indicates a loss or reduction in the B-band O-polysaccharide present. The mucoid cells have cell-surface alterations commonly observed with cystic fibrosis patients, which are connected to the formation of biofilms and alginate matrix. This infers that the mucoid resistant forms may indicate cell-surface alterations that would aid biofilm formation. There were no observable differences between the lipopolysaccharide (LPS) banding patterns of the mucoid and non-mucoid cells. Thus, the observed resistance towards the biocides is not a result of increased phospholipid patches in the LPS thereby facilitating increased entry of the biocide into the cell. The isothiazolone biocides were not discovered to be membrane active. Therefore, the observed alterations in the outer membrane are not a direct result of the action of the biocides but connected to the resistance mechanism. The permeabilisers render the cells susceptible to the biocides hence indicating the resistance is connected to the outer membrane.

Chapter Seven

The Isolation of Bacteria from Contaminated Industrial Products Using an Isothiazolone Biocide Preservative System

Antibiotic resistance has been studied for many years. However, the genetic and biochemical basis of resistance towards antimicrobial agents (for example, disinfectants, antiseptics and preservatives) is less well understood (Russell, 1995; Chopra, 1991). Resistance towards non-antibiotic antimicrobials is an increasing problem and, therefore, research into this area is increasing, leading to an understanding of the mechanisms involved. Resistance is defined as the ability of bacteria to evade the action of an antimicrobial agent. Sensitivity, in comparison, is defined as the ability of an antimicrobial agent to inhibit the growth or kill the bacteria. Tolerance is defined as an ability to grow in the presence of the antimicrobial agent by an adaptive mechanism. It is, therefore, difficult to determine whether a bacterium is demonstrating tolerance or resistance to an antimicrobial agent.

Biocides have a tremendous industrial importance in preventing spoilage of paints, cosmetics and pharmaceutical products by microorganisms. The observed increase in resistance towards biocidal agents is a potential industrial problem. The isothiazolone biocides are widely used in the preservation of paints, pharmaceutical and cosmetic products (Collier *et al,* 1990c; Shepherd *et al,* 1985). The microbial contamination of petroleum products is a considerable problem in refineries and distribution systems. However, a mixture of MIT and CMIT is found to be effective against *Pseudomonas* spp. contaminating these products (Bento & Gaylarde, 1996). Acquired resistance towards biocides is thought to result from genetic changes. These occur either by mutation or the acquisition of genetic material from other bacteria (Russell, 1995). Non-plasmid-encoded acquired resistance towards biocides may result when the bacteria are gradually exposed to sub-inhibitory concentrations of biocide (Russell, 1995). This type of resistance is often

unstable and hence, may be lost when the bacteria are removed from the presence of biocide (Russell, 1995).

Cosmetics and paints do not need to be sterile. However, they must be adequately preserved. Cosmetic products constantly come into contact with possible sources of contamination, for example; saliva, dirty hands and tap water. They are also generally kept in warm and humid environments (Magee *et al,* 1997) which will encourage the growth of microorganisms. Microbial contamination of cosmetic products may result in infection, discoloration, gas production, odour formation and general unfitness for purpose (Magee *et al,* 1997). Although spoilage of the cosmetics is a significant problem, the greatest consideration is the threat to human health (Magee *et al,* 1997). There have been reported cases where mascara has been contaminated by *Pseudomonas aeruginosa* (Magee *et al,* 1997), which has the potential to result in serious eye infections. It is therefore, essential that biocidal products are used as inhibitors or preservatives in the cosmetic industry. However, the task of preservation becomes increasingly difficult with the onset of bacterial resistance to these products.

In the paint industry the potential for risk to human health may not seem initially important. However, if potentially pathogenic bacteria begin to grow within the product, this may pose a potential health risk. The consumer may have broken skin during use of the product, which may result in bacteria gaining entry into the body, or may become infected through inhalation. Spoilage of the paint products by microorganisms may result in the breakdown of emulsions and possible coagulation of the product. This therefore, has significant economic implications and if the contamination occurs during the manufacturing of the product, may result in a large scale problem.

The purpose of this investigation was to identify the nature of microbial contamination in two samples from an industrial source in the paint and coatings industry and to assess any evidence of bacterial resistance towards the isothiazolone biocides. The techniques used have been outlined in the previous chapters. Initially the contaminants were isolated from the samples and identified. The MICs of the isolated bacteria were determined against the isothiazolone biocides and the thiol-interactive agent thiomersal.

The outer membrane proteins were extracted and the profiles were determined, in order to observe any alterations in the cell envelope outer membrane proteins and to establish if T-OMP was present in the outer membrane.

Sample Information

The first sample was taken from a contaminated external coatings product. The product in question is used to protect wood against potential damage from the elements. It is preserved with a 0.5% w/v commercial isothiazolone preservative. The isothiazolone preservative is composed of CMIT (0.5%) and MIT (0.2%), in addition to 2-(2butoxyethoxy(ethoxy)-methanol and (ethylenedioxy) dimethanol. The second sample was taken from a contaminated paint product. The product is used to protect brick work and concrete against damage from the elements. It is preserved with the same preservative system as sample one.

The contaminated products samples were placed in clean, sealed containers, and transported to the laboratory at room temperature. They were stored in the laboratory at 4°C until analysis.

Isolation of microorganism from the test samples

The isolation of bacteria from the samples, in order to identify all the bacteria present, was achieved by two methods. Initially an enrichment technique was used. An aliquot (1 mL) of the sample was added to separate flasks containing 100 mL of nutrient broth or R2A medium. The flasks were incubated for 18 hours at 30°C, 200 osc min^{-1} in an orbital incubator (Gallenkamp INA-305). An aliquot (1 mL) of the culture was serially diluted in the range of 10^{-1} to 10^{-10} in 0.9% w/v sterile saline. The dilutions were plated out in triplicate on appropriate media and incubated for 24 hours at 30°C. The plates were counted, viable counts estimated and colonies sub-cultured for further analysis.

The second technique performed was the filtration technique. An aliquot (0.1 mL) of the sample was made up to a total volume of 20 mL with sterile water. This solution was filtered under pressure using a vacuum pump (Whatman, Maidstone) through a 0.2 μm

nitrocellulose membrane (Whatman, Maidstone). The filter membrane was placed on to the surface of sterile agar plates of nutrient agar (Oxoid CM 3) or R2A agar. The plates were incubated for 24 hours at temperatures of 25°C, 30°C and 37°C. The colonies were counted, viable counts calculated and colonies subcultured for further analysis.

Identification of Isolated bacteria

The bacteria isolated from the test samples were identified by preliminary identification techniques, which included the Gram reaction, cell morphology, motility test, catalase test and oxidase reaction (Chapter 2). This then enabled the appropriate API test (Biomerieux, France; API 20 NE, API 20 E or API 50 CH) to be used in order to identify the genus and species of the bacterium.

Determination of the Minimum Inhibitory Concentrations of the Identified Bacteria

Selected bacteria identified (Table 21) were inoculated into aliquots (25 mL) of appropriate media (Nutrient broth or R2A). The cultures were incubated for 19 hours at the appropriate temperatures (according to their isolation temperature), 200 osc min^{-1} (Gallenkamp, INA). The MIC was determined according to Bloomfield (1991), (described in Chapter 3) against the three isothiazolone biocides BIT, MIT and CMIT and the thiol-interactive agent thiomersal. Control cultures of the Gram negative bacteria were also tested for their MIC's against the four biocides. An aliquot (10 mL) of the cultures was centrifuged for 15 minutes at 5000 g (Microcentaur, MSE). The pellet was washed three times in Tris buffer (0.5 M, pH 6.8) and stored at -18°C for further analysis.

Preparation of Outer Membrane Proteins Profiles

Outer membrane proteins were prepared according to Pugsley (1986; Chapter 5) from the stored pellets. The outer membrane proteins were separated by SDS-PAGE (Laemmi, 1970; Chapter 5) using a 12% separator gel. The protein concentrations were estimated using the BCA assay (Smith *et al*, 1985; Chapter 2).

The estimation of viable counts from the enrichment and filtration techniques are shown in Tables 18 and 19. Results from the enrichment technique illustrated a microbial load of approximately the same size in both the paint product and coatings product when grown on R2A or nutrient agar medium. However, when considering the filtration technique, which gave a more representative estimation of the microbial load in the samples, the contaminated paint product had a higher microbial load. The microbial population in the paint product was too numerous to count in many instances. The greatest counts were observed at 37°C, the temperature of the human body. This is an important indication when considering the possible risk to human health and possible sources of contamination. The bacteria may be pathogenic or be opportunistically pathogenic. This optimum growth temperature may also indicate that the source of contamination is from a human source and, hence, may be occurring during the manufacturing of the product.

Table 26: Viable Counts Obtained from the External Coatings and Paint Product using the Enrichment Isolation Technique.

Product	Medium (CFU mL^{-1})	
	R2A	Nutrient Agar
External Coating	1.24×10^8	2.3×10^8
Paint Product	1.89×10^8	2.04×10^8

Table 27: Viable Counts obtained from the External Coatings and Paint Products using the Filtration Technique.

Product	Isolation Temperature	Medium (CFU mL^{-1})	
		R2A	Nutrient Agar
External Coating	25°C	0	10
	30°C	20	90
	37°C	20	90
Paint Product	25°C	TNTC	TNTC
	30°C	TNTC	TNTC
	37°C	30	120

Table 28: Preliminary Morphological and Biochemical Test Results from the Bacteria Isolated from the Coating and Paint Products.

Sample	Gram Reaction	Morph.	Motility	Oxidase	Catalase	Reference
Coatings	+	Rods	+	nd	-	CP1
Coatings	+	Rods	+	nd	-	CP2
Coatings	+	Rods	+	nd	-	CP3
Coatings	+	Rods	-	nd	-	CP4
Paint	+	Rods	+	nd	-	PP1
Paint	+	Rods	+	nd	+	PP1
Paint	+	Rods	-	nd	+	PP3
Paint	+	Rods	+	nd	-	PP4
Paint	-	Rods	-	-	nd	PP5
Paint	-	Rods	-	-	nd	PP6
Paint	-	Rods	-	-	nd	PP7
Paint	-	Rods	-	-	nd	PP8

morph. = morphology; nd = not done

Table 29: Identification of the Bacteria Isolated from the Coatings and Paint Products using the API Identification Technique

Reference Number	API System	Identification	Percentage I.D
CP1	50 CH	Unacceptable	nr
CP2	50 CH	*Paenibacillus alvei*	nr
CP3	50 CH	Unacceptable	nr
CP4	50 CH	Unacceptable	nr
PP1	50 CH	*Bacillus coagulans*	nr
PP2	50 CH	*Bacillus circulans or Bacillus subtilis*	nr
PP3	50 CH	*Bacillus stearothermophilus*	nr
PP4	50 CH	Unacceptable	nr
PP5	20 E	*Acinetobacter spp.*	87.4
PP6	20 E	*Tatumella ptyseos*	22
PP7	20 E	*Tatumella ptyseos*	99.8
PP8	20 NE	*Pseudomonas flourescens*	30.3

nr = no result

The majority of bacteria isolated from the coatings product appeared to belong to the genus *Bacillus*. Although an acceptable identification was not possible in the majority of the isolated cultures from the coatings product, the aerobic Gram positive rods appeared to posses an endospore, and, therefore, are most likely to belong to the genus *Bacillus*. The possible reason for the poor identification of these bacteria is that the API system is designed to identify medically important bacteria and, as such, is not always successful in the identification of environmental isolates. *Bacillus spp.* are widespread in nature,

commonly isolated from soil and food and few species are pathogenic to humans (Holt *et al,* 1994). They can survive extreme temperatures due to their endospores.

A greater variety of bacteria were isolated from the contaminated paint product these included three *Bacillus spp.*, *Paenibacillus alvei*, *Acinetobacter spp.*, *Tatumella ptyseos* and *Pseudomonas flourescens*. The two species which were of particular interest in this project were *T. ptyseos* and *P. flourescens*, the Gram negative rods. The outer membrane proteins were extracted from their Gram negative cell envelopes, to determine if any observable differences occurred in the strains demonstrating tolerance towards the isothiazolone biocides and sensitive control cultures. It was, therefore, possible to determine whether T-OMP was present. The reference cultures were *T. ptyseos* and *P. flourescens. T. ptyseos* is a rare opportunistic pathogen, its optimum growth temperature is between 25 and 36°C, it is isolated from human clinical specimens, mainly the respiratory tract, some blood isolates and animals. *P. flourescens* is an opportunistic pathogen, it is widespread in nature and can be isolated from a number of environmental or human sources.

Table 30: Determination of the MIC for the bacteria identified from the coatings and paint products against the three isothiazolone biocides and thiomersal.

Reference	Determined MIC ($\mu g\ mL^{-1}$)			
	BIT	MIT	CMIT	Thiomersal
Control Bacteria				
Pseudomonas flourescens	256	64	4	4
Tatumella ptyseos	1	1	1	1
CP1	2	N/A	N/A	8
CP2	4	N/A	N/A	8
CP3	2	N/A	N/A	2
CP4	2	N/A	N/A	2
PP1	2	N/A	N/A	2
PP2	1	N/A	N/A	1
PP3	1	N/A	N/A	2
PP4	2	N/A	N/A	1
PP5	**4**	**64**	**64**	**4**
PP6	**4**	**64**	**4**	**1**
PP7	**2**	**128**	**16**	**1**
PP8	**1**	**64**	**2**	**1**

N/A = Not Applicable; Embolden cultures PP5, PP6, PP7 and PP8 = Gram negative bacteria (refer to Table 29 for the identifications).

The MICs for the species of *Bacillus* isolated from the coatings product (Table 28) were determined against the isothiazolone biocide BIT and the thiol-interactive agent thiomersal to establish whether any degree of resistance towards the biocides was demonstrated (Table 28). It was observed that relatively low concentrations of biocide (between 2 and 8 $\mu g\ mL^{-1}$) inhibited the growth of the bacteria. It was suggested that these concentrations were too low to suggest any real level of tolerance or resistance. Therefore, the bacteria isolated from the contaminated coatings product were not investigated further. The concentrations of biocide required to inhibit the growth of the *Bacillus spp.* isolated from the paint product were between 1 and 2 $\mu g\ mL^{-1}$. This group was also excluded from further study due to the low level of tolerance or resistance demonstrated towards the biocides.

The MICs for the Gram negative bacteria isolated from the contaminated coatings product were determined against all three isothiazolone biocides and thiomersal (Table 28). The concentrations of BIT and thiomersal required to inhibit the growth of the bacteria were low at between 1 to 4 $\mu g\ mL^{-1}$. However, the concentration of MIT required to inhibit the growth of the *Acinetobacter* (PP5), *T. pytseos* (PP6) and *P. flourescens* (PP8) was 64 $\mu g\ mL^{-1}$, and was 128 $\mu g\ mL^{-1}$ for *T. ptyseos* (PP7). The MIC for MIT against the control culture of *T. ptyseos* was 1 $\mu g\ mL^{-1}$ and the MIC against the control *P. flourescens* was 64 $\mu g\ mL^{-1}$. Therefore, the isolated cultures of *T. pytseos* demonstrated resistance towards the biocide MIT. The MICs determined against CMIT for the isolated cultures ranged between 2 and 64 $\mu g\ mL^{-1}$, whereas the MICs for CMIT, against the control cultures were 1 $\mu g\ mL^{-1}$ for *T. ptyseos* and 4 $\mu g\ mL^{-1}$ for *P. flourescens*. The isolated culture of *P. flourescens* (PP8) did not demonstrate any resistance towards CMIT. However, the MIC was 16 $\mu g\ mL^{-1}$ for the isolated culture of *T. ptyseos* (PP8) thereby indicating a significant tolerance towards CMIT. The MIC for *T. ptyseos* (PP6) was 4 $\mu g\ mL^{-1}$, thereby indicating a slight tolerance towards CMIT. The preservation system in the paint and coatings products contains the isothiazolones MIT and CMIT, thus, it is suggested than on exposure to the biocides the bacteria develop tolerance towards the preservation biocides. There is no apparent resistance demonstrated towards the third

isothiazolone biocide BIT. However, the isolated culture of *P. flourescens* (PP8) had a considerably lower MIC against BIT, thereby indicating an increased resistance towards one isothiazolone biocide and increased sensitivity towards another. The observed MIC results for the *Acinetobacter spp.* (PP5) were relatively high for MIT and CMIT in comparison to the other biocides. It would have been interesting to compare these results to a control bacterium. However, this was not possible because the bacterium was not identified to species level.

The outer membrane proteins were extracted from the Gram-negative isolates and the control bacteria, and the protein profiles were determined using SDS-PAGE. Upon examination of the protein profiles (Figure 53) it was observed that OMP shifts occurred between the sensitive and resistant cultures. Eight OMPs (protein numbers 1, 2, 7, 14, 15, 18, 19 and 21) visible in the sensitive cells of *T. ptyseos* are not visible in the resistant profile. It is not possible to accurately identify any the addition of OMPs in the resistant profile, because of the difference in the protein concentration between the sensitive and resistant profile. The OMP profile for the sensitive culture of *P. flourescens* contains 22 visible protein, whereas, the resistant culture of *P. flourescens* contains only 17 visible proteins. The following proteins have present in the sensitive cultures have disappeared from the resistant strain, protein numbers 10, 15 17 19 and 21. However, two OMPs (12 and 16) have appeared in the resistant profile which are not present in the sensitive profile. Therefore, it may be suggested that the increased tolerance towards the biocidal agents may be a result of the alterations in the OMP profiles.

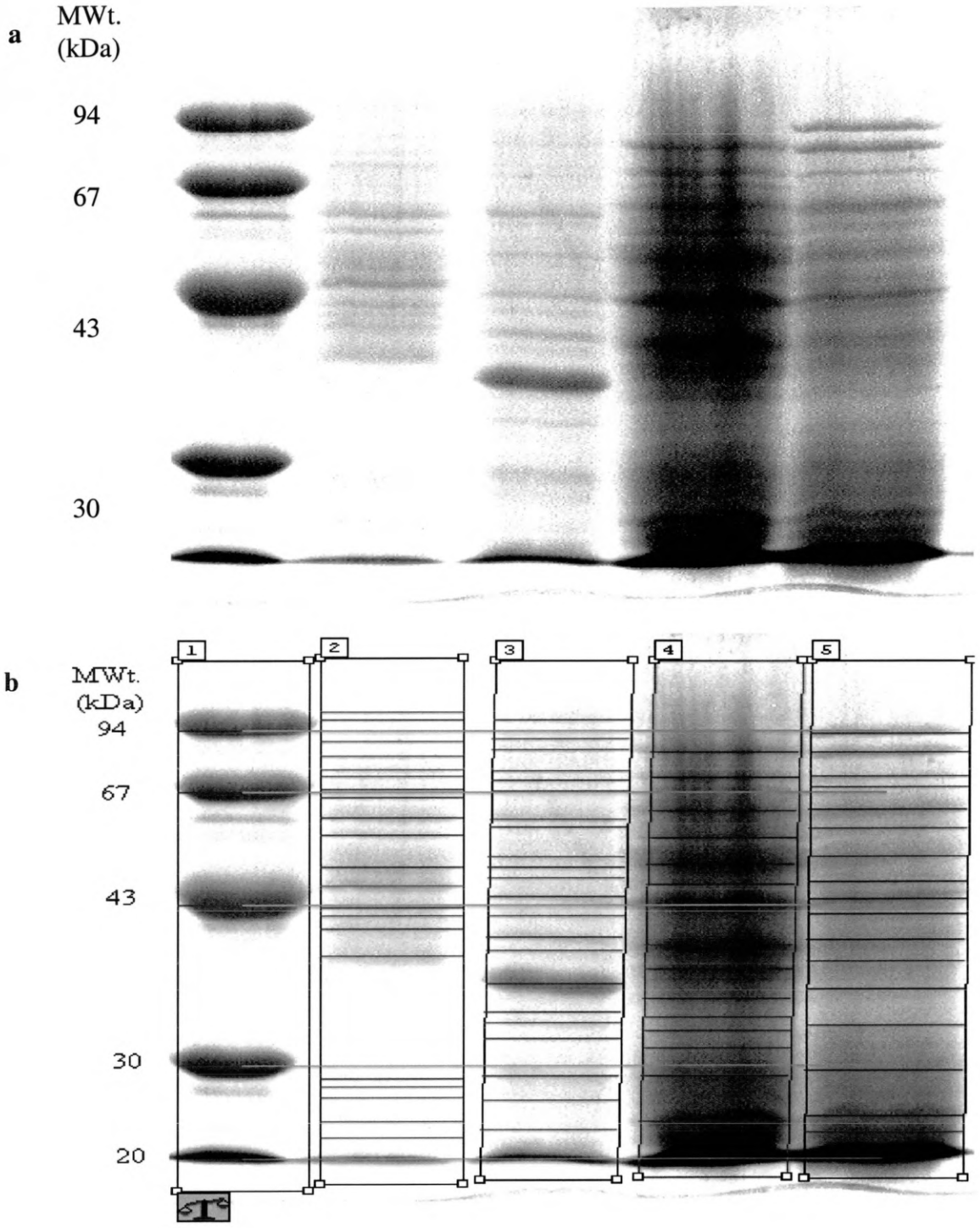

Figure 53 (a) Outer Membrane Protein Profile of the Gram negative bacteria isolated from the coatings and paint products and the control isolates of *Pseudomonas flourscens* and *Tatumella ptyseos*. Lane 1: molecular weight markers; Lane 2: control cells of *Tautmella ptyseos*; Lane 3: control cells of *Pseudomonas flourscens*; Lane 4: isolated cells of *Tautmella ptyseos*; Lane 5: isolated cells of *Pseudomonas flourscens*. **(b)** The same gel image after Phoretix analysis. Black lines on the image indicate the presence of protein bands. White lines on the image indicates computed propagation of molecular weights across the gel. Scales icon indicates the marker lanes. Protein concentrations:- Lane 2: 14 $\mu g\ mL^{-1}$; Lane 3: 19$\mu g\ mL^{-1}$; Lane 4: 26$\mu g\ mL^{-1}$; Lane 5: 21 $\mu g\ mL^{-1}$.

Action taken to prevent the contamination of the coating and paint products

In order to prevent microbial growth in the commercial products action was taken before this investigation was carried out. The preservation system in the coatings product has been altered to a solution of 0.2% w/w stabilised solution of bronopol and Kathon™ (CMIT (0.7-0.9% w) and MIT (0.2-0.3% w) and 0.25% w/w synergistic combination of aromatic and heterocyclic algicidal and fungicidal active agents. In addition, the production plant has been revisited, sterilisation techniques and hygiene regulations have been reviewed. The new system appears to be working, there has been no reports of contaminated products. However, within time the bacteria may develop resistance towards the new preservation system.

Chapter Eight

Discussion: Studies on T-OMP and the Development of Antimicrobial Tolerance in *Pseudomonas aeruginosa* PAO1

Resistance is defined as the ability of a microorganism to evade the action of an antimicrobial agent, whereas sensitivity is defined as the ability of the agent to inhibit the growth of or kill the microorganism. Resistance is divided into two classes intrinsic and acquired. Intrinsic resistance is the in-built mechanism of the microorganism, in which they evade the action of the antimicrobial agent. Intrinsic resistance is particularly important in Gram negative bacteria. This is due to the semi-permeable nature of the outer membrane towards antimicrobial agents. Acquired resistance may be sub-divided into genotypic and phenotypic characters. Phenotypic resistance is classified as being unstable and reversible with no related genetic alterations to the genome. Therefore, the observed resistance will be reversed when the prevailing conditions are removed. Phenotypic resistance often exploits some intrinsic resistance mechanism of the microorganism. Genotypic resistance is defined as the stable acquisition of new genetic information or mutations and it is, therefore, non-reversible.

When resistance is observed towards a particular antimicrobial agent it is more commonly a result of numerous mutations resulting in a small gradual increment in resistance. Alternatively, resistance may be the result of a variety of biochemical mechanisms giving rise to acquired resistance. For example, resistance in Gram negative bacteria may result from the combined effects of the slow permeation of the antimicrobial into the cell, in association with the subsequent biochemical inactivation of the antimicrobial agent. For example, resistance to the ß-lactams. The mechanism of resistance within the cell must be developed whilst maintaining the physiological function of the cell. For example, the modification of the target site is often accompanied by a decrease in the sensitivity towards an antimicrobial, whilst the

target site maintains its function. For example, the overproduction of the target site is a mechanism associated with resistance towards trimethoprim. This results in a greater concentration of the antimicrobial required to inhibit the growth of the bacteria. The cell envelope structure demonstrates high levels of plasticity, which constantly interact with the external environment (Gilbert *et al*, 1987). Therefore, the cell envelope has the ability to adapt to many alterations in the external environment, in order to prevent the antimicrobial agent gaining entry into the cell interior.

The exposure of Pseudomonas aeruginosa PAO1 to the Isothiazolone biocides and Thiomersal.

Initial experiments investigating the development of resistance in *Pseudomonas aeruginosa* PAO1 towards the isothiazolone biocides and thiomersal were performed by repeated passage in MIC/4 of the biocide. The MICs increased for all four biocides when *P. aeruginosa* was cultured in R2A medium and for the three isothiazolone biocides when cultured in CDM. During the induction of resistance towards BIT in R2A medium the original MIC was 56 μg mL^{-1}. Following the first exposure to MIC/4 the MIC increased to 60 μg mL^{-1}. The MIC continued to increase throughout the induction of resistance at a gradual rate, until by passage 11 (the point at which the cells are deemed resistant) the MIC was 98.6 μg mL^{-1} (Figure 16). This is an overall increase of 76% over the original pre-exposed MIC. Upon removal of the cells from the presence of the biocide the MIC immediately decreased to 95 μg mL^{-1}. The cells were passaged three successive times in the absence of biocide, at which point (Passage 14) the MIC decreased to 84 μg mL^{-1}. A similar pattern of resistance was observed when *P. aeruginosa* was exposed to BIT and cultured in CDM. The original MIC for the sensitive cells was 5.73 μg mL^{-1}. The cells were passaged in the presence of MIC/4 of biocide for ten successive passages at which point (Passage 11) the MIC had increased to 9.07 μg mL^{-1} (Figure 17). This is an increase of 58% over the original pre-exposed MIC. Upon removal of

the cells from the presence of the biocide the MIC began to immediately decrease, at passage 14 the MIC was 6.4 $\mu g\ mL^{-1}$.

The initial MIC for the sensitive cells towards MIT during the induction of resistance was 19 $\mu g\ mL^{-1}$. Following the initial exposure to the presence of the biocide the MIC increased to 28.6 $\mu g\ mL^{-1}$. The MIC continued to increase with every successive passage in the presence of biocide. At Passage 11 the MIC was 48.66 $\mu g\ mL^{-1}$ (Figure 22), this is an increase of 156% over the original pre-exposed MIC. Once the cells were removed from the presence of the biocide the MIC immediately began to decrease, and at passage 14 the MIC was 39.3 $\mu g\ mL^{-1}$. The effect of the induction of resistance towards MIT was also significant in CDM. An overall increase of 298% in the MIC from the sensitive cells (MIC of 2.46 $\mu g\ mL^{-1}$) to the resistant cells (MIC of 3.4 $\mu g\ mL^{-1}$, Figure 18) was observed. The initial passage in the presence of biocide demonstrated an increase of 10 $\mu g\ mL^{-1}$. Upon removal of the cells from the presence of the biocide the MIC decreased to 9.0 $\mu g\ mL^{-1}$ by passage 14.

The original MIC for the sensitive cells in the induction of resistance towards CMIT in R2A media was 1.3 $\mu g\ mL^{-1}$, which is significantly lower than the other biocides. This may be due to the different mode of action of CMIT as a result of the highly reactive thiol-acyl chloride formation (Collier *et al*, 1990a; Collier *et al*, 1990c). There was no significant increase observed in the MIC of CMIT until passage five, the fourth subsequent passage in the presence of biocide, at which point the MIC had increased to 1.76 $\mu g\ mL^{-1}$ (Figure 19). This indicates a different mechanism of resistance may be occurring during the induction of resistance towards CMIT, as opposed to that involved in resistance to the other biocides. At passage 11, the MIC had increased to 2.5 $\mu g\ mL^{-1}$, an increase of 92% over the original pre-exposure MIC. Once the cells are cultured in the absence of biocide a significant decrease was observed between passages 11 and 12 to 2.0 $\mu g\ mL^{-1}$. The MIC decreased to 1.8 $\mu g\ mL^{-1}$ by passage 14, indicating that the mechanism of resistance is dependent upon the presence of the biocide. Upon initial

exposure of the cells towards MIC/4 of CMIT cultured in CDM, the MIC increased from 0.25 μg mL^{-1} to 0.3 μg mL^{-1}. However, there was no further significant increase in the MIC until passage 5, at which point the MIC increased to 0.38 μg mL^{-1}. This is the same point at which the development of resistance was observed in the cells cultured in R2A medium. At passage 11, the MIC increased to 0.6 μg mL^{-1} (Figure 20), an increase of 140% over the original pre-exposure MIC. Upon removal of the cells from the presence of the biocide the MIC decreased, until at passage 14 the MIC was 0.15 μg mL^{-1}. This final MIC was less than the original MIC of the sensitive cells, indicating that resistance had been completely reversed and that, subsequently, the cell had enhanced sensitivity towards this biocide. This notable difference regarding the biocide CMIT may be indicative of the different activity of the biocide. The MIC decreased when the cells resistant to CMIT in R2A were placed in biocide free-media. However, this reversal of resistance goes further than the original MIC in the exposure to CMIT in CDM and may be due to the absence of thiol-groups. Hence, the activity of the biocide is not quenched by the presence of thiol-groups in the media. This may also indicate that the resistance mechanism is some form of reprocessing procedure within the cell. The resistance mechanism may be connected to an active metabolic flux of the biocide, which removes the biocide from the cell. Therefore, accounting for the lower observed MIC in the passage 14 cells than the sensitive cells.

The induction of resistance towards thiomersal demonstrated a slightly different pattern than that of the isothiazolone biocides. The initial MIC for the sensitive cells was established at 9.4 μg mL^{-1}, and following the first exposure in the presence of biocide the MIC increased to 10 μg mL^{-1}. The MIC began to increase in a definite step-wise fashion (Figure 21) until at passage 11 the MIC had increased to 17.93 μg mL^{-1}. This is an overall increase of 91% between the sensitive and resistant cells. Upon removal of the cells from the presence of the biocide the MIC continued to increase to 18.0 μg mL^{-1}. At passage 14 the MIC had decreased to 16.8 μg

mL^{-1}. It was not possible to induce resistance towards thiomersal in CDM. This is thought to be a result of the combined effect of nutrient limitation and biocide induced sub-MIC stress. These observations indicate, that the mechanism of resistance developed towards thiomersal, is a different mechanism than that towards the isothiazolone biocides.

The resistance observations indicate that the resistance mechanism is dependent upon the presence of the biocide, but it is a gradual adaptation and not an instantaneous one. Upon the removal of the cells from the presence of the isothiazolone biocides, the MICs begin to decrease, indicating that biocide presence is required for the mechanism of resistance. However, the reversal of the resistance is not immediate in the majority of cases. The only example, whereby the resistance appears to be totally reduced, was the induction of resistance towards CMIT in CDM. This is possibly due to the reactivity of the biocide and the absence of exogenous thiol-groups which increased the rate of the reversal mechanism. Thereby, indicating that the resistance mechanism is connected to an active metabolic efflux of the biocide from the cell. Results indicate that the resistance reversal mechanism is a gradual step-wise adaptation, similar to the mechanism for the development of resistance.

Brözel and Cloete (1994) concluded that a gradual adaptation in the MIC indicates that the mechanism of resistance is not a mutational event, but a specific intracellular mechanism. They observed that T-OMP was lost within the first exposure to the biocide and did not reappear in the 72 hours post-exposure, in the absence of biocide. Their observation of the loss of T-OMP in the first exposure to the biocide indicates that the mechanism of resistance is only related to the loss of the protein and that the protein is not the sole mechanism of resistance. The protein disappeared before the cells reached significant levels of resistance, and hence, this implies that a biochemical system may participate in the resistance mechanism. They observed that the T-OMP did not reappear in the 72 hour post-exposure in the absence of biocide, unlike the findings of this investigation. This may be due to differences in the specific form of biocide

used between these two studies. Brözel & Cloete (1994) induced resistance using the commercial product Kathon™, whereas the pure forms of the biocides were used the present investigation.

The reactivity of the biocides in relation to the observed resistance.

The biocide CMIT is the most reactive of the four biocides used in this investigation (Table 11; Collier *et al*, 1990a; Collier *et al*, 1990c). This greater reactivity is thought to be related to the specific mode of action of CMIT. The initial reaction of CMIT with thiols results in the formation of mixed disulphide adjuncts. Further reaction with thiols results in the formation and subsequent release of oxidised thiol-dimers and reduced open-ring forms of the isothiazolone biocides (e.g. mercaptoacrylamide; Collier *et al*, 1990b) The mercaptoacrylamides react further with the biocides resulting in biocide dimers (Collier *et al*, 1990b). The CMIT mercaptoacrylamide tautomerises producing a highly reactive thiol-acyl (Figure 4; Collier *et al*, 1990b). This is thought to account for the biocidal activity demonstrated towards the eukaryotic microorganism *Schizosaccharomyces pombe* (Collier *et al,* 1990b). However, significant biocidal activity was not observed towards Gram negative bacteria and this is thought to be related to the selective permeability of the Gram negative outer membrane (Collier *et al*,1990b). Following treatment of the cells with EDTA, Brözel and Cloete (1994) observed bactericidal activity towards *P. aeruginosa*. However, following treatment with the permeabilisers sodium polyphosphate (SPP) and trisodium citrate dihydrate (TCD) the sensitivity of the cells was increased towards the biocides (Tables 17 and 18). The MICs decreased when in the presence of permeabiliser agents which enhances the entry of the biocides into the cell interior. However, they did not decrease to the levels observed in the sensitive cells (Tables 17 and 18). This indicates that the outer membrane plays a role in the development of the resistance towards the isothiazolone biocides and thiomersal, but it is not

the only factor participating in the resistance mechanism. This supports the theory of Collier *et al*, (1990b) that the activity of the biocides is rate limited. This is because the OMPs limit the entry of the biocide into the cell interior. The biocides may demonstrate biocidal activity if they are allowed to attain high concentrations in the cell interior. If the biocide is only allowed to slowly enter into the cell interior it will not reach sufficient concentrations to achieve biocidal activity. Therefore, if the LPS is removed the entry rate into the cell interior is increased and hence, permits a greater biocide concentration into the cell resulting in higher levels of bactericidal activity (Brözel & Cloete, 1994).

Alterations in the Outer Membrane Proteins and their association with Antimicrobial Resistance.

Alterations in the outer membrane proteins in Gram negative bacteria have been associated with resistance to a variety of antimicrobial agents. Although it is often postulated that the loss or appearance of the OMPs is directly associated with the observed resistance to the antimicrobial, in many cases it may be a phenomenon associated with the resistance mechanism occurring within the cell. A decrease in the intensity of OMP F (38 kDa) and a prominent increase in a 41 kDa OMP is associated with resistance towards erythromycin in *P. aeruginosa* S-6 (Tateda *et al*, 1994). The role of the 41 kDa protein is unknown. However, it is possible that it is an altered form of OMP F. This is because the total amount of the 38 kDa and the 41 kDa proteins in the treated cells is equivalent to the total amount of the 38 kDa protein in the pretreated cells (Tateda *et al*, 1994). Sub-MIC treatment with aztreonam was found to increase the copy number of the 38 kDa protein (OMP F) in *P. aeruginosa* and was observed to result in alterations in the integrity of the LPS (Magni *et al*, 1994; Cipriani *et al*, 1991). Imipenem is a broad spectrum carbapenem ß-lactam antibiotic, which is highly active against *P. aeruginosa*. It is of particular interest because of its high potency, broad spectrum activity and

the general lack of microbial cross-resistance found with other ß-lactam antibiotics (Ochs *et al*, 1999). It was found that resistance developed at a significant rate and was associated with the loss of protein D2 (41 kDa) (Hung *et al*, 1992; Yoshihara *et al*, 1996). They also observed a decrease in the production of three other outer membrane proteins C, E1 and E2 (23, 35, and 45 kDa respectively). These proteins are also associated with resistance towards ß-lactams, the new quinolones and chloramphenicol. The loss of OMP D expression is a result of the deletion in the OMP D coding region and upstream promoter region (Ochs *et al*, 1999).

There are three types of outer membrane proteins (OMP) in bacteria, which are classified as general, specific and those related to the active transport mechanisms. For example, the active transport systems related to the uptake of vitamin B12 and iron chelation (Kramer, 1999). The active transport systems are energised by conformational coupling to the plasma membrane through proteins spanning the periplasm (Kramer, 1999). The overproduction of Opr J is associated with cross-resistance to the new cephems and quinolones and is most probably associated with alterations in the efflux transport of the antimicrobials (Masuda *et al*, 1995). The loss or overproduction of the OMPs in Gram negative bacteria may not be the direct mechanism of resistance, but it is often an indication of some other resistance mechanism occurring within the cell. Therefore, the OMP profiles in the cells resistant towards the isothiazolone biocides and thiomersal from this study were investigated to determine if any alterations were observed.

The Observation of T-OMP shifts.

The OMP profiles of all the passages of cells exposed to MIC/4 of the isothiazolone biocides and thiomersal were investigated to determine whether any alterations could be related to the observed resistance to the biocides. An outer membrane protein (T-OMP) in the range of 31-36 kDa (Table 17) was observed to disappear in cultures of *P. aeruginosa* exposed to MIC/4

concentrations of the isothiazolone biocides and thiomersal. There are slight variations in the estimated molecular weights of T-OMP, which is thought to be due to variations in the scanning of the gels into the computer and the exact point of estimation of the Rf values by the computer software. The mean of the estimated values for T-OMP is 33 kDa (with a standard deviation of 1.718 kDa). Brözel and Cloete (1994) observed the disappearance of T-OMP to occur at 35 kDa. However, they did not use a computerised system for molecular weight determination. They estimated the Rf values of the proteins against the position of molecular weight makers by hand, this may account for the variation between the results of this investigation and that of Brözel and Cloete (1994). T-OMP was absent in all of the cultures which were deemed resistant to the isothiazolone biocides and thiomersal. However, the exact passage number at which point the protein disappeared varied between different induction systems. T-OMP was observed to disappear at passage four during the induction of resistance towards BIT in R2A media. At this point the cells had been exposed to three successive passages in the presence of biocide and the MIC of the cells was 76 μg mL^{-1}. Therefore, the MIC had increased by 20 μg mL^{-1} from the original pre-exposed MIC. This represents 36% of the overall increase in MIC between the sensitive and resistant cells. In the cells exposed to MIT and thiomersal in R2A media, T-OMP was observed to disappear by the second passage. This is after only one exposure to MIC/4 of biocide, and the MIC has only increased to 28.6 μg mL^{-1} for the MIT exposed cells and 10 μg mL^{-1} for the thiomersal exposed cells. This represents only 32% and 7% respectively of the total increase in MIC. T-OMP was also observed to have disappeared by the second passage in the cells exposed to BIT and MIT in CDM. This only represents 19% of the total increase in MIC for the BIT induction of resistance and 13% of the total increase in the MIC for the MIT induction of resistance. T-OMP did not disappear until the third passage in the induction of resistance towards CMIT in R2A medium and the fourth passage in the induction of resistance towards CMIT in CDM. This is possibly

because the MICs, in this instance, did not begin to increase after the cells initial exposure to biocide. There was no significant increase in MIC until the fifth passage in R2A medium. Although there was a slight increase in the MIC after the initial exposure to the biocide in CDM, there was no significant increase in MIC until the fifth passage. These results suggest that T-OMP is sensitive to sub-MIC concentrations of biocide and that this is exhibited by the early disappearance of this protein in passages well below the deemed resistance level (Passage 11). The protein T-OMP reappears in all cases when resistant cells are passaged in the absence of the biocide. When resistant cells are subsequently passaged in the absence of the biocide T-OMP reappears in the first of such passages. This suggests that the appearance / disappearance of T-OMP is dependent upon the presence of the biocide and may not be directly linked to the resistance *per se*.

The disappearance and reappearance of the protein T-OMP would initially support the theory by Brözel and Cloete (1994) that T-OMP is the entry route into the cell and hence the loss of T-OMP would limit the entry of biocide into the cell. Hence, this may account for the observed development of resistance in *P. aeruginosa* PAO1. However, upon close examination of the T-OMP disappearance and the observed MICs at the individual passage points, it is clear that the cells have developed little resistance to the biocides when the protein T-OMP disappears. In addition, the protein T-OMP reappears in most instances immediately after the removal of the biocide presence, at which point the MICs have decreased very slightly from the resistance levels (Table 12). Hence, the cells will still be significantly resistant to the biocide at this point. These results suggest that the disappearance of T-OMP is connected to the development of resistance, but not the sole mechanism accounting for that resistance. Brözel and Cloete (1994) did not observe the reappearance of T-OMP following the passaging of the resistant cells in biocide-free media. They therefore, concluded that the disappearance of T-OMP was a permanent resistance mechanism or mutation. However, in this study the protein T-

OMP reappeared and the MICs began to decrease when cells were cultured in the absence of biocide. Therefore, it is evident that the resistance mechanism is not permanent when the presence of the biocides is removed.

Brözel and Cloete (1994) observed a wider stress response (that is other resistance mechanisms in association with OMP alterations) on the first exposure to the biocide. This resulted in the concurrent production of minor OMPs which were not detected in the sensitive, resistant or non-exposed cells. They concluded that these proteins did not contribute towards the observed resistance mechanism. Slight variations in the overall OMP profiles were observed in this investigation (Figure 30), and although not regarded as being initially significant in the resistance mechanism they may be of importance in subsequent investigations. A wider stress response is often demonstrated upon exposure to environmental stress conditions (Brown & Williams, 1985). Therefore, alterations in the outer membrane may infer the presence of several distinct resistance factors with similar outcomes. Resistance to the fluoroquinolones has been observed to be associated with alterations in DNA gyrase and cell permeability (Hostacka *et al*, 1995). The unusual presence of three OMPs at 54, 50 and 49 kDa is associated with the decreased cell permeability (Hostacka *et al,* 1995). The mutation *nal*B is associated with the presence of the 49 kDa protein and is also thought to confer cross-resistance to a variety of antimicrobial agents including the quinolones, cephems, carbenicillin and chloramphenicol (Masuda *et al*, 1995).

The two-dimensional gel electrophoresis analysis indicates, that outer membrane proteins which are present in the sensitive cells are lost from those cells resistant to MIT and thiomersal. However, it is not clear as to the exact identity of T-OMP in the two-dimensional gels. Therefore,it is not possible to conclude that T-OMP is lost from the outer membrane. However, the gels do indicate that protein shifts are occurring in the outer membrane, when a tolerance to the biocides is observed.

The effect of nutrient limitation on Pseudomonas aeruginosa.

In conditions of nutrient limitation the cell becomes economical with its metabolic processes in order to redirect the energy towards more essential services, this may result in OMP shifts. The effects of biocide challenge may have similar effects upon exposed bacterial cells. Therefore, the exposure of the cells to biocides may effectively starve the cells resulting in the alteration of OMP profiles. The observation of T-OMP shifts following exposure to biocides may result from the resistance mechanism directed towards the biocides or, the results of nutrient limitation subsequent to biocide challenge. In order to eliminate this second possibility from this investigation, the outer membrane was observed with reference to T-OMP under conditions of nutrient limitation in the absence of biocide. It was observed that under conditions of carbon, nitrogen, phosphorous and potassium limitation the protein T-OMP did not disappear (Figure 43). Therefore, it is possible to conclude that the alteration in the OMP profile, resulting in the loss of T-OMP, is related to biocide challenge and not nutrient limitation.

Cross-Resistance between antimicrobial compounds.

A wide variety of products including toothpastes, cutting boards and carpeting contain the antibacterial agent triclosan (Travis, 2000). Recent studies have reported triclosan-resistant microbes and there is growing concern that the increasing use of this product will encourage the evolution of bacteria impervious to the compound and others (Travis, 2000). Following the introduction of triclosan three decades ago, its use has rapidly increased. It was thought that resistance would not develop towards this compound, because the mode of action against bacteria targets the cell in a number of ways, instead of targeting a single protein (Travis, 2000). It was however discovered that the major mode of action is specific and inhibits the enzyme enoyl-ACP reductase or Fab I involved in fatty acid synthesis (Heath & Rock, 2000; Travis,

2000). A mutation in the gene encoding the enzyme in some bacteria is associated with resistance to triclosan (Travis, 2000). In addition, this mutation is associated with resistance towards antibiotics employed against tuberculosis, for example, isoniazid (Heath & Rock, 2000; Travis, 2000). Hence, the use of triclosan may result in the emergence of drug-resistant strains (Travis, 2000). Suzangar *et al*, (2000) suggested that the slow release of triclosan from cutting boards, could expose bacteria to sublethal concentrations of the agent and promote the development of resistance (Travis, 2000). The widespread use of this agent will promote the development of resistance and reduce the effective useful life of the agent, in addition it may promote cross-resistance towards other antimicrobial agents (Travis, 2000).Therefore, it is apparent that the induction of resistance to one antimicrobial agent may promote resistance towards others. It was suggested that the cultures resistant towards one of the isothiazolone biocides or thiomersal may be resistant to the other biocides. The results of the cross-resistance study (Table 14), investigating the possibility of cross-resistance between the induced resistant cultures of *P. aeruginosa* towards the isothiazolones and thiomersal and the other biocides, indicate the emergence of cross-resistance with this group. A marked increase was observed in the MICs of the cross-resistant test strains towards the other test biocides, over the original MIC prior to biocide exposure. For example, sensitive cells of *P. aeruginosa* were discovered to have an MIC of 56 μg mL^{-1} towards BIT, the test-culture resistant to MIT has an MIC of 70 μg mL^{-1} against BIT (Table 13). This indicated significant cross-resistance between the *P. aeruginosa* cultures resistant to MIT and BIT. The results of all the resistant strains indicate that once resistance has been induced in a culture towards one biocide the resistance is, to some extent, exhibited towards other members of the same group. The cross-resistance is also demonstrated towards other thiol-interactive agents. For example, the isothiazolone resistant strains demonstrate a degree of resistance towards thiomersal and *vice versa* (Tables 13 and 14).

Alterations in the Lipopolysaccharide and Cellular Morphology and their association with Antimicrobial Resistance.

OMP alterations and the integrity of the LPS influences the permeability of antimicrobial agents across the Gram negative outer membrane (Giordano *et al*, 1993). LPS molecules are confined to the outer leaflet of the outer membrane and are tightly associated with the OMPs (Jann & Jann, 1999). It is thought that modifications of the LPS structure play an important role in the regulation of the protein channels. Therefore, increased resistance towards antimicrobial agents may be associated with observed changes in the OMP or LPS, or both (Giordano *et al*, 1993). The LPS profiles of resistant cells were investigated to determine whether any observable alterations could be detected between the sensitive and resistant non-mucoid and mucoid cells. There were no observable alterations in the LPS profiles of the resistant cultures (Figure 57). However, it cannot be concluded that the LPS plays no factor in the mechanism of resistance. This is because alterations in the LPS profiles are not always observed when the LPS participates in the resistance mechanism.

Sub-inhibitory concentrations of erythromycin enhanced the serum sensitivity of some strains of *P. aeruginosa* (Tateda *et al*, 1994). This observation was accompanied by cellular morphology alterations and changes in the cell surface hydrophobicity. Wild-type rough mutants demonstrate an absence of heptose. They tend to be more hydrophobic and are, therefore, more permeable to hydrophobic antimicrobials (Rocchetta *et al*, 1999). This is thought to be due to the hydrophobic nature of the phospholipid bilayer patches, which replace the heptose. Nikaido and Varra (1985) suggested that reorganisation of the outer membrane, instead of substitution, accounts for the observed replacement of proteins with phospholipids. They also suggested that this reorganisation, rather than the alteration of LPS causes an increase in the hydrophobic permeability in the rough mutants.

Evans *et al*, (1991) observed that mucoid strains of *P. aeruginosa* were associated with decreased sensitivity towards the quinolone antibiotic ciprofloxacin. *P. aeruginosa* strain Z61 demonstrates a six-fold increase in permeability towards the ß-lactam nitrocefin. This is also accompanied by a 4 to 10000-fold increase in the susceptibility towards 30 different antimicrobials. Although there were no observed alterations in the OMP profiles (Angus *et al*, 1982). However, alterations were observed in the lipid A component of LPS, which is thought to favour an opening of the functional pores.

Two different morphological types of *P. aeruginosa* PAO1 were observed on the gradient plates in the routine maintenance of resistant cultures (Figure 45). One type was mucoid and the other non-mucoid. It is thought that the emergence of mucoid forms is accompanied by alterations in the LPS, because they are observed to express smaller quantities of B-band O-polysaccharide, whilst maintaining the levels of A-band O-polysaccharide (Rocchetta *et al*, 1999). The B-band polysaccharides are highly anionic and extend through the layers of the A-band polymers and OMPs (Rocchetta *et al*, 1999). The non-mucoid forms are devoid of alginate and are thought to contain predominately surface B-band O-polysaccharide and demonstrate low surface hydrophobicity (Rocchetta *et al*, 1999). The cell surface hydrophobicity characteristics of the two morphological types were investigated in order to establish if this absence could account for the difference. The non-mucoid cells exhibited a higher cell surface hydrophobicity than that observed with the mucoid cells resistant to the biocides MIT, CMIT and thiomersal in R2A medium (Figures 46 and 47). This suggests a loss or reduction in the B-band O-polysaccharide, whilst the A-band O-polysaccharide remains the same. The non-mucoid cells exhibit a higher hydrophilicity than that observed with the mucoid cells resistant to BIT in R2A medium (Figures 46 and 47). This indicates that there was no loss of the B-band polysaccharides in this instance. The non-mucoid cells demonstrated low surface hydrophobicity and surface charges, which adhere to glass and polystyrene more effectively

than those with a B-band absence (Rocchetta *et al*, 1999). Cell surface alterations and alginate production are commonly associated with bacterial strains isolated from cystic fibrosis patients and hence, may be a stress response related to the resistance mechanism. This is because the cell surface alterations and alginate may enable the cell to evade the action of the antimicrobial, by preventing the entry into the cell or producing a protective coating.

Efflux pumps

Active efflux proteins of wide specificity are common in wild type bacteria and contribute significantly to the intrinsic resistance observed in *P. aeruginosa* (Ma *et al*, 1994). Poole *et al*, (1993) demonstrated an efflux system involving three proteins Mex A, Mex B and OMP M, which are critical to the intrinsic resistance mechanism in *P. aeruginosa*. It has been established that a knockout mutation involving genes of any of the three proteins results in a four-fold to ten-fold increase in susceptibility towards the quinolones, ß-lactams (except imipenem), tetracycline and chloramphenicol. Multiple drug resistant pumps (MDR's) function to effectively protect the cells from commonly used drugs, which are numerous in type and function (Ouellette *et al*, 1997). The results of this study suggest that the observed resistance towards the isothiazolone biocides and thiomersal is associated with the switching-on of a MDR once the cells are exposed to sub-MIC levels of the biocides. It is also suggested, that the disappearance of T-OMP is associated with this switching-on mechanism. A prominent increase was observed in a 41 kDa protein following sub-MIC exposure of *P. aeruginosa* S-6 to erythromycin (Tateda *et al*, 1994). The role of this protein is unknown, however, it is suggested that it is an altered form of a 38 kDa protein (Tateda *et al*, 1994). Such a phenomenon could explain the observed loss of T-OMP in these studies. It is possible that the metabolic commitment and amino-acids involved in the construction of T-OMP are diverted towards the development of a resistance mechanism upon direct biocide exposure. This resistance

mechanism may involve the construction and operation of a MDR efflux pump, as yet unobserved, or alternatively the development of some internal resistance mechanism or pathway. The first evidence of the MDR in the Gram negative bacteria was reported by Lomovskaya and Lewis (1992). They observed that the MDR protein *Emr*AB in *Escherichia coli* protected the cells against nalidixic acid and thiolactomycin (Lewis *et al*, 1997). A MDR has recently been suggested by Maira-Latran *et al*, (2000) in a biofilm formation of *E. coli*. This MDR is thought to protect the cells against the antimicrobial agents iodine, chlorine, peroxygens and gluteraldehyde. Köhler *et al,* (1996) observed that the overexpression of OMP M and OMP J was associated with resistance towards trimethoprim and sulphamethoxazole, which is thought to be due to the expression of the mexABOprM efflux system.

Schweizer (Chuanchuen *et al*, 2000) has discovered that almost all strains of *P. aeruginosa* demonstrate resistance towards triclosan due to the action of efflux pumps (Travis, 2000). The pumps are found to remove triclosan and other toxic substances from the cell. Therefore, it is possible that the observed resistance in *P. aeruginosa* towards the isothiazolone biocides and thiomersal is a result of some resistance mechanism being switched on in the cells.

Conclusion.

This study was designed to extend and elucidate the work of Brözel and Cloete (1994) and to revisit the topic of resistance in *Pseudomonas aeruginosa* and the associated loss of T-OMP with the use of purified forms of the active ingredients of Kathon™. The physiological alterations of the cells involved in biocide exposure were closely examined using contemporary methods and techniques, which in turn have lead to an extension and improvement of our knowledge surrounding the development of resistance towards biocides and concurrent shifts in OMPs, particularly the T-OMP. The mechanism of resistance to the isothiazolones and thiomersal is a gradual adaptive process dependent upon the presence of the biocide. The loss of

the protein T-OMP (a 33 kDa protein) is associated with the development of resistance, but does not appear to be the sole reason for its occurrence. The protein disappears following the immediate exposure of the cells to the biocide and subsequently reappears upon the removal of the biocide presence, hence indicating the disappearance / reappearance of T-OMP is just a phenomenon related to the presence / absence of biocide and not the direct cause of resistance. This may imply that the metabolic energy required for the production of the protein is redirected into the resistance mechanism in order to remove the biocide from the cell interior. The morphological differences observed in the resistant cultures and the reversal of the MIC below sensitive MIC levels in the cells resistant to CMIT in CDM indicate a more complex resistance mechanism than that observed by Brözel and Cloete (1994). Results of this study suggest that this mechanism may be an efflux system for the removal of the biocide from the cell.

Suggestion for Further Work

This project could have been extended further with additional financial funding and permitted time. The suggestions for future work are:

- Purification of the protein T-OMP and subsequently sequence its N-terminal amino acids.
- The raising of polyclonal and monoclonal antibodies to the T-OMP in order to facilitate the isolation, purification and characterisation of its protein structure and consequently determine its role in the outer membrane.
- Construction of DNA probes in order to screen *P. aeruginosa* libraries in order to clone and sequence the gene encoding T-OMP.
- Investigate the possibility of an efflux pump mechanism within the cell, which would account for the observed resistance towards the biocides.
- Determine whether the observed resistance and loss of T-OMP would be observed in a *P. aeruginosa* biofilm.

References

AL-Adham, I.S.I., Dinning, A.J., Austin, P. & Collier, P.J. Cell membrane effects of some common biocides. (1998) *Journal for Industrial Microbiology* **21**, 6-10.

Ames, G.F.L., Spudich, E.N. & Nikaido, H. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. (1974) *Journal of Bacteriology* **117**, 406-416.

Angus, B.L., Carey, A.M., Caron, D.A., Kropinski, A.M.B. & Hancock, R.E.W. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic susceptible mutant. (1982) *Antimicrobial agents & Chemotherapy*, **21**, 229-309.

Ayres, H.M., Payne, D.N., Furr, J.R. & Russell, A.D. Effect of permeabilizing agents on antibacterial activity against a simple *Pseudomonas aeruginosa* biofilm. (1998) *Letters in Applied Microbiology* **27**, 79-82.

Ayres, H.M., Furr, J.R. & Russell, A.D. Effect of permeabilisers on antibiotic sensitivity of *Pseudomonas aeruginosa*. (1999) *Letters in Applied Microbiology* **28**, 13-16.

Barker, J., Brown, M.R.W., Collier, P.J., Farrell, I. & Gilbert, P. Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: Physiological status and susceptibility to chemical inactivation. (1992) *Applied and Environmental Microbiology.* **58**, 2420-2425

Bellido, F., Martin, N.L., Siehnel, R.J. & Hancock, R.E.W. Revaluation, using intact cells of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. (1992) *Journal of Bacteriology* **174**, (16) 5196-5203

Bento, F.M. & Gaylarde, C.C. Microbial Contamination of Stored Diesel Oil in Brazil. (1996) *Revisita de Microbiologica* **27**, (3) 192-196.

Bloomfield, S.F. Methods for assessing antimicrobial activity. *In Mechanisms of Action of Chemical Biocides, their Study and Exploitation.(1991) Eds: Denyer, S.P. & Hugo, W.B. Blackwell scientific publications, London (p. 1-20).*

Brock, T.D & Madigan, M.T. Cell Biology (1991) *In Biology of Microorganisms sixth edition Prentice-Hall International, Inc. USA (p.39-91).*

Brown, N. Opportunistic pathogens in hospitals-all change please. (1999) *Microbiology Today, Quarertly magazine of the Society for General Microbiology* **26**, 106-108.

Brown, M.R.W. & Gilbert, P. Sensitivity of biofilms to antimicrobial agents. (1993)*Journal of Applied Bacteriology* (suppl.) **74**, 87s-97s.

Brown, M.R.W. & Williams, P. The influence of environment on envelope properties affecting survival of bacteria in infections. (1985) *Annual Review of Microbiology* **39**, 527-556.

Brözel, V.S. & Cloete, T.E. Resistance of *Pseudomonas aeruginosa* to isothiazolone. (1994) *Journal of Applied Bacteriology* **76**, 576-582

Cochran, W.R., McFeters, G.A. & Stewart, P.S. Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramide. (2000) *Journal of Applied Microbiology* **88**, 22-30.

Collier, P.J. Biocides and preservative tolerance development: what it is and how to prevent it? The available weaponry: overview of biocides and preservatives. (1997) *97th General Meeting of the ASM presentation.*

Collier, P.J., Austin, P., & Gilbert, P. Uptake and distribution of some isothiazolone biocides into *Escherichia coli* ATCC 8739 and *Schizosaccharomyces pombe* NCYC 1354. (1990b) *International Journal of Pharmaceutics.* **66**, 201-206.

Collier, P.J., Austin, P., & Gilbert, P. Isothiazolone biocides: enzyme-inhibiting pro-drugs. (1991) *International Journal of Pharmaceutics* **74**, 195-201.

Collier, P.J., Ramsey, A.J., Austin, P., & Gilbert, P. Growth inhibitory and biocidal activity of some isothiazolone biocides. (1990a) *Journal of Applied Bacteriology.* **69**, 578-584.

Collier, P.J., Ramsey, A., Waigh, R.D., Douglas, K.T., Austin, P. & Gilbert, P. Chemical reactivity of some isothiazolone biocides. (1990c) *Journal of Applied Bacteriology.* **69**, 578-584.

Chamberland, S., Malouin, F., Rabin, T., Scholloordt, T., Parr, T.R. & Bryan, L.E. Persistence of *Pseudomonas aeruginosa* during ciprofloxacin therapy of a cystic fibrosis patient: transient resistance to quinolones and protein F deficiency. (1990) *Journal of Antimicrobial Chemotherapy* **25**, 995-1010.

Chuanchen, R., Beinlich, K. & Schweizer, H.P. Multipledrug efflux pumps and triclosan resistance in *Pseudomonas aeruginosa.* (2000) Abstract in *100th General Meeting of the American Society for Microbiology, L.A.*

Chapman, J.S. Resistance to Biocides- the 'other' antimicrobials. *Personal Communication, Rohm and Haas Co., USA.*

Chapman, J.S. Characterising bacterial resistance to preservatives and disinfectants. (1998) *International Biodeterioration & Biodegradation* **41**, 241-245.

Chapman, J.S., Diehl, M.A. & Fearnside, K.J. Preservative tolerance and resistance. (1997) *International Journal Cosmetic Science* **19** 1-10.

Chopra, I. Bacterial resistance to disinfectants, antiseptics and toxic metal ions. (1991) *In Mechanisms of Action of Chemical Biocides their Study and Exploitation Eds. Denyer, S.P. & Hugo, W.B. Blackwell Scientific Publications, London (p. 45-64)*

Cipriani, P., Giordano, A, Magni, A. & Filadoro, F. Influence of aztreonam-netilmicin association on outer membrane proteins of *Pseudomonas aeruginosa* strains. (1991) *Microbiologica* **14**, 301-306.

Cook, F.K. & Pierson, M.D. Inhibitors of bacterial spores by antimicrobials. (1983) *Food Technology* **37** 114-126.

Costerton, J.W., Stewart, P.S. & Greenberg, E.P. Bacterial biofilms: A common cause of persistent infections. (1999) *Science* **284**, 1318-1322.

Das, J.R., Bhakoo, M., Jones, M.V. & Gilbert, P.B. Changes in the biocide susceptibility of *Staphylococcus aureus* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. (1998) *Journal of Applied Microbiology* **84**, 852-858.

Decad, G.M. & Nikaido, H. Outer membrane of Gram-negative bacteria XII. Molecular sieving function of cell wall. (1976) *Journal of Bacteriology* **128**, 325-336.

Denyer, S.P. Mechanisms of action of antibacterial biocides. (1995) *International Biodeterioration & Biodegradation* **36**, (3-4) 227-245.

Dinh, T., Paulsen, I.T. & Saier, M.H. A family of extracytoplasmic proteins that allow transport of larger molecules across the outer membrane of Gram negative bacteria. (1994) *Journal of Bacteriology* **176** 3825-3831.

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

Diver, J.M., Schollaardt, T., Rabin, H.R., Thorsen, C. & Bryan, L.E. Persistence mechanisms in *Pseudomonas aeruginosa* from cystic fibrosis patients undergoing ciprofloxicin therapy. (1991) *Antimicrobial Agents and Chemotherapy* **35**, 1538-1546.

Domenico, Reich, J., Madonia, W. & Cunha, B.A. Resistance to bismuth among gram negative bacteria is dependent upon iron and its uptake. (1996) *Journal of Antimicrobial Chemotherapy* **38**, 1031-1040.

Ellman, G.L. (1964) *Analytical Chemistry* **60**, 9917

Evans, D.J., Brown, M.R.W., Allison, D.G. & Gilbert, P. Susceptibility of *Escherichia coli* & *Pseudomonas aeruginosa* biofilm to a profloxacin effect of specific growth rate. (1991) *Journal of Antimicrobial Chemotherapy* **27**, 177-184.

Foley, I. & Gilbert, P. Antibiotic resistance of biofilms. (1996) **Biofouling 10**, (4) 331-346.

Fuller, S.J., Denyer, S.P., Hugo, W.B., Pemberton, C., Woodcock, P.M. & Buckley, A.J. The mode of action of 1,2-benzisothiazol-3-one on *Staphylococcus aureus*. (1985) *Letters in Applied Microbiology* **1**, 13-15.

Fuchs, G. & Kroger, A. Growth and nutrition. (1999) *In Biology of the prokaryotes Eds. Lengeler, J.W., Drews, G. & Schegel, H.G.,Oxford (p.88-108).*

Ghannoum, M., Thompson, M., Bowman, W. & Al-Khalil, S. Mode of action of the antimicrobial compound 5-Bromo-5-nitro-1,3-dioxane (Bronidox). (1986) *Folia Microbiologica* **31**, 19-31.

Gilbert, P. Microbial resistance to preservative systems. (1988) *In Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries, eds. Bloomfield, S.F., Baird, R. Leak, R.E. & Leech, R. Ellis Horwood, Chichester, 171-194.*

Gilbert, P. & Brown, M.R.W. Some perspectives on preservation and disinfection in the present day. (1995) *International Biodeterioration and Biodegradation* **36**, (3-4) 219.

Gilbert, P., Brown, M.R.W. & Costerton, J.W. Inocula for antimicrobial sensitivity testing: a critical review. (1987) *Journal of Antimicrobial Chemotherapy* **20**, 147-154.

Gilbert, P. Collier, P.J., Andrews, J., Brown & M.R.W. Influence of post-growth procedures on the properties of microorganisms (out of the test-tube into the frying pan) (1995) *In Microbiological Quality Assurance. A guide towards relevance and reproducibility of inocula. Eds. Brown, M.R.W. & Gilbert, P. CRC Press, New York (p. 121-132).*

Giordano, A., Magni, A., Trancassini, M. & Cipriani, P. Outer membrane proteins and lipopolysaccharide changes after exposure of *Pseudomonas aeruginosa* to antibacterial drugs. (1993) *Microbiologica* **16**, 281-286.

Gotoh, N., Itoh, Nobuko, I. Yamada, H. & Nishino, T. Evidence for the location of OprM in the *Pseudomonas aeruginosa* outer membrane. (1994) *FEMS Microbiology Letters* **122**, 309-312.

Grabert, E., Wingender, J. & Winkler, U.K. An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa.*(1990) *FEMS Microbiology Letters* **68**, (1-2) 83-88.

Hacker, J. Prokaryotes in medicine. (1999) *In Biology of the prokaryotes Eds. Lengeler, J.W., Drews, G. & Schegel, H.G., Blackwell, Oxford (p.815-847).*

Hammond, S.M., Lambert, P.A., & Rycroft, A.N. The bacterial cell surface. (1984) *Croom Helm Ltd., London (p.57-119)*

Hancock, R.E.W. Alterations in the outer membrane permeability. (1984) *Annual Review Microbiology* **38**, 237-64.

Hancock, R.E.W. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. (1998) *Clinical Infectious Diseases* **27**, (Suppl) S93-S99.

Hancock, R.E.W., Siehnel, R. & Martin, N. Outer membrane proteins of *Pseudomonas.* (1990) *Molecular Microbiology* **4**, (7) 1069-1075.

Heath, R.J. & Rock, C.O. A triclosan-resistant bacterial enzyme. (2000) *Nature* **406**, 145-146.

Helander, I.M. & Mattila-Sandholm, T. Fluorometric assessment of Gram-negative bacterial permeabilization. (2000) *Journal of Applied Microbiology* **88**, 213-219.

Hosacka, M., Gotoh, N. & Nishino, T. Purification of a 54-kilodalton protein (OprJ) produced in NfxB mutants of *Pseudomonas aeruginosa* and production of a monoclonal antibody specific to OprJ. (1995) *Antimicrobial Agents and Chemotherapy* **39**, 8 1731-1735.

Hostacka, A. & Karelova, E. Outer membrane proteins and elastase of *Pseudomonas aeruginosa* after the postantibiotic effect induced by amikacin. (1997a) *Chemotherapy* **43**, 118-122.

Hostaka, A & Karelova, E. Postantibiotic effects of norfloxcin and its influence on profiles of outer membrane proteins on *Pseudomonas aeruginosa.* (1997b) *Pharmazie* **52**, (3) 238-239.

Huang, H., Siehnel, R.J., Bellido, F., Rawling, E. & Hancock, R.E.W. Analysis of two gene regions involved in the expression of the imipenem-specific, outer membrane porin protein Opr D of *Pseudomonas aeruginosa.* (1992) *FEMS Microbiology Letters* **97**, 267-274.

Hugo, W.B. The mode of action of antiseptics. (1967) *Journal of Applied Bacteriology*, **30**, 17-50.

Jann, K. & Jann, B. Assembly of Cellular Surface Structures. (1999) *In Biology of the Prokaryotes eds. Lengeler, J.W., Drews, G. & Schegel, H.G. Blackwell Science, Oxford (p. 555-569)..*

Jarrell, S.B. Basic Statistics (1994) *W. C. Brown Publishers (p.148-325).*

Köhler, T., Kok, M., Michea-Hamzehpour, M., Plesiat, P., Gotoh, N., Nishino, T., Curty, L.K., Pechere, J. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in *Pseudomonas aeruginosa.* (1996) *Antimicrobial Agents and Chemotherapy* **40**, (10) 2288-2290.

Kragelund, L., Leopold, K. & Nybroe, O. Outer membrane protein heterogeneity within *Pseudomonas fluorescens* and *Pseudomonas putida* and use of an Opr F antibody as a probe for rRNA homology group I Pseudomonads. (1996) *Applied & Environmental Microbiology* **62**, (2) 480-485.

Kramer, R. Multiple Roles of Prokaryotic Cell Membranes.(1999) *In Biology of the Prokaryotes eds. Lengeler, J.W., Drews, G. & Schegel, H.G. Blackwell Science, Oxford (p. 68-87).*

Kroll, R.G. & Patchett, R.A. Biocide-Induced Perturbations of Aspects of Cell Homeostasis: Intracellular pH, Membrane Potential and Solute Transport. *In Mechanisms of Action of Chemical Biocides, their Study and Exploitation. (1991) Eds: Denyer, S.P. & Hugo, W.B. Blackwell scientific publications London (p. 189-202).*

Kropinski, A.M., Kuzio, J., Angus, B.L. & Hancock, R.E.W. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersuceptible mutant of *Pseudomonas aeruginosa* (1982) *Antimicrobial Agents & Chemotherapy.* **21**, 310-319.

Laemmi, U.K. Clevage of structural proteins during the assembly of the head of bacteriophage T4.(1970) *Nature* **227**, 680-685.

Lawrence, J.V. & Maier, S. Correction for the inherent error in optical density readings. (1977) *Applied & Environmental Microbiology* **33**, (2) 482-484.

Lee, Y.H., Brown, M.R.W., & Cheung, H.Y. Defined minimal media for the growth of phototrophic and auxotrophic strains of *Bacillus sterarothermophilus*. (1982) *Journal of Applied Bacteriology* **53**, 179-187.

Lewis, K., Hooper, D.C. & Ouellette, M. Multidrug resistance pumps provide broad defence. (1997) ASM News **63**, (11) 605-610.

Lewis, S.N. & Miller, G.A. Derivatives of 3-isothiazolones with active halogen compounds. (1974) *US patent* **3**, 835,150.

Lomovskaya, O. & Lewis, K. EMR, an *Escherichia coli* locus for multidrug resistance. (1992) *P. Natl. Acad. Science, USA* **89** 8938-8942.

Lowry, O.H., Rosenborough, N.H., Farr, A.L. & Randall, R.J. Protein Measurement with the Folin Phenol Reagent. (1951) *Journal of Biological Chemistry* **193**, 265-273.

Ma, D., Cook, D.N., Hearst, J.E. & Nikaido, H. Efflux pumps and drug resistance in Gram-negative bacteria. (1994) *Trends in Microbiology* **2**, (12) 489-493.

Magee, M.A., Sutton, S.V.W. & Brannan, D.K. Preservative efficiency, microbial content and disinfection testing. (1997) *In Cosmetic Microbiology: A Practical Handbook ed. D.K. Brannan. CRC Press New York (p. 95-126).*

Magni, A., Giordano, A., Papa, F., DeVito, D. & Cipriani, P. Effects of continuous exposure to ciprofloxacin on the outer membrane of *Pseudomonas aeruginosa.* (1994) *Microbiologica* **17**, 307-312.

Maira-Litrán, T., Allison, D.G., & Gilbert, P. Expression of the multiple antibiotic resistance operon (mar) during growth of *Escherichia coli* as a biofilm. (2000) *Journal of Applied Microbiology* **88**, 243-247.

Mann, C.M., Cox, S.D. & Markham, J.L. The outer membrane of *Pseudomonas aeruginosa* NCTC 6749 contributes to its tolerance to the essential oil of Melaleuca alternifolia (tea tree oil). (2000) *Letters in Applied Microbiology* **30**, 294-297).

Masuda, N., Sakagawa, E. & Ohya, S. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa.* (1995) *Antimicrobial Agents & Chemotherapy* **39**, 645-649.

Marger, M.D. & Saier, M.H. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. (1993) *Trends in Biochemical Science* **18** 13-20.

Mayer, F. The prokaryotic cell (1999) *In Biology of the Prokaryotes eds. Lengeler, J.W., Drews, G. & Schegel, H.G. Blackwell Science, Oxford (p. 20-44)..*

McMurray, L.M., Oerthinger, M. & Levy, S.B. Triclosan targets lipid synthesis. (1998) *Nature* **394** 531-532.

McMurray, L.M., McDermot, D.F. & Levy, S.B. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. (1999) *Antimicrobial Agents & Chemotherapy* **43 711-713.**

Mitsuhashi, S. Drug resistance in bacteria: history, genetics and biochemistry. (1993) *International Medical Research* **21** 1-14.

Neidhardt, F.C., Ingram, J.L. & Schaechter, M. Structure and function of the bacterial cell (1990) *Physiology of the Bacterial Cell. A Molecular Approach. P.30-59. Sinauer Associates, Massachusetts, USA.*

Nicas, T.I. & Hancock, R.E.W. Outer membrane permeability in *Pseudomonas aeruginosa.* Isolation of a porin protein-F deficient mutant. (1983) *Journal of Bacteriology*, **153**, 281-285.

Nikado, H. Outer membrane of *Salmonella typhimurium.* Transmembrane diffusion of some hydrophobic substances. (1976) *Biochimica et Biophysica Acta* **433**, 118-132.

Nikaido, H. Bacterial resistance to antibiotics as a function of outer membrane permeability. (1988) *Journal of Antimicrobial Chemotherapy* **22** Suppl. A17-22.

Nikaido, H. Non-specific and specific permeation channels of the Pseudomonas aeruginosa outer membrane (1992). *In Pseudomonas: molecular biology & biotechnology. eds. Galli, E.,*

Silver, S. & Witholt, B. American Society For Microbiology, Washington D.C, Oxford (p.146-153)..

Nikaido, H. Porins and specific channels of bacterial outer membranes. (1992) *Molecular Microbiology* **6** 435-442.

Nikaido, H. Multidrug efflux pumps of Gram negative bacteria. (1996) *Journal of Bacteriology* **178** 5853-5859.

Nikaido, H. & Nakae, T. The outer membrane of Gram-negative bacteria. (1979) *Advances in Microbial Physiology* **20**, 163-250.

Nikaido, H. & Saier, M.H. Transport proteins in bacteria-common themes in their design. (1992) *Science* **258** 963-942.

Nikaido, H. & Vaara, M. Molecular basis of the permeability of the bacterial outer membrane. (1985) *Microbiological Reviews*, **49**, 1-32.

Ochs, M.M., Mcusker, M.P., Bains, M. & Hancock, R.E.W. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin Opr D selective for imipenem and basic amino acids. (1999) *Antimicrobial Agents & Chemotherapy* **43**, (5) 1085-1090.

O'Farrell, P.H. High resolution two-dimensional electrophoresis of proteins. (1975) *The Journal of Biological Chemistry* **250**, (10) 4007-4021.

Osborn, M.J. & Wu, H.C.P. Proteins of the outer membrane Gram-negative bacteria. (1980) *Annual Review of Microbiology* **34**, 369-422.

Ouellette, M., Lewis, K. & Hooper, D.C. Eukaryotic microbial multidrug resistance pumps. (1997) *ASM News* **63**, (12) 664-667.

Owen, R.A. & Hartman, P.E. Glutathione: A protective agent in *Salmonella typhimurium* and *Escherichia coli* as measured by mutagenicity and by growth delay assays. (1986) *Environmental Mutagenesis* **8**, 659-673.

Paul, S., Bezbarauah, R.L., Roy, M.K. & Ghosh, A.C. Multiple antibiotic resistant (MAR) index and its reversion in *Pseudomonas aeruginosa.* (1997) *Letters in Applied Microbiology* **27**, 169-171.

Paulsen, I.T., Brown, M.H. & Skurray, R.A. Proton-dependent multidrug efflux systems (1996) *Microbiological Reviews* **60** 575-608.

Paulsen, I.T., Slinwinski, M.K., & Saier, M.H. Microbial genome analysis: global comparisons of transport capabilities based on phylogenesis, bioenergenetics and substrate specificity (1998) *Journal of Molecular Biology* **277** 573-592.

Pettersson, A., Poolman, J.T., Vander Ley, D & Tommassen, J. Response of *Neisseria meningitidis* to iron limitation. (1997) *Antonie van Leeuwenhook* **71**, 129-136.

Poole, K., Kredes, K., McNally, C. & Neshat, S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. (1993) *Journal of Bacteriology* **175**, (22) 7363-7372.

Preston, M.A. & Penner, J.L. Structural and antigenic properties of lipopolysaccharide from serotype reference strains of *Camplyobacter jejuni*. (1987) *Infection and Immunity* **55**, (8) 1806-1812.

Pugsley, A.P., Moreno, F. & Lorenzo, V. Microcin-E492-insensitive mutants of *Escherichia coli*. (1986) *Journal of General Microbiology* **132**, 3253-3259.

Rattan, A. Mechanisms of resistance to fluoroquinolones. (1999) *National Journal of India* **12** 162-164.

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

Rocchetta, H. L., Burrows, L. L. & Lam, J. S. Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa.* (1999) *Microbiology and Molecular Biology Reviews* **63**, (3) 523-553.

Rosenberg, M., Gutnick, D. & Rosenberg, E. Adherence of bacteria to hydrocarbon: A Simple method for measuring cell-surface hydrophobicity. (1980) *FEMS Microbiology Letters* **9**, 29-33.

Rozwarski, D.A., Grant, G.A., Barton, D.H.R., Jacobs, W.R. & Sacchettini, J.C. Modifications of the NADH of the izoniazid target (InhA) from *Mycobacterium tuberculosis.* (1998) *Science* **297** 98-102.

Russell, A.D. Factors influencing the efficiency of antimicrobial agents (1992a) *In Principles and Practice of Disinfection, Preservation and Sterilisation Eds. Russell, A.D., Hugo, W.B. & Ayliffe, G.A.J. 2nd edition Blackwell Scientific , Oxford, P. 246-253.*

Russell, A.D. Types of antimicrobial agents. (1992b) *In Principles and Practice of Disinfection, Preservation and Sterilisation Eds. Russell, A.D., Hugo, W.B. & Ayliffe, G.A.J. 2nd edition Blackwell Scientific, Oxford, P. 59-60.*

Russell, A.D. Mechanisms of bacterial resistance to biocides. (1995) *International biodegradation and Biodeterioration* **36**, (3-4) 247-265.

Russell, A.D. Plasmids and bacterial resistance to biocides. (1997) *Journal of Applied Microbiology* **82**, 155-165.

Russell, A.D. & Furr, J.R. Susceptibility of porin-and lipopolysaccharide-deficient strains of *Escherichia coli* to some antiseptics and disinfectants. (1986) *Journal of Hospital Infection* **8**, 47-56.

Russell, A.D. & Furr, J.R. Comparative sensitivity of smooth, rough and deep rough strains of *Escherichia coli* to chlorohexidine, quaternary ammonium compounds and dibromopropamidine isethionate. (1987) *International Journal of Pharmaceutics* **36**, 191-197.

Russell, A.D., Furr, J.R. & Pugh, W.J. Sequential loss of outer membrane lipopolysaccharide and sensitivity of *Escherichia coli* to antibacterial agents. (1987) *International Journal of Pharmaceutics*, **35**, 227-233.

Russell, A.D., Mallard, J.Y. & Furr, J.R. Possible link between bacterial resistance and use of antibiotics and biocides. (1998) *Antimicrobial Agents & Chemotherapy* **42**, (8) 2151.

Sawer, I.K., Berry, M.I. & Ford, J.L. Effect of medium composition, agitation and the presence of EDTA on the antimicrobial activity of crypoleine. (1997) *Letters in Applied Microbiology* **25**, 207-211.

Shepherd, J.A., Woodcock, P.M. & Gilbert, P. Interaction of 2-Bromo-2-nitropropan-1,3-diol with some respiration and dehydrogenase enzyme systems in *E. coli*. (1985) *Journal of Pharmacy and Pharmacology* **37**, Suppl. 95.

Shepherd, J.A., Buerby, M.R., Pemberton, P., Gilbert, P. & Waigh, R.D. The reaction of bronopol (2-bromo-2-nitropropan-1,3-diol) with thiols. (1987) *Journal of Pharmacy and Pharmacology* **39**, suppl. 116.

Singer, M. Laboratory procedures for assessing the potential for antimicrobial agents as industrial biocides. (1976) *Process Biochemistry* **July / Aug** 30-35.

Skillman, L.C., Sutherland, I.W. & Jones, M.V. The role of exopolysaccharide in dual species biofilm development. (1999) *Journal of Applied Microbiology Symposium Supplement* **85**, 13s-18s.

Sleytr, U.B. & Messner, P. Crystalline surface layers of bacteria. (1993) *Annual Review of Microbiology* **42** 359-393.

Smit, J., Kanno,Y. & Nikaido, H. Outer membrane of *Salmonella typhimurium*: Chemical analysis and freeze-fraction studies with lipopolysaccharide mutants. (1975) *Journal of Bacteriology* **124**, 942-958.

Smith, P.K., Krohn, I., Hermanson, A.K., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M. & Klenk, D.C. Measurement of protein using Bicinchronic acid (1985) *Analytical Biochemistry* **150**, 76-85.

Son, R., Rusul, G. & Karim, M.I.A. Conjugal transfer of plasmids and antibiotic resistance among *Escherichia coli* isolated from animals in a rural area in Sarawak (Malaysia). (1997) *Journal of Applied Microbiology* **82**, 240-244.

Sondossi, M., Riha, V.F., Rossmoore, H.W. & Sylvestre, M. Factors involved in bactericidal activities of formaldehyde and formaldehyde condensate / isothiazolone mixtures. (1993) *International Biodeterioration and Biodegradation* **32**, 243-261.

Sox, T.E. Mechanisms of action of cosmetic preservatives. (1997) *In Cosmetic Microbiology: A Practical Handbook ed. D.K. Brannan. CRC Press New York (p.163-178)..*

Stickler, D.J.B., Thomas, J.C., Clayton, C & Chawla, J.A. Studies on the genetic basis of chlorohexidine resistance. (1983) *British Journal of Clinical Practice Symposium* **25**, (Supplement) 23-28.

Sugawara, E., Steiert, M., Rouhani, S. & Nikaido, H. Secondary structure of the outer membrane proteins OMP A of *Escherichia coli* and Opr F of *Pseudomonas aeruginosa.* (1996) *Journal of Bacteriology* **178**, (20) 6067-6069.

Sunairi, M., Iwabuchi, N., Yoshizawa, Y., Murooka, H. & Nakajima, M. Cell-Surface hydrophobicity and scum formation of *Rhodococcus rhodochrous* strains with different colonial morphologies. (1997) *Journal of Applied Microbiology* **82**, 204-210.

Suzanger, S., Allison, D.G. & Gilbert, P. An evaluation of biocide-containing material for their surface colonisation-resistance and other properties. (2000) *100th General Meeting of the American Society for Microbiology, L.A.*

Tateda, K., Ishii, Y., Hirakata, Y., Matsumoto, T., Ohno, A. & Yamaguchi, K. Profiles of outer membrane proteins and lipopolysccharides of *Pseudomonas aeruginosa* grown in the presence of sub-MICs of macrolide antibiotics and their relation to enhanced serum sensitivity. (1994) *Journal of Antimicrobial Chemotherapy* **34**, 931-942.

Travis, J. Popularity of germ fighter raises concern. (2000) *Science News* **157**, 342-343.

Trueman, J.R. The halogens. (1971) *In Inhibition and destruction of the microbial cell. Eds. Hugo, W.B. 137-183. London Academic Press.*

Waigh, R.D. & Gilbert, P. Mechanisms of chemical reactions with bimolecules. (1991) *In Mechanism of action of chemical biocides: their exploitation and study eds. Denyer, S.P. & Hugo, W.B, London (p.251-262)*

Walker, J. M. The bicinchonic acid assay for protein quantification. (1984a) *In Methods in Molecular Biology Volume 32. Basic Protein and Peptide Protocols. Th Humana Press Inc., New Jersey (p. 5)*

Walker, J. M. Quantification of proteins on polyacrylamide gels (nonradioactive). (1984b) *In Methods in Molecular Biology Volume 32. Basic Protein and Peptide Protocols. Th Humana Press Inc., New Jersey (p.106-112)*

Walker, J. M. Drying gels. (1984c) *In Methods in Molecular Biology Volume 32. Basic Protein and Peptide Protocols. Th Humana Press Inc., New Jersey (p. 157-161).*

Weiss, M.S., Abel, O., Weckesser, J., Welte, W., Schultz, E. & Schulz, G.E Molecular architecture and electrostatic properties of a bacterial porin. (1991) *Science* **254** 1627-1630.

Wood, P., Jones, M., Bhakoo, M. & Gilbert, P. A novel strategy for control of microbial biofilms through generation of biocide at the biofilm-surface interface. (1996) *Applied & Environmental Microbiology* **62**, (7) 2598-2602.

Wylie, J.L. & Worobec, E.A. The Opr B porin plays a central role in carbohydrate uptake in *Pseudomonas aeruginosa.* (1995) *Journal of Bacteriology* **177**, (11) 3021-3026.

Yamano, Y., Nishikawa, T. & Komatsu, Y. Outer membrane proteins responsible for the penetration of ß-lactams and quinolones in *Pseudomonas aeruginosa.* (1996) *Journal of Antimicrobial Chemotherapy* **26**, 175-184.

Yoneyama, H., Ocakton, A., Gotoh, N., Nishino, T. & Nakae, T. (1990) Subunit swapping in the mex-extrusion pumps in *Pseudomonas aeruginosa. Biochemical and Biophysical Research Community* **244** 898-902.

Yoshihara, E., Gotoh, N., Nishino, T., & Nakae, T. Protein D2 porin of *Pseudomonas aeruginosa* outer membrane bears the protease activity. (1996) *FEBS Letters* **394**, 179-182.

Yoshimura, F. & Nikaido, H. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. (1982) *Journal of Bacteriology* **152**, 636-642.

Zgurskaya, H.I. & Nikaido, H. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *E. coli.* (1999)*Proc National Academic Science, USA* **96** 7190-7195 (2000).

Zgurskaya, H.I. & Nikaido, H. Multidrug resistance mechanisms: drug efflux across two membranes. (2000) *Molecular Microbiology* **37** 219-255.

Appendix